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**THE EFFECT OF CIS-5,8,11,14,17-EICOSAPENTAENOIC ACID
UPON THE CONTRACTILE MECHANISMS LINKED TO
CALCIUM INFLUX AND THE MOBILISATION OF
INTRACELLULAR CALCIUM IN AORTIC SMOOTH MUSCLE**

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Doctor of Philosophy

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The effect of cis-5,8,11,14,17-eicosapentaenoic acid upon the contractile mechanisms linked to calcium influx and the mobilisation of intracellular calcium in aortic smooth muscle

Nicola Bretherton

Ph.D. Thesis 1998

Summary

Epidemiological studies previously identified cis-5,8,11,14,17-eicosapentaenoic acid (EPA) as the biologically active component of fish oil of benefit to the cardiovascular system. Although clinical investigations demonstrated its usefulness in surgical procedures, its mechanism of action still remained unclear.

It was shown in this thesis, that EPA partially blocked the contraction of aortic smooth muscle cells to the vasoactive agents KCl and noradrenaline. The latter effect was likely caused by reducing calcium influx through receptor-operated channels, supporting a recent suggestion by Asano *et al* (1997). Consistently, EPA decreased noradrenaline-induced contractures in aortic tissue, in support of previous reports (Engler, 1992b).

The observed effect of EPA on cell contractions to KCl was not simply due to blocking calcium influx through L-type channels, consistent with a previous suggestion by Hallaq *et al* (1992). Moreover, EPA caused a transient increase in $[Ca^{2+}]_i$ in the absence of extracellular calcium. To resolve this it was shown that EPA increased inositol phosphate formation which, it is suggested, caused the release of calcium from an inositol phosphate-dependent internal binding site, possibly that of an intracellular membrane or *superficial* sarcoplasmic reticulum, producing the transient increase in $[Ca^{2+}]_i$. As it was shown that the cellular contractile filaments were not desensitised to calcium by EPA, it is suggested that the transient increase in $[Ca^{2+}]_i$ subsequently blocks further cell contraction to KCl by activating membrane-associated potassium channels. Activation of potassium channels induces the cellular efflux of potassium ions, thereby hyperpolarising the plasma membrane and moving the membrane potential farther from the activation range for calcium channels. This would prevent calcium influx in the longer term and could explain the initial observed effect of EPA to block cell contraction to KCl.

Keywords: fish oil; calcium channels; inositol phosphate; potassium channels

To my parents, Val and Reece

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This thesis is an account of original work undertaken in the Department of Pharmaceutical Sciences, Aston University. The study was carried out during my employment as a part-time Research Technician for Dr. Keith Wilson.

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Chapter 1

General Introduction

1.1. The Beneficial Effects Of Fish Oils In Cardiovascular Disease.

Hypertension is recognised as one of the major contributors to death through cardiovascular diseases, including cardiac failure, coronary artery disease and stroke. Attempts to reduce the incidence of cardiovascular disease have led investigators to suggest that this may be possible through changes in lifestyle and particularly changes in diet, but dietary intervention trials in hypertension have often failed to show this. Although there is scientific evidence which links the amount of saturated fat in the diet with the risk of hypertension (Sacks 1989) the mechanism by which this occurs is poorly understood.

Epidemiological studies on Greenland Eskimos since the late 1940s revealed a reduced prevalence of cardiovascular-related diseases in this population (Ehrstrom, 1951; Kromann & Green, 1980; Knapp & Fitzgerald, 1989) and through experimental and clinical intervention trials, attempts have since been made to elucidate the reasons for this. Diet, especially fish and other marine animal consumption, has been of interest for many investigators (Bang *et al*, 1976). A study which analysed death certificates in the Greenland Eskimo population suggested that Eskimos do have a lower than expected rate of myocardial infarction (Dyerberg & Bjerregaard, 1987) and another epidemiological study suggested that in Dutch male subjects, the inclusion of 30 g of fish in the daily diet reduced mortality rate by 50 % over a 20 year period. Another study (DART) was conducted in England in 2033 male survivors of myocardial infarction (Burr, *et al*, 1989). The men were advised to increase the amount of fish in their diets and after two years, were shown to have a 29 % decrease in mortality. In a very recent study, Albert *et al* (1998) investigated prospectively, the association between fish consumption and the risk of sudden cardiac death. He used male physicians, aged 40-84 who were free of myocardial infarction, cerebrovascular disease and cancer at the start of study. The patients ate one fish meal at intervals ranging from less than monthly, to more than weekly and the incidence of sudden cardiac death was measured. The study showed that at a threshold consumption level of one fish meal weekly, dietary fish intake was associated with a reduced risk of sudden death.

Attempts to identify the components of dietary fish responsible for reduced mortality from cardiovascular disease have identified the biologically active components to be fish oils and in particular, the long chain polyunsaturated fatty acids (PUFAs) in the oils (Leaf & Weber, 1988). Experimental trials have shown PUFAs to have benefits on the cardiovascular system; they lower blood pressure

(Lockette *et al*, 1982; Norris *et al*, 1986; Knapp, 1988; McMilan, 1989; Bonaa *et al*, 1990; Mano *et al*, 1995; Joly *et al*, 1995), decrease the ratio of low density to high density lipoprotein levels (Leaf & Weber, 1988) and decrease platelet aggregation (Leaf & Weber, 1988). It follows, therefore, that it may be beneficial for PUFA-containing medicines to be prescribed as a preventative measure in the treatment of cardiovascular disease. Indeed, one particular study demonstrated that hypercholesterolaemic patients, who suffer from an excess of cholesterol in the bloodstream were administered MaxEpa 5 capsules which contain PUFAs for 3 months. Following arterial biopsies the blood vessels were found to have an improved relaxatory response, demonstrating that PUFAs significantly improved small arterial function in these hypercholesterolaemic patients (Goode *et al*, 1997).

Clinical investigations to date have also shown that PUFAs have promise in two major indications associated with the cardiovascular system; preventing restenosis after coronary angioplasty and preventing venous graft occlusion following coronary artery bypass surgery. In angioplasty, the coronary vessel walls that have been injured during the procedure to repair or unblock them, respond with an inflammatory reaction, leading to restenosis. Restenosis is the recurrence of abnormal narrowing of the artery but has been shown to be prevented by combined administration of different PUFAs (Horrobin, 1993). Venous bypass graft surgery is often followed by occlusion of the vein within the first year following surgery. PUFAs have been used to prevent reocclusion, although a significant effect of this treatment is not always observed (Parks & Rudel, 1990; Simopoulos, 1988).

Experimental and clinical investigations have also been made with PUFAs on animal models of human cardiovascular-related diseases, often to identify the effects on *secondary hypertension* and also to determine whether PUFAs offer protection during a myocardial infarction. Secondary hypertension has an identifiable underlying cause such as kidney disease and if diagnosed early, can be cured by removing the underlying cause. Hobbs *et al* (1996) examined whether dietary fish oil prevented the development of renal damage associated with secondary hypertension using a rat model of human hypertension, *i.e.* the stroke-prone spontaneously hypertensive rat fed a high salt diet. The animal diet contained 5 % (by weight) fish oil and the clinical development of proteinuria, which is the presence of abnormal quantities of protein in the urine and which may indicate damage to the kidneys and the development of hypertension were measured. The study demonstrated that in the fish oil-fed animals, there was no development of proteinuria or hypertension, thereby demonstrating that fish oil prevented the development of renal damage. Three studies which examined the effect of PUFAs on protection against myocardial infarction ligated a coronary or cerebral artery in dogs or rats that had been fed fish oil. Much of the cellular

damage or death (*necrosis*) associated with an infarction occurs when blood can reflow through the vessel after the occluding thrombus moves or breaks down. The return of oxygenated blood to the tissue deprived of oxygen (*ischemia*) damages the vessel because of the free radicals formed. However, the amount of myocardial necrosis in animals that had been fed fish oil was significantly lower than animals who had not been fed fish oil.

The results of these studies demonstrate that dietary fish oil supplementation has wide-ranging therapeutic implications in the treatment of cardiovascular-related diseases, either as a preventative measure, or as a follow-up measure to clinical intervention. This, combined with the ever increasing desire to replace foods in the diet which contain large amounts of cholesterol and saturated fatty acids, lends support to further investigation of the effect of PUFAs, particularly because, even though they are drugs, they are likely to be viewed as nutritional supplements by the public and, unlike synthetic agents, are not subject to the popular fears of toxicity. Consequently, it is important to determine the precise mechanism of action of the biologically active components in the fish oils if they are to be administered as a dietary supplement.

1.2. The n-3 Family Of Fatty Acids And Their Metabolism

Fatty acids can be classified into two broad groups according to their chemical structure. Those having no double bonds are classed as saturated fatty acids and those with at least one double bond are unsaturated fatty acids. It is possible to further subdivide the unsaturated fatty acids into monounsaturated fatty acids or polyunsaturated fatty acids (PUFAs). There are four families of PUFAs; the non-essential, palmitoleic (n-7) and oleic (n-9) families and the essential, linoleic (n-6) and linolenic (n-3) families*. Linoleic acid (18:2n-6) and α -linolenic acid (18:3n-3), the precursors of the two essential fatty acid families, are not synthesised *de novo* by animal or human cells and therefore must be provided by dietary intake†. Longer-chain, unsaturated fatty acids are also supplied in the diet, however, they can be synthesised *de novo* by alternate desaturation-elongation reactions of the precursor acids (Sprecher, 1981), as outlined in figure 1.1.

* The term in brackets denotes the position of the first double bond from the methyl end of the fatty acid.

† The number before the colon gives the carbon number and the figure after denotes the number of double bonds.

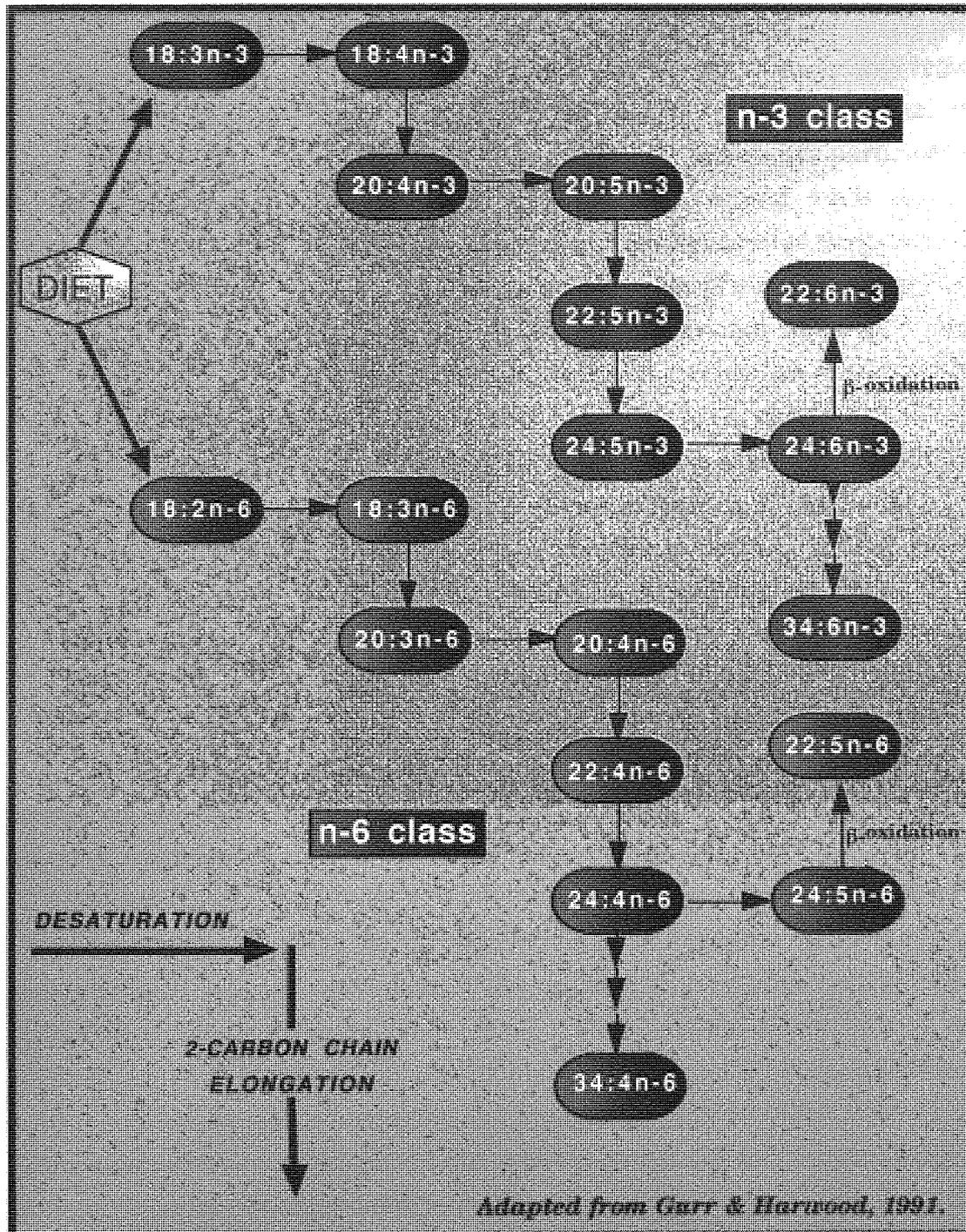


Figure 1.1. Diagrammatic representation of the outline of major pathways of n-3 and n-6 fatty acid biosynthesis by desaturation and chain elongation in animal tissues.

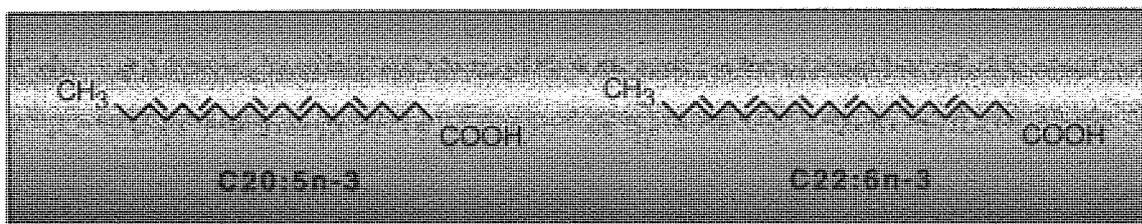


Figure 1.2. Schematic of the chemical structure of eicosapentaenoic (C20:5n-3) and docosahexaenoic (C22:6n-3) acids.

Essential fatty acids are major components of the phospholipids which affect the integrity and fluidity of intracellular and plasma membranes. In this regard, they modulate the activity of membrane-bound receptors, enzymes, molecule carriers and ionic channels. In addition, some essential fatty acids participate in the normal intravascular and cellular metabolism of cholesterol, while some are implicated in the physiology of the reproductive and cardiovascular systems and in immune function, through their conversion to a wide range of eicosanoids, including prostanoids (prostaglandins and thromboxanes) and leukotrienes which have potent biological effects. Attempts to elucidate the beneficial mechanism of action of the fish oils led to the identification of the essential long chain n-3 PUFAs, eicosapentaenoic acid (EPA: 20:5n-3) and docosahexaenoic acid (DHA: 22:6n-3) as the biologically active components of fish oil, shown in figure 1.2. Both acids can be synthesised from the precursor α -linolenic acid (refer to figure 1.1) which can be found in green leaves and some plant oils, including linseed, rapeseed and soybean (refer to Leaf & Weber, 1988).

The main human dietary sources of EPA and DHA are fatty fish and supplements such as cod liver oil and n-3 fatty acid capsules. Intake of these nutrients in human populations ranges from non to more than 10 g daily (Drevon, *et al.*, 1993) but it has been proposed that recommended dietary intake of EPA and DHA is only 350-400 mg daily (Bézar *et al.*, 1994).

The availability of essential fatty acids is affected by the digestion and absorption of dietary lipids or *triacylglycerols*. In humans, the digestion of consumed triacylglycerols commences in the stomach by the action of gastric lipase. However, this enzyme operates best at a pH of 5 to 6, so has a limited role in the adult stomach which is at pH 2. As the partially digested food passes along the digestive tract into the duodenum, the intestinal mucosa releases hormones including secretin and cholecystokinin which control pancreatic and intestinal secretion. Secretin also stimulates secretion of bile by the hepatic cells of the liver. Bile is partially an excretory product and partially a digestive secretion consisting of water, bile salts, cholesterol, lecithin, bile pigments and ions and is stored in the gall bladder. Cholecystokinin induces the release of the stored bile into the duodenum and the bile salts it contains emulsify the triacylglycerols into a suspension of droplets. Lipolysis continues in the small intestine, into which pro-lipase is secreted by the pancreas in response to secretin. The pro-lipase is converted into active lipase and in the presence of bile salts and co-lipase, lipase catalyses the hydrolytic removal of two of the three fatty acids from the glycerol of the droplets to yield a mixture of long and short-chain fatty acids and monoacylglycerols. Short-chain fatty acids diffuse into the epithelial cells lining the finger-like projections or *villi* of the intestinal cells and then into the blood

capillaries contained within the villi. Long-chain fatty acids and monoacylglycerols are carried in micelles which are spherical aggregates formed from bile, until the micelles reach the epithelial cell surface of the villi. The fatty acids and monoacylglycerols dissolved inside the micelles diffuse into the epithelial cells, leaving the micelles behind in chyme. Within the epithelial cells, the fatty acids and monoacylglycerols are further digested by lipase to glycerol and fatty acids, then recombined to form triacylglycerols in the endoplasmic reticulum of the epithelial cells. The triacylglycerols, while still in the endoplasmic reticulum, aggregate into globules along with hydrolysed dietary phospholipids and hydrolysed cholesteryl esters. The hydrolysis products are coated with proteins (to prevent sticking together of globules) to form chylomicrons which leave the epithelial cells and enter the lacteals of villi. From here, they are transported in the lymphatic system to the thoracic duct and enter the cardiovascular system at the left subclavian vein. Finally, they arrive at the liver through the hepatic artery.

Within a few hours after consuming a meal that contains a large amount of fat, most chylomicrons are removed from blood as they pass through blood capillaries in the liver. This is accomplished by lipoprotein lipase, an enzyme found in capillary endothelial cells. As the chylomicrons pass along the capillaries, lipoprotein lipase breaks down the triacylglycerols in the chylomicrons into fatty acids and glycerol. The fatty acids diffuse into liver and fat cells and recombine with glycerol produced by the cells, to re-form triacylglycerols. The fatty acid components of the lipids entering the liver have several different metabolic pathways, outlined in figure 1.3.

In order to be transported in blood and utilised by body cells, triacylglycerols are combined with protein transporters (apoproteins) to make them soluble. This combination of lipid and protein is known as a *lipoprotein*. In addition to chylomicrons, there are several other types of lipoproteins, known as high-density lipoproteins (HDL), low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL).

Lipid digestion and absorption processes have been reviewed several times and it has been observed that the structure of PUFAs and their position in dietary triacylglycerols and phospholipid molecules are important determinants in their bioavailability. *In vitro* certain long-chain PUFAs of marine oils have been shown to be resistant to pancreatic lipase hydrolysis. This was attributed to the presence of a double bond between carbon 2 and carbon 5 of the acyl chain (when counted from the carboxylic end) (Bottino *et al.*, 1967). This is the case with DHA, in which the first double bond is located at the fourth carbon atom. The resistance to lipolysis seemed independent of the fatty acid in the glycerol moiety of triacylglycerol. It follows therefore that this property could impair absorption of

DHA from marine oils when transposed *in vivo*. However, this appears to be a controversial point (Carrier *et al*, 1991) in that absorption could be delayed but not decreased, so that availability would not be affected.

The availability of EPA and DHA is also reportedly affected when they are given in the form of ethyl esters (instead of natural glyceryl esters). Their absorption is impaired, possibly due to the lack of the monoacylglycerols necessary for triacylglycerol re-synthesis in enterocytes. However, hydrolysis does take place, probably less rapidly, but completely (Lawson & Hughes, 1988).

Essential fatty acids and their metabolic products have dual roles as membrane components and as precursors of a variety of 20-carbon, oxygenated fatty acids with potent biological activities, *i.e.* eicosanoids. Eicosanoids are implicated in the control of many physiological processes and are among the most important mediators and modulators of the inflammatory reaction. Dyerberg initiated the idea that the n-3 fatty acids could prevent thrombosis and atherosclerosis as a result of induced changes in eicosanoid metabolism (Dyerberg *et al*, 1978). Since then, the potential beneficial effects of n-3 fatty acids have been demonstrated in clinical studies associated with cardiovascular epidemiology (refer to the Third International Conference on Preventive Cardiology, 1994), although the underlying mechanism of action of the n-3 fatty acids has still not been elucidated. However, it has been shown that when n-3 fatty acids are included in the diet, they and their metabolic products compete with another PUFA which is the main source of the eicosanoids, *i.e.* arachidonic acid.

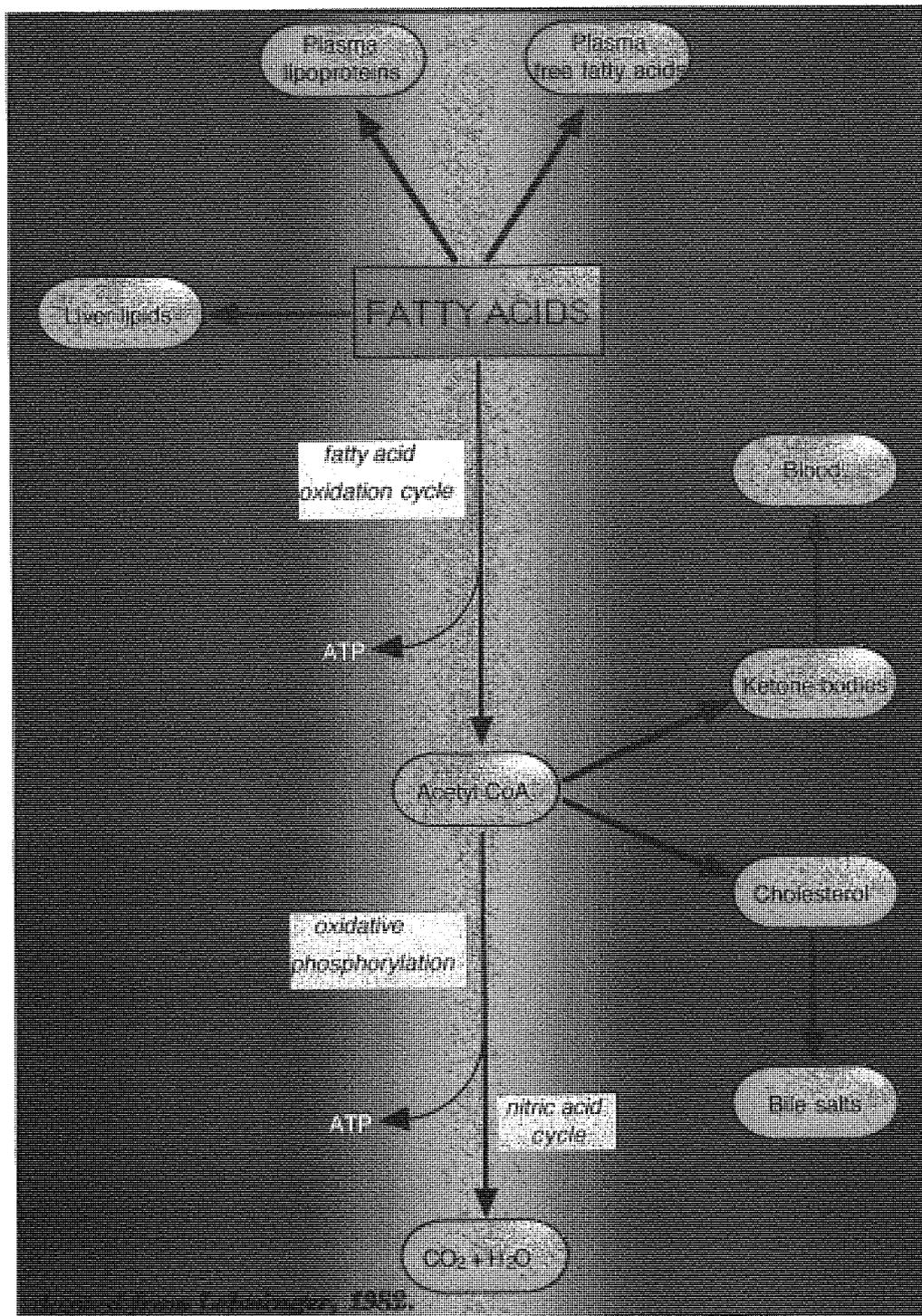


Figure 1.3. Diagrammatic representation of fatty acid metabolism in the liver.

n-3 Fatty acids and their metabolic products compete with arachidonic acid in several ways;

- by inhibiting the synthesis of arachidonic acid from its precursor (Damron & Bond, 1993)
- by competing for the 2-acyl position of cell membrane phospholipids which reduces plasma and cellular levels of arachidonic acid and has also been suggested to alter membrane fluidity (Goodnight, 1982)
- by reducing the circulating plasma and cellular levels of arachidonic acid or by competing with arachidonic acid as the substrate for metabolising enzymes, such as the cyclo-oxygenase enzyme which metabolises the fatty acids to prostanoids (Siess *et al.*, 1980).

In addition to n-3 fatty acids competing with arachidonic acid, the products of the n-3 fatty acids, EPA and DHA are less active than those of arachidonic acid (Leaf & Weber, 1988). It follows, therefore, that the effects induced by arachidonic acid and its eicosanoid derivatives may be decreased in the presence of EPA and DHA, which generally leads to a more vasodilatory and anti-platelet-aggregatory status of the cardiovascular system.

However, administration of n-3 or n-6 metabolites alone could cause undesirable effects as it implies modification in the availability of the different substrates for prostanoid production. This would lead to an imbalance in prostanoid biosynthesis, which might be an important risk factor for coronary heart disease. This was postulated following the results of a study on PUFA metabolism by Bordoni *et al.*, (1996). In the investigation, the cells from heart tissue taken from rats were cultured and then experimented upon. These primary heart cell cultures from ventricular myocytes were taken from 2-4 day old Wistar rats. The cultures were treated with combinations of n-3 and n-6 PUFAs and the results showed that supplementation with both n-3 and n-6 fatty acids maintained the n-6/n-3 lipid ratio similar to control cells, thereby maintaining balance in prostanoid biosynthesis.

In view of the reported actions of PUFA metabolites, questions have been raised over whether it is simply the metabolic products from the breakdown of the fatty acids which contribute to their beneficial cardiovascular effects or whether the fatty acids themselves have a more direct action. In attempts to elucidate the answer, investigations have examined the effects of fatty acids and/or their metabolites in certain pathological conditions. One of the conditions receiving much attention is that of hypertension.

1.3. n-3 Fatty Acids Associated With The Reduction Of Hypertension.

Many clinical trials and dietary intervention studies have focused upon determining whether the administration of n-3 fatty acids and fish oils produce

reductions in blood pressure or *hypertension* (Lockette *et al*, 1982; McMilan, 1989; Bønaa *et al*, 1990; Mano *et al*, 1995; Joly *et al*, 1995). These studies compared dietary supplementation with oils known to contain EPA or DHA or their precursors, such as menhaden or borage oil, with the effects of supplementation with corn, soybean or linseed oil. The studies generally utilised men and women with persistently elevated diastolic blood pressure between 90 and 104 mmHg, which had no particular organic cause and was previously untreated, termed *stable mild essential hypertension*.

A population-based intervention trial by Bønaa *et al* (1990) indicated that systolic and diastolic blood pressure was decreased in the presence of n-3 fatty acids and that EPA was more important than DHA in lowering blood pressure. There were no significant changes in total plasma levels of phospholipid, however, the decrease in blood pressure was proportional to the increased ratio of plasma phospholipid n-3/ n-6 fatty acid concentration.

In other studies, the spontaneously hypertensive rat was used as a model for stable mild essential hypertension (Schoene & Fiore, 1981; Singer *et al*, 1990) to confirm that dietary enrichment with n-3 fatty acids induced a significant decrease in blood pressure (Engler, 1990). The stroke-prone spontaneously hypertensive rats were fed a 5% corn oil diet or 5% menhaden oil diet for 15 weeks. Systolic blood pressure decreased in rats on the fish diet compared to rats on the corn diet (Smith *et al*, 1992). It is clear, therefore, that investigations on both human populations and animal models demonstrate that fish oil n-3 fatty acids are effective in reducing hypertension.

Arterial blood pressure is controlled by factors that affect either the cardiac output, the blood volume, the peripheral resistance or any of these in combination. It follows, therefore, that the mechanism of action of the n-3 fatty acids to reduce blood pressure may be mediated by an action on the blood pressure control mechanisms which are extrinsic to the cardiovascular system or on the heart itself, on the vasculature, or on both the heart and vasculature.

1.4. The Role Of n-3 Fatty Acids On The Myocardium

Whereas most studies have focused on the potential beneficial effects of the n-3 PUFAs, EPA and DHA on plasma lipid levels, on platelet lipid levels and on platelet function, less attention has been given to the possible effects of these fatty acids on the myocardium itself. There are reports which suggest a beneficial effect of the n-3 fatty acids on the regulation of the automaticity of cardiac contraction and the termination of lethal tachyarrhythmias which is a condition in which the heart beats with an irregularly fast rhythm (Kang & Leaf, 1994; 1995) but few reports devoted to the actual mechanism of action of the n-3 fatty acids.

Studies with PUFAs have shown that the effect of EPA and DHA may have an electrophysiological basis on the myocardium (Kang & Leaf, 1996a). It was demonstrated that these PUFAs decreased the contraction rate of spontaneously beating, isolated rat neonatal cardiac muscle cells or *myocytes* (Kang & Leaf 1994) and that EPA reduced the membrane electrical excitability of cardiac myocytes (Kang & Leaf, 1996b).

The electrophysiological mechanism of the anti-arrhythmic effect has been associated with an effect of the PUFAs on the permeability of the sodium ion channel in the cardiac myocytes. This would provide an electrophysiological basis for the anti-arrhythmic effects of the fatty acids because inhibition of sodium entry through sodium channels in the cells decreases the intracellular sodium ion concentration, which returns the cell to its polarised (resting) state, reduces the intracellular calcium ion concentration and as a consequence, reduces contraction rate. Whether this is a specific or non-specific action of the PUFAs remains unclear, although it has been shown that PUFAs mediate a reversible, structure-specific, concentration-dependent suppression of ventricular, voltage-activated sodium currents (Xiao, 1995). Studies using [benzoyl-2,5-H-3] batrachotoxinin A 20 alpha-benzoate ([H-3] BTXB), a radioligand which binds to the sodium channel, revealed that the PUFAs which demonstrate an anti-arrhythmic effect, including EPA and DHA, inhibit the binding of [H-3] BTXB to the channel protein in a dose-dependent fashion (Kang & Leaf, 1996a). From these studies, therefore, it might be concluded that the protective effect of PUFAs is due to a specific action of the PUFAs on the sodium ion channel.

However, it has also been suggested that PUFAs mediate a specific effect on calcium ion channel permeability. Hallaq *et al* (1992) reported that PUFAs blocked ouabain toxicity (0.1 mM) in cardiac myocytes. Ouabain is a cardiac glycoside and as such, inhibits the sodium/potassium pump located in the cell membrane. This results in increased intracellular sodium concentration, which in turn induces a secondary rise in intracellular calcium ion concentration, causing depolarisation. During depolarisation, influx of calcium through calcium ion channels increases, therefore, when the effect of EPA (5 μ M) and DHA (5 μ M) produced a similar result to the calcium channel blocker, nitrendipine (0.03 nM) to prevent this ouabain toxicity, this suggested an effect of the PUFAs to block calcium entry at the calcium channel. Furthermore, it was shown that EPA and DHA inhibited the specific binding of [³H]-nitrendipine to the calcium ion channel in the cardiac myocytes (Hallaq *et al*, 1992). Consistent with this effect, Pepe *et al* (1994) showed that PUFAs blocked the effects of dihydropyridines, compounds which bind selectively to the calcium channel and favour either the opening (as shown by BAY K 8644) or non-opening (as shown by nitrendipine) state of the channel. In this study, cardiac

myocytes were loaded with indo-1, a fluorescent indicator used to measure intracellular concentrations of calcium. The PUFAs caused in a decrease in intracellular calcium concentrations. These results led to suggestions that n-3 fatty acids may acutely bind to a membrane locus associated with the dihydropyridine calcium binding site (Pepe *et al*, 1994) particularly as the effects of non-dihydropyridine calcium blockers (including verapamil and diltiazem) were not affected by PUFAs.

Contrary to this suggestion, however, the n-6 PUFA arachidonic acid has been shown to stimulate a transient increase in the intracellular calcium concentration in rat cardiac myocytes following treatment of the cells with ethylene-glycol-tetra-acetic acid (EGTA). EGTA is a calcium ion chelator often used *in vitro* to bathe cells or tissues, in order to prevent any extracellular calcium from entering the cells or tissues (Damron & Bond, 1993). So the result from this study suggests that far from reducing entry of calcium into the cell, PUFAs may acutely increase intracellular concentrations of calcium.

It would appear, therefore, that whilst each of the reports highlighted suggest a specific mechanism of action of the PUFAs, no clear conclusion can be drawn on what this mechanism is. Contrary to a specific mechanism, other reports suggest that PUFAs exert a non-specific influence on the cardiovascular system. This was demonstrated by Stubbs & Smith (1984) who used high concentrations of PUFAs (10-50 μM or greater) and suggested that they non-specifically reduced the phase transition of membrane lipids and altered local lipid viscosity. This suggestion was expanded by Yeagle (1989) who showed that dietary lipids influenced the lipid composition of the cardiac myocyte membranes, although the mechanism by which this affected the cell function was not clear. It seem appropriate, however, that the presence of PUFAs in the phospholipids of cell membranes may modify the function of membrane-bound proteins, by altering the micro environment within which these proteins act. These proteins include receptors, transport pathways and enzymes and there is a role for each of these groups of proteins in cardiac function. The effect of incorporating n-3 PUFAs into cell membrane phospholipids to see whether this may offer a protective effect against tachyarrhythmias in cardiac myocytes has been investigated previously; McLennan (1993a and *et al* 1985, 1993b) demonstrated that dietary lipid modulation of cardiac myocyte membranes in rats and non-human primates does alter myocardial substrate vulnerability to arrhythmic stimuli. However, this has not necessarily been shown to occur as a direct result of the altered PUFA composition of the cell membranes (Weylandt *et al*, 1996).

It would appear, therefore, that information on the specific and/or non-specific antiarrhythmic effects of the n-3 PUFAs on cardiac function is far from

conclusive. Similar conclusions may be drawn from those studies which have attempted to elucidate the role of n-3 PUFAs on the vasculature.

1.5. The Role Of n-3 Fatty Acids On The Vasculature

An alternative suggestion to the antiarrhythmic effects of n-3 fatty acids in the prevention of cardiovascular disease is that of their effect against atherosclerosis (Lervang *et al*, 1993; McLennan 1993a; McLennan *et al* 1985, 1993b).

Atherosclerosis is a disease of the lining or *intima* of arteries caused by plaques of lipid and connective tissue matrix proteins. The plaque forms the substrate on which thrombosis develops subsequent to rupture. The complexity of the disease has given rise to many treatments available for atherosclerosis, several of which decrease plasma low density lipoproteins (LDL). The function of LDL is to transport cholesterol to peripheral tissues, where it is used in steroid hormone synthesis, bile acid production and manufacture of cell membranes. Most tissues of the body contain LDL receptors and following binding of LDL with its receptor, it is taken into the cell by receptor-mediated endocytosis. Within the cell, the LDL is broken down and the cholesterol contained within the LDL particle is released to serve the cells needs. However, as blood levels of LDL increase or if there are too few LDL receptors owing to environmental and genetic factors, the risk of the LDL particle depositing cholesterol in the smooth muscle cells of arteries increases. therefore, LDL is important in potentiating the formation of the atherosclerotic plaque. The treatments which decrease plasma LDL include

- bile acid-binding resins which sequester bile acids leading to increased expression of LDL receptors on liver cells (hepatocytes)
- fibrates which stimulate lipoprotein lipase, thereby increasing hydrolysis of triacylglycerol to liberate free fatty acids and increase hepatic LDL uptake
- 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, which stimulate the synthesis of LDL receptors in hepatocytes
- nicotinic acid which inhibits hepatic triacylglycerol production and VLDL secretion.

All these effects serve to lower blood LDL levels (Brett, 1989).

Thrombosis is the unwanted formation of a haemostatic plug or thrombus within the blood vessels or heart. Drugs used to treat this pathological condition may affect thrombosis in three distinct ways; by modifying blood coagulation (fibrin formation); by modifying platelet adhesion and activation; by affecting the processes involved in fibrin removal (fibrinolysis) (Collen, 1993).

These parameters have been investigated in attempts to elucidate the antiatherosclerotic mechanism of action of the n-3 PUFAs. Leaf and Weber (1988) reviewed the cardiovascular effects of n-3 fatty acids based on studies performed in the mid-1970s by workers such as Dyerberg (1981) and Bang *et al* (1976). The review summarised the principal antiatherosclerotic effect of the n-3 fatty acids; to reduce the circulating levels of triacylglycerols and LDL. However, examination of the reports to which they refer revealed that the effects of fish oils on LDL and HDL levels were inconsistent (Davis *et al*, 1987; Roach *et al*, 1987). Therefore, it would seem reasonable to suggest that the effect of n-3 PUFAs cannot be simplified in the manner described by Leaf & Weber.

In a subsequent report by Goodnight (1991) the antiatherosclerotic effect of fish oil was attributed to an inhibition of plaque formation; the initial stage of plaque formation involves injury to blood vessel endothelial cells. This alters prostanoid biosynthesis, thereby decreasing production of prostacyclin which is a vasodilator and an inhibitor of platelet aggregation. With dietary supplementation of n-3 PUFAs, there is competition with n-6 PUFAs for incorporation (de Jonge *et al*, 1996) into cell membranes. Cyclooxygenase or lipoxygenase activity on the membrane-associated, n-3 PUFAs would result in the formation of the 3-series prostaglandins and thromboxanes or the 5-series leukotrienes instead of the 2- and 4- series products normally formed from the n-6 PUFA, arachidonic acid. The implication of this is that prostaglandin I₃ is similar in potency as prostaglandin I₂ (prostacyclin) as a vasodilator and platelet aggregation inhibitor, thromboxane A₃ is much less active than thromboxane A₂ as a vasoconstrictor and platelet-aggregating agent and leukotriene B₅ is much less active than leukotriene B₄ as a chemoattractant of monocytes, resulting in a tendency favouring the anti-atherogenic state of the blood vessels. This incorporation and subsequent metabolism of n-3 PUFAs was proposed to explain their beneficial effects (Fischer & Weber, 1984; Dyerberg & Jorgensen 1980; Lorenz *et al*, 1983).

It can, therefore, be inferred from Goodnight's suggestion (1991) that eicosanoid metabolism appears to be important in the antiatheromatous action of the n-3 PUFAs, an idea initiated by Dyerberg (1981). However, it should be noted that generalising this effect may be inappropriate as species differences exist; *in vitro* the n-3 PUFA EPA is only slightly converted into cyclooxygenase products in vascular tissue of the rat or the rabbit (Hornstra *et al*, 1981) or in cultured murine aortic cells (Morita *et al*, 1983). Furthermore, the effect of EPA is unaffected by the cyclooxygenase inhibitor, indomethacin or the lipoxygenase inhibitor, nordihydroguaiaretic acid in perfused rabbit ear arteries (Juan & Sametz, 1986) or the isolated aorta of the rat (Engler, 1990). Therefore, these results demonstrate (a) EPA is only slightly converted into cyclooxygenase products and (b) prostaglandin

synthesis is inhibited, yet the beneficial effect of EPA is observed *in vitro* (Lockette *et al.*, 1982; Engler, 1990; Weiner *et al.* 1986) so cannot be solely attributed to the production of eicosanoids.

An alternative beneficial cardiovascular effect of n-3 PUFAs, common to all species examined, is that they mediate relaxation in isolated blood vessels, including coronary arteries (Shimokawa & Vanhoutte, 1989), perfused rabbit ear artery (Juan & Sametz, 1986), rat thoracic aorta (Lockette *et al.*, 1982; Smith *et al.*, 1992; Engler *et al.*, 1990;1994; Engler, 1992a; 1992b) and the rat and monkey mesenteric vascular bed (Mano *et al.*, 1995). The relaxatory effect has been attributed to a number of mechanisms. One proposed mechanism is that the n-3 fatty acids decrease vascular reactivity to constrictor substances (Juan & Sametz, 1986; Yin, 1991; Mano *et al.*, 1995), leading to a decrease in blood pressure (Lockette *et al.*, 1982; Norris *et al.*, 1986; Knapp & Fitzgerald, 1989; McMilan, 1989; refer to Bønaa *et al.*, 1990; Mano *et al.*, 1995; Joly *et al.*, 1995). However, Chin *et al.* (1993) measured no change in arterial pressure in the brachial artery of normotensive human subjects following dietary supplementation with n-3 fatty acids, despite measuring attenuated constrictor responses to infused noradrenaline (100 ng min⁻¹) or angiotensin II (8, 16 or 32 ng min⁻¹). This was attributed to the presence of a hypotensive effect of fish oils only in subjects with high blood pressure or hypercholesterolaemia.

Another proposed mechanism of n-3 PUFAs is on cell hypertrophy and proliferation. A fatty acid-enriched diet has been shown to decrease the abnormal smooth muscle cell hypertrophy or proliferation observed in response to surgical intervention procedures including vein-grafts (Smith *et al.*, 1989) and balloon-injured blood vessels (Weiner *et al.* 1986). Balloon injury occurs when a catheter incorporating a small balloon is introduced into a blood vessel, especially the coronary artery and is then inflated in order to clear an obstruction or dilate a narrowed region. Furthermore, the deposition of platelets into the site of injury of the endothelium and constriction of the carotid artery caused by balloon injury were reduced by the chronic supplementation of pigs' diet with fish oil (Lam *et al.*, 1995). However, in another investigation, an effect of n-3 fatty acids on cell hypertrophy was considered unlikely following an effective decline in blood pressure which was not accompanied by any decrease in marmoset mesenteric blood vessel weights (Mano *et al.*, 1995).

Following dietary fish oil supplementation, there is substitution of n-6 with n-3 fatty acids in the fatty acyl chain of cell membranes (Smith *et al.*, 1992), including vascular smooth muscle cells (Lockette *et al.*, 1982). Therefore, it follows that other mechanisms of action of n-3 fatty acids on the vasculature might include the modification of membrane-bound protein function, including that of receptors,

transport pathways and enzymes. In support of this, it has previously been shown that n-6 fatty acids (Ordway *et al*, 1989; Kim & Clapham, 1989) and n-3 fatty acids (Bregestovski *et al*, 1989; Smith *et al*, 1992, Asano *et al*, 1997; Gardener *et al*, 1998) have a direct effect on potassium ion channels situated in vascular membranes, activation of which results in membrane stabilisation towards the non-contractile state, by drawing the membrane potential closer to the potassium equilibrium potential (Halliday *et al*, 1995). Also, EPA (214 μM) decreased the phenylephrine (5 μM)-stimulated phospholipase C β activity in rat cardiac myocytes (de Jonge *et al*, 1996). Both these studies suggest that n-3 fatty acids may mediate an action on membrane-bound receptor-signalling pathways.

Yet another proposed mechanism of action of the n-3 fatty acids on the vasculature involves the release of endothelium-derived relaxing factor, now known to be nitric oxide (Furchgott & Zawadzki, 1980), a field of study which has received widespread attention over the past two decades.

1.5.1 The role of n-3 PUFAs as an endothelium-dependent or endothelium-independent mediator

Nitric oxide is formed from nitrogen derived from the terminal guanidino atoms of L-arginine in cells and tissues and the oxygen from molecular O₂. A physiological function of nitric oxide was first discovered in the vasculature when it was shown that the endothelium-derived relaxing factor described by Furchgott & Zawadzki in 1980 could be quantitatively accounted for by the formation of nitric oxide by endothelial cells. Nitric oxide is synthesised in an enzyme-catalysed reaction by nitric oxide synthases which are central to the control of nitric oxide biosynthesis. Nitric oxide synthases are widespread throughout vascular and nervous tissues and there are several isoforms of the enzyme, some of which are *inducible*, *i.e.* are expressed in response to pathological stimuli, whilst others are *constitutive*, *i.e.* are present under physiological conditions. In vascular tissues, constitutive isoforms exist, especially in the endothelium. The activity of these isoforms is controlled following an increase in the intracellular calcium concentration, by the intracellular calcium-calmodulin complex which subsequently stimulates endothelial nitric oxide biosynthesis. Nitric oxide has both *autocrine* (acts on the cells which produced it) and a *paracrine* effects (acts on neighbouring cells), however, it generally appears to function as a paracrine mediator. In vascular tissues, it diffuses readily from sites of synthesis in the endothelium (see figure 1.4) and activates guanylate cyclase in neighbouring smooth muscle cells to produce cyclic GMP. Through actions on protein kinases, ion channels, cyclic nucleotide phosphodiesterases and other proteins, there is a decreased intracellular calcium response to vasoconstrictor stimuli with inhibition of vascular tone and

growth (Furchgott & Vanhoutte, 1989) and a decreased pro-aggregatory response, since nitric oxide prevents the activation of platelets (Radomski *et al*, 1991). Therefore, the therapeutic implications for nitric oxide are far reaching in angina pectoris, hypertension and cardiac failure. It has been suggested that n-3 PUFAs increase the release of nitric oxide from the endothelium (Shimokawa & Vanhoutte, 1989; Shimokawa *et al*, 1987; Schini *et al*, 1993) by enhancing production of nitric oxide synthase (Joly *et al*, 1995).

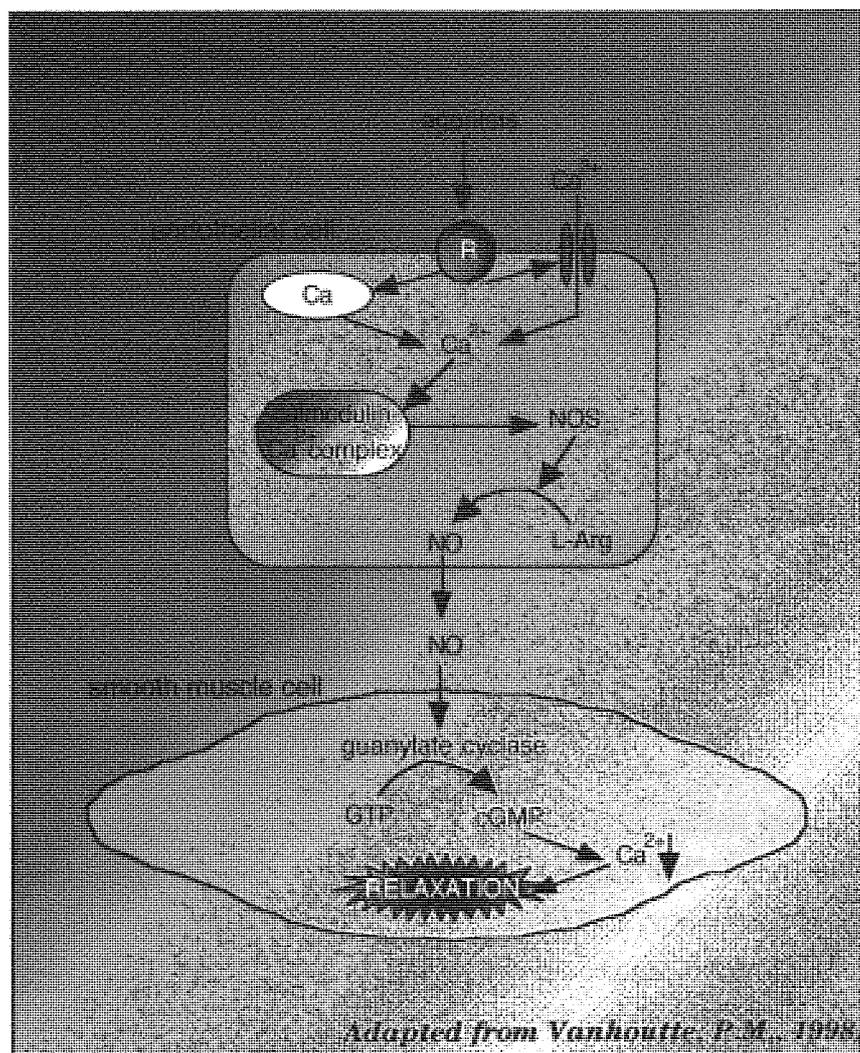


Figure 1.4 Diagrammatic representation of the effect of nitric oxide (NO) on vascular smooth muscle cell relaxation. Ca²⁺, calcium ions; NO, nitric oxide; NOS, nitric oxide synthase; cGMP, cyclic GMP; R, membrane receptor; L-Arg, L-arginine.

A report following investigation into the effect of fish oil fatty acids on nitric oxide production, demonstrated a potentiation of the hyporeactivity induced by *in vivo* endothelial injury in the rat carotid artery by chronic treatment with fish-oil (Joly *et al*, 1995). In this report, balloon injury decreased the contractility of endothelium-devoid carotid arteries to phenylephrine, a synthetic compound related to adrenaline, used as a vasoconstrictor, in control and fish oil-fed rats. Methylene blue, a soluble guanylate cyclase inhibitor, but not N-G-nitro-L-arginine, a nitric oxide synthase inhibitor, nor superoxide dismutase, which protects nitric oxide from degradation, significantly decreased contractions from control or fish oil-fed rats, suggesting that a nitric oxide-cGMP-associated mechanism of action may be involved. N-G-nitro-L-arginine or methylene blue in balloon-injured carotid arteries partly restored contractions to phenylephrine in arteries from control or fish oil-fed rats, whereas superoxide dismutase further depressed the contractility to phenylephrine in balloon-injured arteries from control diet-fed rats but not fish oil-fed rats. 3-Morpholino-sydnonimine (SIN-1, a nitric oxide donor) evoked similar concentration-dependent relaxations in control and balloon-injured carotid arteries from both control and fish oil-fed rats. Superoxide dismutase potentiated significantly the production of cGMP produced by balloon injury in the carotid arteries of control but not fish oil-fed rats. Therefore, this report demonstrated that *in vivo* balloon injury caused the production of nitric oxide in the injured blood vessel wall, leading to decreased contractility to phenylephrine and accumulation of cGMP. It could be concluded that chronic fish oil feeding potentiated the L-arginine nitric oxide pathway in the injured vessel, leading to enhanced hyporeactivity to phenylephrine.

However, not all investigations on n-3 PUFAs which have demonstrated beneficial cardiovascular effects have been associated with increased nitric oxide production by the endothelium. For instance, it has previously been shown that cultured bovine endothelial cells release larger amounts of non-prostanoid relaxing substances after they have been treated chronically with EPA and that the increase is not accompanied by an increase in cyclic GMP production, suggesting a factor other than nitric oxide is involved (Boulanger, *et al* , 1989). Numerous investigations on n-3 PUFAs have also demonstrated that the beneficial cardiovascular effects of PUFAs are not associated with an endothelium-dependent action. Indeed, Mano *et al* (1995) concluded from their studies on the n-3 fatty acids, that the mechanism of action was unrelated to endothelial cell-mediated relaxation and other reports have demonstrated that the beneficial effect of the n-3 fatty acids is apparent in tissues which are devoid of endothelium (Engler, 1990; Bretherton *et al*, 1995; 1996a; 1996b; Hallaq *et al*, 1992; Pepe *et al*, 1994; Bregestovski *et al*, 1989; Smith *et al*, 1992, Asano *et al*, 1997; Gardener *et al*, 1998).

However, the role of the endothelium in the effect of n-3 fatty acids appears to be a controversial issue.

P.M. Vanhoutte, currently of the Institute de Recherche Internationales Servier, France, has produced many reports linking n-3 PUFAs with an endothelium-dependent mechanism of action. The endothelium is a major regulator of vascular tone because it releases vasoactive substances including nitric oxide, endothelium-derived hyperpolarising factor, prostacyclin, endothelin and endothelium-derived contracting factors (Vanhoutte, 1996). Vanhoutte proposed that n-3 PUFAs have a similar mechanism of action as several endothelium-dependent vasodilators which induce endothelium-dependent hyperpolarisation of vascular smooth muscle cells (Nagao & Vanhoutte, 1993) and also, that in normal, atherosclerotic and hypercholesterolaemic arteries, it is endothelium-dependent relaxations and reductions in endothelium-dependent contractions which occur (Shimokawa & Vanhoutte, 1988; Harris-Hooker *et al*, 1983) in response to n-3 fatty acids. In support of Vanhoutte's research work, other investigations have been made. These have often involved collaboration with Vanhoutte and examined supplementation of pigs' diets with fish oil; one recent study used porcine coronary artery vascular smooth muscle cells after the pigs were fed on 4.35 % (vol/wt) NC 020 fish oil for 6-7 weeks and examined the effect of the fish oil on endothelium-dependent hyperpolarisation (Nagao *et al*, 1995). The study measured the membrane potential of the smooth muscle cells in vessels which were slit longitudinally, pinned to the bottom of an organ bath chamber with endothelium uppermost. The results showed that serotonin, a compound normally present in blood platelets and serum, which acts as a neurotransmitter but also induces endothelium-dependent relaxation of smooth muscle cells, induced transient, concentration-dependent hyperpolarisations of coronary arterial smooth muscle cells with intact endothelium. The conclusion to be drawn, therefore, was that the fish oil significantly potentiated the serotonin-induced endothelium-dependent hyperpolarisation. However, this study only examined the effect of fish oil on vessels with endothelium present. No account was taken of the effect of fish oil on endothelium-denuded vessels. Serotonin, through an action on 5-HT₂ receptors, causes smooth muscle contraction, therefore the effect of fish oil on this response would have made an interesting observation. Another study which also failed to examine the effect of removing the endothelium from blood vessels was that of KIM *et al*, (1992), in which they fed male Yorkshire pigs for 4 weeks with chow supplemented with EPA (3.5 g/day) or DHA (1.5 g/day). Isometric tension recordings of isolated porcine basilar arteries were made. Bradykinin, a peptide released in the blood which acts on the endothelium to cause dilation of blood vessels, induced relaxations in the arteries, which was obviously endothelium-

dependent. The relaxation was augmented by EPA or DHA treatment, so the conclusion made by KIM *et al* from their study was that EPA or DHA augmented endothelium-dependent relaxation.

Some studies which also suggest that PUFAs mediate an endothelium-dependent action used isolated blood vessels removed from alternative species of animals, prior to the acute treatment of fish oil fatty acids. However, similar to the previous reports outlined, the investigations do not compare their results with those obtained in the absence of an endothelium. One such study was that of Chataigneau *et al*, (1998) in which, guinea-pig isolated internal carotid arteries were exposed to saturated or unsaturated fatty acids and endothelium-dependent hyperpolarisation was determined. Acetylcholine ($1 \mu\text{M}$), a compound which occurs throughout the nervous system, in which it functions as a neurotransmitter, but which also exerts an endothelium-dependent relaxation in blood vessel smooth muscle cells, evoked a hyperpolarisation in the presence of a nitric oxide synthase inhibitor (N-omega-nitro-L-arginine; $100 \mu\text{M}$) and a cyclooxygenase inhibitor (indomethacin; $5 \mu\text{M}$). Some of the fatty acids produced a hyperpolarisation of the resting membrane potential and partially inhibited the acetylcholine-induced hyperpolarisation.

Another investigation used to support the endothelium-dependent nature of the effect of n-3 PUFAs, reported that EPA concentration-dependently relaxed rabbit and cat aortic rings with endothelium but not without (Yamagisawa & Lefler, 1987). Ibuprofen, a cyclooxygenase inhibitor, did not but methylene blue, a guanylate cyclase inhibitor, did totally abolish the EPA-induced relaxation indicating endothelium-dependent smooth muscle relaxatory mechanism. However, this same report also demonstrated that isolated perfused cat coronary arteries, exerted a dilator effect to EPA ($3\text{-}300 \mu\text{M}$) which was endothelium-independent and not affected by ibuprofen. Therefore it was concluded that EPA exerted an endothelium-dependent and an independent relaxation, depending on the vessel used in the investigation. Another report, which at first glance appears to support a role for an endothelium-dependent mechanism of action of n-3 PUFAs, is that reported by Schini, *et al* (1993). They reported that EPA potentiated the production of nitric oxide evoked by interleukin-1-beta, a cytokine involved in the inflammatory response, but the nitric oxide production was not in endothelial cells, as described earlier, but in cultured vascular smooth muscle cells. They demonstrated that incubation of cells with interleukin-1-beta evoked a time and concentration-dependent release of nitrite, an oxidation product of nitric oxide, which was significantly potentiated by simultaneous exposure to EPA in a concentration-dependent manner. Nitrite production was significantly inhibited to a similar extent in the presence or absence of EPA by nitro-L-arginine, an inhibitor of nitric

oxide synthase, transforming growth factor beta 1, which regulates cell proliferation and differentiation, platelet-derived growth factor and thrombin, which is an enzyme involved in the blood clotting mechanism. Smooth muscle cells exposed to interleukin-1 beta and EPA prior to addition of the smooth muscle cells to indomethacin-treated platelets, inhibited the aggregation caused by thrombin, but not that of oxyhaemoglobin or methylene blue. Untreated cells or cells exposed to EPA alone had no such effects. Therefore, these observations suggest that EPA potentiates the production of nitric oxide evoked by interleukin-1 beta, but it is endothelium-independent as the nitric oxide is produced in vascular smooth muscle.

So it would appear, therefore, that PUFAs can be shown to exert endothelium-dependent and endothelium-independent actions, depending upon the compounds used in the experimental design, the presence of an endothelium and also, the tissues selected for use. Some studies, however, have attempted to determine whether the effect of PUFAs is by an endothelium-independent mechanism, by comparing the effect of PUFAs in vessels with and without endothelium, often using compounds which act directly on smooth muscle tissue.

The vasorelaxant action of n-3 PUFAs shown by Juan *et al* (1987), Engler (1989; 1990; 1992a, b), Engler *et al.*, (1990; 1994) and Asano *et al* (1997) have been supported by other reports which demonstrate an endothelium-independent link between n-3 PUFAs and their beneficial effect. Sometimes, the PUFAs are associated with an action on membrane-bound receptor-signalling pathways; it was been shown that pretreatment for 3 days with 60 μM DHA impairs α_1 -adrenoceptor-mediated contractile responses and inositol phosphate formation in rat cardiomyocytes (Reithman *et al*, 1996). The role of α_1 -adrenoceptor-mediated contractile responses and inositol phosphate formation are explained in detail in 1.8. In the report by Reithman *et al* (1996), pretreatment of cells with DHA (60 μM) for up to 72 h did not affect contraction amplitude, but prevented the increased contraction velocity of cells caused by noradrenaline (100 μM), a hormone which causes vasoconstriction and timolol (10 μM), a beta blocker, isoprenaline (10 μM), a synthetic derivative of adrenaline- or ouabain (10 μM). DHA (60 μM) pretreatment also decreased the noradrenaline (100 μM) - and timolol (10 μM)-induced inositol phosphate formation to 55 ± 17 % the value in the absence of DHA pretreatment. However, pretreatment of the cells for only 6 h with DHA reduced the decrease in inositol phosphate formation to 17 ± 10 % and inconsistently with the effect at 72 h, arachidonic acid (60 μM) no longer significantly decreased inositol phosphate formation. The researchers studied whether a decrease in the number or affinity of α_1 -adrenoceptors may be one of the mechanisms of the DHA-induced decrease in inositol phosphate formation and found that pretreatment with DHA (0.6, 6 or 60 μM) for 3 days did not alter [^3H]prazosin (an α_1 -adrenoceptor agonist) binding to,

nor maximal binding capacity of the cells. They also examined whether the attenuation of the isoprenaline-stimulated increase in contraction velocity was due to a decreased adenylate cyclase activity, but pretreatment with DHA (60 μM) for 3 days increased adenylate cyclase activity. Therefore, this report showed that DHA impaired α_1 -adrenoceptor-mediated contractile responses and inositol phosphate formation in rat cardiomyocytes, an effect which was endothelium-independent.

Another report which demonstrated the endothelium-independent mechanism of action of n-3 PUFAs was the investigation into the effect of EPA on the intracellular mechanisms associated with changes of intracellular calcium concentration and increases in membrane potential, which in turn modulate the contractile status of the cells (Asano *et al*, 1998). For between 6 h and 7 days, 30 μM EPA was exposed to rat vascular smooth muscle (A7r5) cells. In this study, fura-2, a fluorescent indicator, was used to take measurements of intracellular calcium ion concentrations, and patch-clamping measurements of resting membrane potential were also taken. Also, the fatty acid composition of cellular phospholipids was determined. The EPA and docosapentaenoic acid (DPA) content of the phospholipid fraction of the cells increased in a time-dependent manner. Arachidonic acid decreased and the ratio of EPA to AA significantly increased demonstrating that the n-3/n-6 fatty acid ratio increased, consistent with other studies (Swanson *et al*, 1989). Resting intracellular calcium ion concentrations decreased from 170 ± 46 nM in controls to 123 ± 29 nM after EPA (30 μM) exposure for 7 days. EPA also decreased the peak and sustained rise of intracellular calcium ion concentration induced by vasopressin (100 nM) and endothelin-1 (100 nM), both of which cause vasoconstriction or platelet-derived growth factor (PDGF; 5 ng ml⁻¹), which causes proliferation of vascular endothelial and smooth muscle cells. EPA decreased the transient rise induced by these agonists in the absence of extracellular calcium. Resting membrane potential was significantly higher in EPA-treated cells. 7-day treatment with EPA inhibited PDGF-induced cell migration. Therefore, from this multifactorial investigation, it was concluded that the effect of the n-3 fatty acid EPA, was not dependent upon the endothelium. Instead, it was suggested that cellular incorporation of EPA attenuates intracellular mechanisms associated with changes of intracellular calcium ion concentrations and that EPA also increases membrane potential, both of which are beneficial in treating cardiovascular disease.

Another study in support of an endothelium-independent mechanism of action of n-3 fatty acids was that reported by Hirafuji *et al* (1998). In this investigation, the effect of DHA was examined on intracellular calcium dynamics in vascular smooth muscle cells taken from normotensive (Wistar-Kyoto) and stroke-prone, spontaneously hypertensive rats. DHA (30 μM) exposed to the cells for 2

days in the culture medium, suppressed peak intracellular calcium concentrations induced by serotonin (5-HT), angiotensin II, a vasoconstrictor, and potassium chloride (KCl), another vasoconstrictor, in the cells from the normotensive, but not the stroke-prone, spontaneously hypertensive rats. DHA did not spur the 5-HT induced increase in inositol phosphate levels. Therefore this investigation demonstrated that DHA suppressed receptor-mediated calcium ion influx in vascular smooth muscle cells and furthermore, that the effect of n-3 PUFAs was independent of an action associated with the vascular endothelium.

It can be concluded, therefore, from the numerous reports which have attempted to elucidate the mechanism of action of the n-3 fatty acids on the vasculature, that the precise site of action has not been clearly defined. However, the general tendency of the reports is to suggest that the n-3 fatty acids mediate their beneficial cardiovascular-related effects either directly on the vascular smooth muscle cells, or indirectly on the vascular smooth muscle, by an endothelium-dependent action, therefore it is important to establish the precise mechanism before their routine therapeutic use could become commonplace.

1.6. The Beneficial Dose Of n-3 Fatty Acids

The reported beneficial effects of fish oil fatty acids have led to questions arising over the optimum dose of n-3 fatty acids which should be included in the diet. It would seem logical to select a dose, dependent upon the effect sought and the duration of the desired effect (Leaf & Weber, 1988). Epidemiological studies of Greenland Eskimos reported that this population ingest enough marine food to obtain 5-10 g of n-3 fatty acids daily, presumably for a lifetime. However, recommended daily intakes of essential fatty acids are difficult to propose since optimal requirements are not precisely known. In a recent review on the metabolism and availability of essential fatty acids in animals and humans (Bezard *et al*, 1994) it was proposed by (Kinsella, 1991) that no more than 1-2% of calories from linoleic acid, the major essential fatty acid was required to relieve symptoms of fatty acid deficiency. Bezard *et al* (1994) proposed that human consumption of the essential fatty acids should exceed this amount, possibly to an intake of 3-6% of calories. This effectively amounts to a level of 3-6 g daily, although values of 7-10g were proposed by Lemarchal *et al*. (1992). Bezard *et al*. (1994) proposed that for linolenic acid which is the EPA and DHA precursor, 0.5-1% of calories should be consumed, whilst for EPA or DHA themselves, 0.4% was reasonable (Bezard *et al*, 1994).

In experimental studies associated with tissue and/or cellular preparations, it is appropriate to select for use, a physiological concentration of PUFA to be used. However, *in vivo* it is difficult to determine the local concentration of released fatty

acids at a precise moment in time in order that the physiological concentration is identified. In a recent review by Sumida *et al* (1993), the concentrations of PUFAs used in studies which examined the modulatory effects of fatty acids on the enzymes and proteins involved in membrane signal transduction, ranged from as low as 1.4 μM to 400 μM . However, it should be noted that Knapp (1988) pointed out that the amount of n-3 PUFAs used in animal and human studies tend to be unrealistically high. Nevertheless, it has been documented that concentrations of 50-100 μM of arachidonic acid were obtained locally after pancreatic islet cell stimulation and in activated platelets (Sumida *et al.*, (1993). An alternative method of determining the concentration of PUFA to be used was documented by Asano *et al.*, (1998), who used normal plasma concentrations of the PUFAs to select the experimental dose. In this report, it was highlighted in the discussion that the normal plasma concentration of EPA is 120 μM , compared with 382 μM in plasma taken from persons who have consumed a meal rich in n-3 PUFAs, such as mackerel meat and a concentration greater than 500 μM was identified following treatment of persons with a highly purified EPA-ethyl ester at an oral dose of 1800 mg daily.

The concentration of PUFAs in essential fatty acid supplements was also considered relevant in attempting to identify the optimum concentration to be administered. These supplements, including MaxEPA capsules, generally contain approximately 0.3 g EPA and DHA in 1.0 ml capsule form, however, it is possible to obtain capsules containing up to 50% n-3 PUFAs. This compares with cod-liver oil which is about 20% n-3 fatty acids, but due to its vitamin A and D content, the quantities which can be safely ingested are limited.

Perhaps another question surrounds the type of PUFA to be added into the diet as supplements. Since DHA is a major component of the grey matter of the cerebral cortex and of photoreceptor membranes of the retina and since deprivation of linolenic acid has been associated with impaired learning and visual activity (Neuringer *et al*, 1986), it was estimated that the need for n-3 PUFAs is increased in the early developmental stage of life, which amounts to 0.7-1.2% of calories (Lemarchal *et al*, 1992). DHA undergoes some retroconversion to EPA within 2-4 h after ingestion of pure DHA (Leaf & Weber, 1988) and a series of desaturation, elongation and β -oxidation steps are able to convert EPA to DHA (refer to figure 1.1). However, as EPA has been reported as having a greater beneficial biological activity on the cardiovascular system than DHA at the present time, then it suggested that any therapeutic intervention may be best served by the addition of EPA to any treatment programme.

Another consideration, before supplementation with PUFAs is readily advocated, is the likelihood of lipid peroxidation and the associated toxic effects which likely accompanies supplementation of PUFAs in the body. One of the

characteristic reactions of lipids which are exposed to oxygen is the formation of peroxides. In biological tissues, uncontrolled lipid peroxidation causes membrane destruction and is increasingly regarded as an important event in the control or development of diseases due to the formation of free radicals which can damage proteins, other lipids and vitamins. Under experimental conditions, it has been shown that PUFAs are easily oxidised in culture medium and in some cases the resultant hydroperoxide levels increase to cytotoxic levels in the medium and the cells (Shiina *et al*, 1993). To assist in overcoming this potential problem, however, it might be considered appropriate to use triacylglycerol forms of EPA which can be used to avoid the experimental problems experienced. Furthermore, since fish has been an important and common food for humans for millennia, perhaps dietary supplementation with an n-3 PUFA-containing fish meal could be advocated. It should be noted, however, that in doing this, concerns have been raised for the potential of mercury and chlorinated hydrocarbon accumulation in fish caught in coastal waters or lakes, although it is known that these toxins can be readily removed in industrial processing.

In elucidating the mechanism of action of the n-3 PUFAs, prior to advocating dietary supplementation, it seems appropriate to identify where the likely mechanisms might be associated. In the cardiovascular system, there are intracellular mediators involved in the modulation of its susceptibility to extrinsic stimuli. Some of the intracellular mediators are described in the following sections.

1.7. Calcium And Vascular Smooth Muscle

Calcium plays a critical role in intracellular signalling and is the primary regulator of the smooth muscle contraction-relaxation cycle (Morgan, 1987). The free intracellular calcium ion concentration ($[Ca^{2+}]_i$) is nominally in the region of 0.1 μM and this must rise to around 10 μM in order for contraction of the vascular muscle to occur (Janis & Triggle, 1983). The extra cellular concentration of calcium is around 10,000 times greater, in the region of 10 mM and even in 1883, Ringer demonstrated that calcium was an essential component in fluid bathing an isolated heart preparation in order that functional viability be maintained. Subsequently, Godfraind and Kaba (1969) investigated the effects of calcium removal from the bathing medium on responses of the rat aorta to KCl or adrenaline and observed that while responses to KCl were totally dependent on the presence of extra cellular calcium, incubation in calcium-free Krebs solution reduced but failed to abolish completely the response to adrenaline. Godfraind & Kaba concluded from these results that there were two sources of activator calcium in the rat aorta. It is now well known that it is the intracellular calcium ion concentration ($[Ca^{2+}]_i$) which is important in the cellular response and this is regulated by the entry and exit of

calcium across either the plasma cell membrane (Benham & Tsien, 1987) and/or the intracellular sarcoplasmic reticulum membrane (Berridge & Irvine, 1989). Calcium entry from the extra cellular solution occurs through voltage-dependent or receptor-operated channels (Van Breemen, 1989) and is under the control of the membrane potential and agonists such as hormones, autocooids and neurotransmitters. Other mechanisms, such as sodium/calcium exchange also operate. Calcium entry into the cytosol from the sarcoplasmic reticulum is under the control of second messengers (Van Breeman, 1989 and review by Gurney & Clapp, 1994). Calcium is removed from the cytosol by sequestration into the sarcoplasmic reticulum or by the action of the calcium activated ATP-ase associated with the plasma membrane (Berridge & Irvine, 1989).

It is also well recognised that a biphasic form of the contractile response of the rat aorta is observed following selective agonist stimulation. This response consists of an initial phasic contraction followed by a sustained, tonic contraction (Cauvin & Malik, 1984; Scarborough & Carrier, 1984). In 1972, Godfraind & Kaba extended their observations of 1969 and reported that the biphasic form of the contractile response of the rat aorta to adrenaline was the consequence of a rapid rise in calcium due to calcium release from intracellular stores, while the slow, sustained contraction was dependent upon calcium entry from the extra cellular solution. Since then the contribution of both processes to the tonic contraction has become well recognised (Scarborough & Carrier, 1984).

1.7.1. Stimulation of calcium influx

Plasmalemmal calcium permeability is regulated by the voltage-dependent channels and by chemical gating of receptor-operated channels (Bolton, 1979). Net influx is also affected by the action of the calcium extrusion pump (Eggermont *et al*, 1988). At least six voltage-dependent channels are now recognised and identified according to their activation voltages, conductance, inactivation rate and agents which block them. The channels are identified as the T (transient or *fast* channel), which opens on depolarisation of the cell membrane to -40 mV, the L (long lasting or *slow* channel), which opens on depolarisation of the cell membrane to -20 mV, the N (neuronal) channel (Nowycky *et al*, 1985), the P and the Q channels and the R-type channels. The T and L channels are found in cardiac and vascular smooth muscle. These tissues are devoid of N channels which are found only in nerves. The P and Q channels seem to be restricted to central nervous system neurones. The R channels include the remaining voltage-operated calcium channels. Calcium influx is brought about by depolarisation of the cell membrane. The membrane potential is determined by ionic concentration gradients and permeabilities according to the Goldman equation and is regulated close to the potassium equilibrium potential by

the opening or closing of potassium channels (Bolton, 1979). It is also affected by electrogenic ion pumps and exchange carriers (Bolton, 1979). Electrical depolarisation activates voltage gated channels, thereby allowing calcium influx.

Receptor-operated channels were first postulated in smooth muscle by Bolton (1979), van Breeman (1989) and Somlyo & Somlyo (1970). Although the indirect evidence for receptor-operated channels is strong, ligand-gated calcium currents have so far eluded identification by numerous investigators. However, Benham & Tsien (1987) described a receptor-operated calcium current which was evoked in arterial smooth muscle cells by ATP without involvement of a readily diffusible messenger, thereby indicating that ROCs do exist in vascular smooth muscle and are likely coupled to excitatory receptors either directly or *via* G-proteins.

1.7.2. Intracellular calcium storage and release

Calcium uptake by the sarcoplasmic reticulum is accomplished by a calcium-magnesium ATPase that undergoes calcium-dependent phosphorylation and is regulated through cyclic AMP-mediated phosphorylation (Sumida *et al*, 1984). cyclic GMP also stimulates the sarcoplasmic reticulum calcium pump in vascular smooth muscle (refer to Van Breeman & Saida, 1989 for review).

Calcium is released from intracellular storage sites in response to cell stimulation by a number of physiological agonists, including noradrenaline, angiotensin, histamine, vasopressin and prostaglandins (refer to Van Breeman & Saida, 1989). In vascular smooth muscle, the second messenger inositol 1,4,5-trisphosphate (IP₃) plays a major role in this process which, in the aorta is during the initial phase of contraction (see section 1.8). To release intracellularly stored calcium, IP₃ or calcium (also caffeine, ryanodine and ATP) must bind to receptors which are linked to calcium channels on the sarcoplasmic reticulum. The sarcoplasmic reticulum stores from which calcium is released contain pumps to sequester calcium, binding proteins (such as calsequestrin and calreticulin) to store calcium and specific receptor-coupled channels to release calcium back to the cytosol (Berridge, 1993a).

In the review by Van Breeman & Saida (1989) it was reported that a portion of the sarcoplasmic reticulum is located close to the inner surface of the plasmalemma, referred to as *superficial* sarcoplasmic reticulum. Sarcoplasmic reticulum lying in the interior of the smooth muscle cells was referred to as *deep* sarcoplasmic reticulum. They described this two-compartment model to explain the differences in the release of stored calcium in response to various stimuli. More recently, Berridge (1993b) reported that there are indeed different storage compartments for calcium and these can be identified on the basis of the receptor-

coupled channel they possess, *i.e.* *myo*-inositol-1,4,5-trisphosphate or ryanodine receptor channels.

In 1992, Engler examined whether the reduction of noradrenaline-induced contractions in rat aortic tissues by DHA or EPA was related to the inhibition of intracellular calcium mobilisation (Engler, 1992b). Engler reported that DHA or EPA was able to reduce the noradrenaline-induced contractile response in calcium-free, EGTA containing buffer, which led to the suggestion of a role of n-3 fatty acids in intracellular calcium regulation. It has also been reported that fatty acids are able to regulate the activities of proteins located within the plasma and intracellular cell membranes and that elevated levels of DHA in sarcoplasmic reticulum phospholipids are associated with a lower relative activity of the calcium-magnesium ATPase and reduced maximal uptake of calcium in cardiac sarcoplasmic reticulum (Swanson *et al.*, 1989) which may serve therefore, to increase $[Ca^{2+}]_i$. In a recent review on the role of fatty acids as modulators and messengers (Sumida *et al.*, 1993), a fatty acid-induced increase in $[Ca^{2+}]_i$ has also been proposed and attributed to *myo*-inositol-1,4,5-trisphosphate-independent calcium release from intracellular stores.

1.8. Phosphatidyl Inositol Hydrolysis And Vascular Smooth Muscle

Evidence for a link between the effects of hormones and the metabolism of phosphoinositides was first provided by Hokin and Hokin in 1953 in which they suggested that the prime target of phospholipase C was phosphatidylinositol (PtdIns). More recently, a wide variety of receptor-mediated events have been shown to involve the stimulation of membrane bound phospholipase C and the subsequent hydrolysis of phosphoinositides (Berridge 1993a). However, it is now generally considered that it is the polyphosphoinositide, phosphatidylinositol-4,5-bisphosphate (PtdIns[4,5]P₂) which is hydrolysed following receptor stimulation, resulting in the liberation of the two second messengers *myo*-inositol-1,4,5-trisphosphate (Ins[1,4,5]P₃ or IP₃) and 1,2-diacylglycerol (Berridge *et al.*, 1983).

The cycle of events known as the PtdIns cycle is outlined diagrammatically in figure 1.5. A basal rate of PtdIns[4,5]P₂ hydrolysis occurs in the cell under resting conditions. Receptor-stimulated phospholipase C-mediated hydrolysis of PtdIns[4,5]P₂ results in the formation of diacylglycerol, which exerts its effects by activating protein kinase C and Ins[1,4,5]P₃ which acts by mobilising calcium from intracellular stores (Berridge & Irvine, 1984). Since it was established that Ins[1,4,5]P₃ was the second messenger involved in calcium mobilisation, a number of different inositol phosphates have been discovered in cells (Downes, 1988). A more detailed diagram of some of the metabolic pathways linking the enormous

number of inositol phosphates found in eukaryotic cells is shown in the review by Berridge, 1993.

The nomenclature adopted for the family of inositol phosphates in this thesis is consistent with that reported in Berridge (1993b), based on the convention published by the International Union of Pure and Applied Chemists (IUPAC). *myo*-inositol is generally abbreviated to Ins.

Ins[1,4,5]P₃ binds to a receptor on the membrane of the endoplasmic reticulum, activating a calcium channel in the endoplasmic reticulum, thereby releasing a flood of calcium into the cell and raising the free concentration 10- to 100- fold (see Fink & Kaczmarek, 1988; Berridge, 1993a).

Ins[1,4,5]P₃ is dephosphorylated to Ins[1,4]P₂, Ins[4]P and eventually, free inositol. Diacylglycerol is phosphorylated to form diacylglycerol 3-phosphate (phosphatidic acid) which interacts with cytidine triphosphate to form cytidine diphosphate diacylglycerol which recombines with inositol to replenish the pool of PtdIns, and thereby completing the cyclic process.

In 1971, an interesting breakthrough was made by Allison & Stewart when they discovered that lithium ions produced a reduction in the concentration of *myo*-inositol in rat cerebral cortex. This was associated with an increase in the Ins[1]P concentration due to inhibition by lithium of *myo*-inositol-1-phosphatase (Hallcher & Sherman, 1980). Similar conclusions were drawn from the work of Berridge *et al* (1982) who examined the effect of lithium in rat brain slices, rat parotid fragments and insect salivary glands. In a review article published by Berridge in 1993, lithium is also reported to inhibit inositolpolyphosphate-1-phosphatase, which therefore also serves to reduce the dephosphorylation of Ins[1,4]P₂. Therefore, lithium reduces the supply of inositol, the key substrate for the phosphoinositide cascade and consequently rendering it useful in pharmacological studies of agonist-receptor effects linked to PtdIns hydrolysis by allowing amplification of the pharmacological response.

Since the original observation of the activation of protein kinase C by arachidonic and other unsaturated fatty acids, an effect which was not inhibited by cyclooxygenase or lipoxygenase inhibitors, many studies have shown the importance of unsaturated fatty acids in the mechanism of action of protein kinase C. In a recent review of the role of fatty acids in signal transduction, it was reported that fatty acids regulate cell enzyme and protein activities positively or negatively. This would have a bearing on Ins[1,4,5]P₃ and subsequently, the contractile state of the cells if, as the review suggests, levels of phospholipases, including that of phospholipase C were increased by fatty acids (Sumida *et al*, 1993).

1.8.1. α -adrenoceptors and phosphoinositide hydrolysis.

External signals arriving at a cell engage surface receptors to initiate signalling pathways which transmit information from one component to another until the final effector system is activated. The formation of $\text{Ins}[1,4,5]\text{P}_3$ as the focal point for one such pathway was described in the previous section and the surface receptor involved is the α -adrenoceptor.

The existence of two kinds of adrenoceptor, α and β , (Alquist, 1948), made on the basis of the rank orders of potency of a series of structurally related catecholamines in organ bath experiments, was later extended to α_1 - and α_2 -adrenoceptors (Langer, 1974) and β_1 - and β_2 -adrenoceptors (Lands *et al*, 1967). Aided by binding and functional experiments, evidence has since been provided for a number of subtypes of α_1 -, α_2 and β -adrenoceptors

α_1 -Adrenoceptors produce changes in cellular activity by increasing $[\text{Ca}^{2+}]_i$. The receptor couples to the pertussis toxin-insensitive, guanine nucleotide-binding protein (G protein), G_q (Smrcka *et al*, 1991). G proteins in general contain three different subunits, G_α , G_β and G_γ , only the first of which binds and hydrolyses GTP (Clapham & Neer, 1993). The G_q protein has guanosine 5'-diphosphate (GDP) attached to the α subunit. The combination of the receptor and G_q protein causes the release of GDP, thereby allowing $G_{q\alpha}$ to combine with GTP, separate from the G_β and G_γ subunits and stimulate phospholipase C to hydrolyse PIP_2 . This gives both IP_3 and diacylglycerol (Berridge, 1993a), as described.

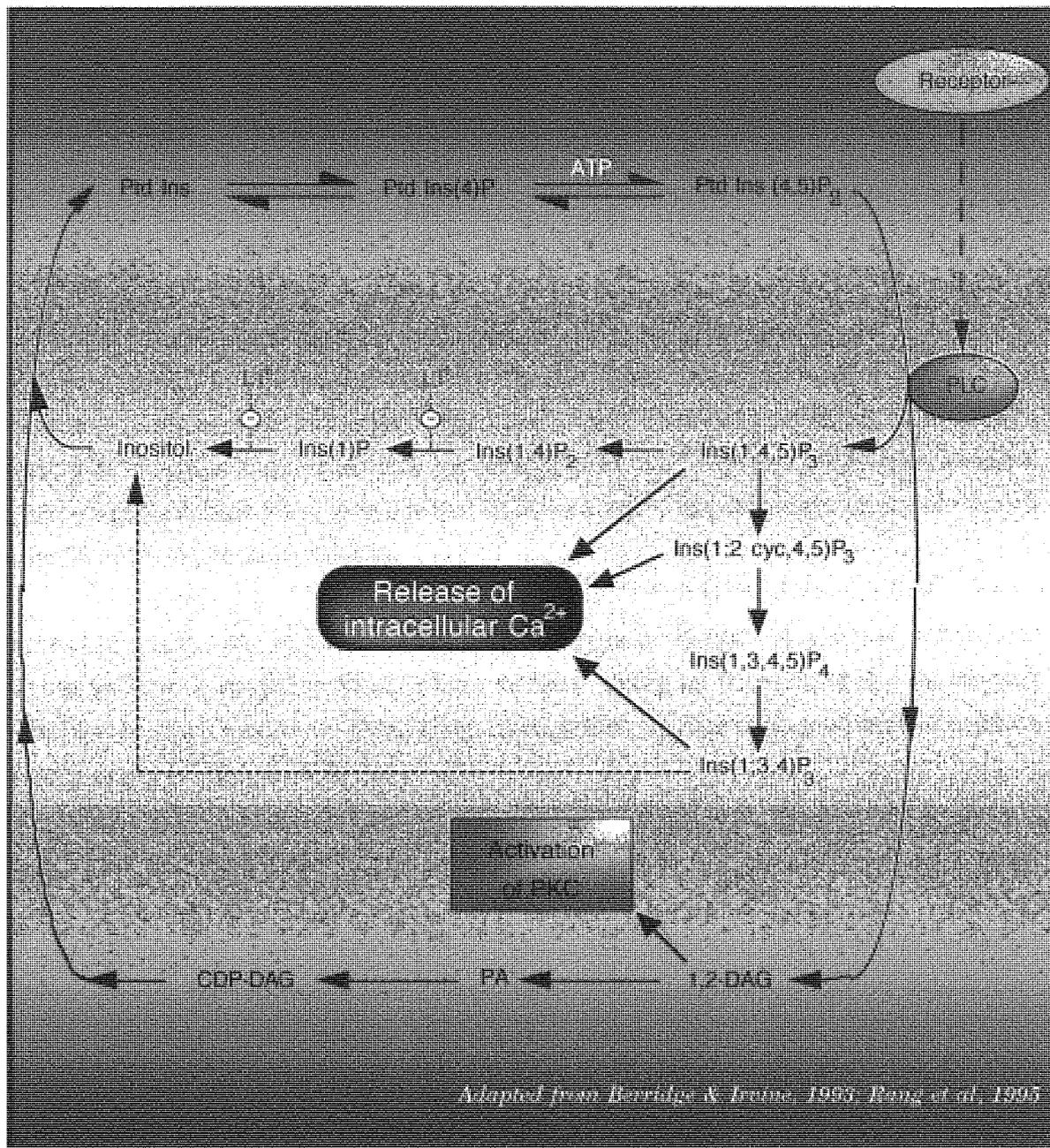


Figure 1.5. The phosphatidylinositol cycle.

Phosphatidylinositol (PtdIns) is phosphorylated to produce phosphatidyl-inositol phosphate (PtdIns(4)P) and subsequently, phosphatidyl inositol bisphosphate (PtdIns(4,5)P₂). The agonist acts at the receptor to stimulate the hydrolysis of PtdIns(4,5)P₂ in the membrane by phospholipase C (PLC) resulting in the production of the two intracellular messengers inositol trisphosphate (Ins(1,4,5)P₃) which is released into the cytosol and diacylglycerol (DAG) which remains in the membrane and activates protein kinase C (PKC). Ins(1,4,5)P₃ is then cycled back, via inositol bisphosphate (Ins(1,4)P₂) and inositol phosphate (Ins(1)P) to free inositol for re synthesis to PtdIns. DAG is phosphorylated to form phosphatidic acid (PA) which interacts with cytidine triphosphate (CTP) to form cytidine diphosphate diacylglycerol (CDP-DAG). This recombines with inositol to replenish the pool of PtdIns. Lithium (Li⁺) inhibits the enzyme inositol 1-phosphatase, preventing the conversion of Ins(1)P to free inositol. Dotted lines indicate a multi-stage process.

1.9. Potassium Channels And Vascular Smooth Muscle

Electrical excitability of cells was identified in the work of Hodgkin, Huxley & Katz, published in 1949-52. Electrical excitability depends on the existence of voltage-gated ion channels in the cell membrane. Ion channels are membrane proteins which act as gates, separating the extra cellular and intracellular compartments and are identified on the basis of the ion they are selective for. The role of potassium channels is to stabilise the membrane by drawing the membrane potential closer to the potassium equilibrium potential and further from firing threshold. Potassium currents have been characterised in isolated cells from a variety of vascular smooth muscle (see Halliday *et al*, 1995) and from these studies, it is recognised that there are several different types of potassium channels which exist in vascular smooth muscle.

The opening probability of the calcium-activated potassium channels ($K_{Ca^{2+}}$) increases with rises in $[Ca^{2+}]_i$ and depolarisation (Beech & Bolton, 1989) and serves to have a negative feedback on calcium entry by increasing the membrane potential to more negative values than those which trigger voltage-operated calcium (particularly L-) channels to open.

The ATP-sensitive potassium channels (K_{ATP}) are regulated by purine derivatives associated with cellular metabolism; intracellular ATP promotes closure, and high ADP favours opening of K_{ATP} (see Randall & McCulloch, 1995). They are also regulated by prostanoids, G-proteins and adenosine (Ashcroft & Ashcroft, 1990). In vascular smooth muscle, activation of K_{ATP} results in a hyperpolarisation of the plasma membrane, resulting in a decrease in voltage-dependent calcium entry.

In 1989, Ordway *et al* used the patch-clamp technique to demonstrate that a number of fatty acids themselves, as opposed to the products of the cyclooxygenase, lipoxygenase and cytochrome P450 pathways, directly activate potassium channels in smooth muscle cells, thereby suggesting that fatty acids may be important regulators of cell membrane excitability. In smooth muscle cells, an increase in fatty acids would therefore result in hyper polarisation and subsequent decrease of contractile activity. In 1990, Kim & Duff studied the ability of free fatty acids, including arachidonic acid, to modulate the K_{ATP} channel in rat cardiac myocytes using the patch-clamp technique and demonstrated inhibition of the channel by the fatty acids. Conversely, Muller *et al* (1992) demonstrated that arachidonic acid activated K_{ATP} channel activity and similarly, Asano *et al* (1997) confirmed that K^+ currents in the foetal rat aortic smooth muscle cells, A7r5, were activated by n-3 fatty acids, but they were unable to clarify which type of K^+ channel is activated. In an attempt to elucidate which K^+ channel may be affected, Gardener *et al* (1998),

suggested an inhibitory effect of EPA ($10 \mu\text{M}$) on a voltage-sensitive, delayed rectifier K^+ current, on a large conductance Ca^{2+} -activated K^+ channel and on K_{ATP} channels.

Alternative methods of study were adopted by Smith *et al* (1992) who measured potassium efflux from cells using the isotopic tracer, rubidium chloride, ^{86}Rb . They examined the effect of a fish-oil supplemented diet on potassium efflux in the aorta of WKY and SHR rats, and compared the effects with those of diltiazem or sodium nitroprusside. They demonstrated that diltiazem and sodium nitroprusside, by reducing $[\text{Ca}^{2+}]_i$ also served to reduce potassium efflux, as determined using ^{86}Rb , and that this decrease was similarly observed in fish-oil fed rats. They concluded that fish oil modulates calcium metabolism in vascular smooth muscle to lower $[\text{Ca}^{2+}]_i$, decrease activation of calcium-activated potassium channels and thus decrease rubidium efflux through these channels.

Introduction To The Thesis

The aim of the present study was to investigate the effect of the n-3 polyunsaturated fatty acid, cis-5,8,11,14,17-eicosapentaenoic acid (EPA) upon the contractile mechanisms linked to both calcium influx and the mobilisation of intracellular calcium in vascular smooth muscle. The initial objective therefore, was to identify and subsequently develop for use, a suitable system of vascular smooth muscle in which calcium regulation could be studied. A direct effect of EPA upon smooth muscle cells is postulated to exist (Juan *et al*, 1987; Engler *et al*, 1990; Engler, 1992 a, b, c; Asano *et al*, 1997, 1998) therefore, to avoid the complexities associated with vascular strip preparations, vascular smooth muscle cells were isolated to allow direct assessment of cellular mechanics and pharmacology (Bolton *et al*, 1985; Yamada *et al*, 1992).

After establishing a viable system of primary cultured aortic smooth muscle cells (AOCs) (chapter 3) the next objective was to examine whether the n-3 fatty acids affected the contractile mechanisms in the AOCs. Increased blood pressure can be associated with increased contractility of the vasculature. Therefore, a population-based intervention trial by Bønaa *et al* (1990) was performed to determine whether blood pressure could be lowered by n-3 fatty acids. The results from this study indicated that EPA was more important than DHA in lowering systolic and diastolic blood pressure, therefore, EPA was selected as the n-3 fatty acid for investigation throughout this thesis. In selecting the concentration of EPA to be used in the investigation, consideration was taken of the n-3 fatty acid concentration used in previous studies; the concentration of PUFAs used in studies on membrane signal transduction has ranged from as low as 1.4 μM up to higher concentrations of 400 μM . However, it has been documented that the physiological concentration of n-3 fatty acids may be somewhere inbetween these values; 50-100 μM for arachidonic acid (Sumida *et al*, 1993) and 120 μM for EPA (Asano *et al*, 1998). Furthermore, previous investigations into the vasorelaxatory mechanism of action of the n-3 PUFAs, performed by colleagues working in the same laboratory as the author have used EPA and DHA at a concentration of 50 μM , therefore, the concentration of EPA used throughout all investigations in this thesis was 50 μM .

Examination for an effect of EPA in the AOCs was initially performed by determining whether pre-contracted AOCs would relax when exposed to EPA and also by determining whether pre-treatment of the AOCs with EPA would prevent contraction following exposure of the cells to the vasoactive agents noradrenaline or potassium chloride (KCl) (chapter 4). In addition, the effect of EPA was compared with that of nicardipine, a dihydropyridine calcium L-type channel blocker and phentolamine, a non-selective α -adrenoceptor antagonist in the imidazoline group to

compare any effect of EPA with an action associated with calcium influx or the mobilisation of intracellular calcium respectively. EPA partially blocked the contraction of AOCs to KCl and noradrenaline (chapter 4), suggesting that the action of EPA may be related to the regulation of $[Ca^{2+}]_i$ as a result of a decrease in the influx of extracellular calcium or by the blockade of intracellular calcium release or alternatively be due to the desensitisation of the cellular contractile filaments to calcium (Somlyo & Himpens, 1989).

The next objective, therefore, was to determine whether the observed anti-vasoconstrictor effect of EPA in chapter 4, was associated with block of calcium influx and as such, with a decreased concentration of intracellular calcium in the AOCs. The effect of EPA was subsequently found to be associated not simply with a block of calcium influx through L-type calcium channels (chapter 5), consistent with a previous suggestion by Hallaq *et al* (1992) but was shown to reduce calcium influx through receptor-operated channels (chapter 5), supporting a recent suggestion by Asano *et al* (1997). The latter effect could account for the observed effect of EPA to partially block the contraction of AOCs to noradrenaline (chapter 4), but not for the same effect shown for KCl. Furthermore, notwithstanding the evidence to decrease calcium influx, EPA caused a transient increase in $[Ca^{2+}]_i$ in the AOCs, in the absence of extracellular calcium (chapter 5). This was shown using the fluorescent calcium-sensing indicator fura-2 and suggested an effect of EPA to mobilise intracellular calcium. This effect was in contrast to that anticipated, so the next objective was to examine the effect of EPA upon intracellular calcium mobilisation. Calcium entry into the cytosol from the sarcoplasmic reticulum is under the control of second messengers (Van Breeman, 1989), including inositol 1,4,5-trisphosphate (IP₃; Berridge, 1989) which is formed *via* hydrolysis of membrane phosphoinositides following α_1 -adrenoceptor stimulation (Han *et al*, 1987). Therefore, the effect of EPA upon inositol phosphate formation was investigated whereupon it was shown that EPA increased inositol phosphate formation (chapter 6). This might explain the observed transient increase in $[Ca^{2+}]_i$ however, it still did not explain the effect of EPA to block cell contraction to KCl (chapter 4).

It has previously been shown that $[Ca^{2+}]_i$ can phosphorylate the myosin light chain of the contractile filaments and so mediate cell contraction (Kitajima *et al*, 1996; Abe *et al*, 1995), or alternatively $[Ca^{2+}]_i$ can activate membrane ion channels, ion pumps, exchange carriers and enzymes without mediating cell contraction (Abe *et al*, 1995). In the event that a membrane-associated protein was activated, thereby blocking calcium entry through voltage-dependent calcium channels, then this latter effect could account for the effect of EPA to block cell contraction to KCl (chapter 4).

Calcium entry through the voltage-dependent calcium channels may be inhibited by the activation of membrane-associated potassium channels which hyperpolarise the plasma membrane, moving the membrane potential farther from the activation range for calcium channels. In so doing, calcium influx is subsequently reduced. Therefore the next objective in this study was to examine the effect of EPA on membrane potassium permeability. This was studied using the isotope ^{86}Rb chloride (^{86}Rb) as the marker for potassium efflux. EPA induced ^{86}Rb efflux from the AOCs (chapter 7), suggesting that the previously observed transient increase in $[\text{Ca}^{2+}]_i$ to EPA might subsequently block calcium influx as shown in chapter 5 by activating membrane-associated potassium channels. This could account for the effect of EPA to block cell contraction to KCl (chapter 4).

The next objective was to ascertain that the effect of EPA was not also to affect the sensitivity of the contractile filaments to $[\text{Ca}^{2+}]_i$ as it has previously been reported that the lack of contraction following an increase in $[\text{Ca}^{2+}]_i$ may occur if the contractile filaments are desensitised to calcium (Somlyo & Himpens, 1989). This was investigated using intact aortic tissue mounted for isometric tension recording and bathed in a calcium-free solution containing a high concentration of potassium to activate the voltage-dependent calcium channels. Upon addition of calcium to the bathing solution, the tissue would be expected to contract due to the influx of calcium into the tissue. However, in the event that the contractile filaments were desensitised, then the addition of calcium would not phosphorylate the myosin light chain of the contractile filaments, so failing to produce the resultant contraction. The results of this investigation (chapter 8) showed that EPA did not cause a rightward shift in the concentration-response curve to calcium, suggesting that EPA did not desensitise the contractile filaments to calcium.

It seemed, therefore, that a final objective remained for the present study. This was to confirm that the observed effects of EPA in AOCs were apparent in an intact smooth muscle preparation, as EPA and DHA have previously been shown to inhibit the contraction induced by α -adrenoceptor agonists in rat aortic rings (Juan & Sametz, 1987; Engler *et al*, 1990; Engler, 1992a). Therefore, the final investigation examined the effect of EPA upon noradrenaline-induced contractures in aortic tissue (chapter 8) and showed that EPA decreased the effect of noradrenaline, consistent with the effect observed in the AOCs.

The results obtained in answer to the objectives outlined above are described in detail in chapters 3 to 8. Chapter 2 describes the methodologies used to achieve these objectives.

Chapter 2

Experimental Methods

2.1. Primary culture of aortic smooth muscle cells

2.1.1. Tissue Preparation

All solutions and instruments were sterilised by autoclaving, filter sterilising or soaking for 30 min in 70% ethanol. Male Wistar rats (200-250 g) were stunned and killed by cervical dislocation. The fur was soaked with 70% ethanol and removed from the chest region. The underlying skin was removed and the internal organs were displaced to expose the aorta. The thoracic aorta was carefully dissected free from the spine and placed into 5 ml culture medium (refer to appendix 1 for composition) at 25°C. The procedure was repeated with up to ten rats.

In a laminar flow hood (Holten HLB2436 BS) the aorta was washed three times in culture medium at 25°C and then bathed in 10 ml culture medium at 25°C. Under a light microscope (Zeiss, standard 14, 491074), the *adventitia* was mechanically removed using forceps. The author was unable to locate any detailed documentation regarding the best method to remove the *adventitia*, so adopted the following procedure:

- at one end of the aorta, a pair of forceps was used to gently hold the tissue in place
- at the same end of the aorta, a second pair of forceps was used to tease the *adventitia* away from the underlying elastic lamina and the layers of smooth muscle
- the *adventitia* was stripped away, along the length of the aorta
- the *adventitia* was then discarded.

Preliminary attempts to remove the *adventitia* had revealed that with practice, this *by eye* method was effective.

The remaining tissue was washed three times in culture medium at 25°C and bathed in 10 ml culture medium at 25°C. The tissue was cut longitudinally to form a strip, pinned open and the endothelium was removed by gently rubbing with a sterile swab. The strip was washed three times in calcium-free physiological solution at 25°C (see 2.9.2.1 for composition) and sectioned into small (~1 mm²) pieces.

2.1.2. Preparation Of The Culture Medium

The culture medium used was prepared as the following composition; D-val-substituted minimum essential medium (refer to appendix 1), supplemented with foetal bovine serum (10% v/v), gentamicin ($100 \mu\text{g ml}^{-1}$), amphotericin B ($2.5 \mu\text{g ml}^{-1}$), L-glutamine (2 mM) and HEPES (10 mM). All supplements were sterilised by autoclaving or filter sterilising using 0.2 or $0.45 \mu\text{m}$ pore size filters.

2.1.3 Aortic Smooth Muscle Cell Dispersal

In order to determine a suitable method for obtaining the maximum number of viable aortic smooth muscle cells, cells were dispersed by one of the following procedures; all solutions and instruments were sterilised by autoclaving, filter sterilising or soaking for 30 min in 70% ethanol:

2.1.3.1. Primary explant and mechanical disaggregation of the aorta

Aorta pieces were transferred to a petri dish containing 5 ml culture medium and were maintained at 37°C , under an atmosphere of 5% CO_2 in air over a period of up to 14 days. Culture medium was changed every 2 or 3 days. After 14 days in culture, the explants were removed. Culture medium was replaced with 1 ml trypsin (25 mg ml^{-1}) and EDTA (10 mg ml^{-1}) solution. After 5 min incubation at 37°C , the solution was collected and centrifuged at 1000 rpm for 5 min. The pellet was re suspended in 10 ml culture medium and viable cell numbers were determined using a haemocytometer slide. Cells which excluded trypan blue (0.4% v/v) were counted.

2.1.3.2. Enzymatic disaggregation of the aorta

The small ($\sim 1 \text{ mm}^2$) pieces from 10 aorta were placed into 10 ml enzyme solution (see 2.3.3.1.) at 37°C . After 30 or 60 min incubation with repeated agitation, 10 ml foetal bovine serum (FBS) was added to the digest which was then centrifuged at 1000 rpm for 5 min. The pellet was resuspended twice in 20 ml culture medium. The tissue was triturated through a wide bore pipette. Cell numbers were determined using a haemocytometer slide. Cells which excluded trypan blue (0.4% v/v) were counted.

2.1.3.3. Successive enzymatic disaggregation of the aorta

In some dispersal procedures, the enzyme disaggregation methodology was repeated. This was to determine whether incubation of the tissue in two successive enzyme solutions was more effective in dispersing viable cells. The enzyme solution used in this second incubation contained a different enzyme to that of the first incubation solution.

2.1.3.3.1. Preparation of enzyme solutions

All enzyme solutions were prepared in 10 ml calcium-free physiological solution at 25°C and contained one of the following:

- trypsin (0.01% w/v)
- elastase (0.05% w/v)
- collagenase (5.4 units ml⁻¹)
- collagenase (5.4 units ml⁻¹), trypsin inhibitor, bovine serum albumin (BSA; 2 mg ml⁻¹)
- papain (7 units ml⁻¹), dithiothreitol (2.5 mM) and BSA (2 mg ml⁻¹).

The enzyme solution was sterilised using a 0.45 µm diameter filter.

Having established that the use of two successive enzyme dispersal solutions produced the maximum number of viable cells (see results 3.2.2), all subsequent experiments were made on cells isolated by the following procedure:

Following preparation of the tissue, small (~1 mm²) pieces of aorta were placed into 10 ml calcium-free physiological solution containing papain (7 units ml⁻¹), dithiothreitol (2.5 mM) and BSA (2 mg ml⁻¹). After 20 min incubation with repeated agitation, FBS (10 ml) was added to the partially digested tissue and centrifuged at 1000 rpm for 5 min. The pellet was re suspended in 10 ml calcium-free solution containing collagenase (5.4 units ml⁻¹), trypsin inhibitor (0.75 mg ml⁻¹) and BSA (2 mg ml⁻¹). After 15 min incubation with repeated agitation, FBS (10 ml) was added to the digest and centrifuged at 1000 rpm for 5 min. The pellet was re suspended twice in 20 ml culture medium. Single cells were obtained by trituration of the tissue through a narrow bore pipette.

Cells were adjusted to a density of 2x10⁴ cells ml⁻¹ with counting performed using a haemocytometer slide only. Cells which excluded trypan blue (3% v/v) were counted.

2.1.4. Aortic Smooth Muscle Cell Culture

Cells were cultured in 13 mm diameter wells at 37°C in a humidified atmosphere of 5% CO₂ in air. Culture medium was replaced every 2 or 3 days.

2.1.5. Determination Of A Growth Curve For Aortic Smooth Muscle Cells In Culture

After 24 h the culture medium from three wells was removed by aspiration and the cells detached from the well using a 'trypsinisation' procedure, in which 1 ml calcium-free physiological solution containing trypsin (25 mg ml⁻¹) and EDTA (10 mg ml⁻¹) at 37°C was added to a well. After 5 min incubation at 37°C with repeated agitation, the solution from the well was added to 10 ml FBS and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the pellet was re suspended in 20 ml culture medium at 37°C. Cell numbers were determined

using a haemocytometer slide. Cells which excluded trypan blue (0.4% v/v) were counted. The procedure was repeated at 24 h intervals.

2.1.6. Immunohistochemical Identification Of Aortic Smooth Muscle Cells

Cells were cultured on three glass slides, in culture medium for 7 days at 37°C under an atmosphere of 5% CO₂ in air and culture medium was replaced every 2 or 3 days. The alpha isotype of smooth muscle actin was identified using a commercially available α -smooth muscle actin, immunohistochemical kit (Sigma, IMM-2), whereby cells on a slide were washed three times in phosphate buffered solution at 25°C and fixed for 10 min at -20°C in acetone. A primary antibody against smooth muscle actin, followed by biotinylated secondary antibody was applied to the cells on the slide. The peroxidase reagent was then added, to form a stable avidin-biotin complex with the secondary antibody. Antibody deposition was visualised by the addition of substrate containing hydrogen peroxide and the chromogen 3-amino-9-ethyl-carbazole (AEC). The bound peroxidase catalyses the oxidation of the AEC to form a red-brown insoluble precipitate at the antigen sites. Mayer's Hematoxylin was used as a counter stain. The procedure was repeated using cells dispersed on two separate occasions.

2.1.7. Determination Of Aortic Smooth Muscle Cell Viability

Initially, the size of cells obtained from separate dispersals was compared, to determine whether inter-dispersal variation existed. Following a dispersal procedure and after 2 h in culture, the cells on a coverslip were washed three times in physiological solution (PSS) (see 2.32.1 for composition) and were equilibrated in 1 ml PSS for 2 min. The PSS was maintained at a room temperature of 22-25 °C as no perfusion system to maintain a temperature of 37 °C was available. Also, the PSS used throughout these studies contained propranolol (1 μ M) to inhibit β -adrenoceptors, ascorbic acid (50 μ M) to prevent oxidation of catecholamines and EDTA (10 μ M) to chelate heavy metal impurities.

Ten single cells, attached to the coverslip, were identified under an inverted light microscope (Zeiss) and cell length and width (taken as the widest point of the cell) was measured with an eye piece graticule. Similarly, cell size measurements were repeated at 24 h intervals for 20 days. The procedure was repeated using cells dispersed on two other occasions.

Subsequently, cells cultured for 10 days were used to examine the effect of PSS containing KCl (60 mM) on cell length and width to determine physiological viability. The time course of the contraction of cells in response to 60 mM KCl was recorded on video-tape and measurements of length were made on six cells taken

from each of 4 separate isolations. In each case, cells were exposed to KCl for 300 sec.

In some experiments, cell length and width was measured before and 1 min after the addition of KCl (60 mM) to the PSS. The cells were washed three times in PSS and cell length and width measured again. These experiments were repeated at 24 h intervals following disaggregation and culture, up to 20 days.

All experiments were repeated using noradrenaline (10 μ M) instead of KCl.

2.1.8. The Effect Of EPA, Nicardipine or Phentolamine Upon Cell Shape.

Cells cultured in petri dishes, on coverslips for 10 days, were used to examine the effect of EPA (50 μ M) upon cell shape. One coverslip was removed from the petri dish and transferred to another dish containing 5 ml PSS at room temperature. After 2 min equilibration, cell length and width were measured, as described in the previous section, using an eye piece graticule. KCl (60 mM) was then added to the PSS and after 1 min, cell length and width were measured again. The effect of EPA upon the KCl response was examined by adding a single dose of EPA (50 μ M) to the PSS after 1 min of the equilibration.

In some experiments, noradrenaline (1 μ M) replaced the use of KCl, whilst nicardipine (0.1 μ M) or phentolamine (1 μ M) was used in other experiments instead of EPA.

2.1.9. Determination Of The Reversibility Of The Responses.

This study involved equilibrating the cells grown on a coverslip in 5 ml PSS at room temperature for 1 min, then measuring cell length. The cells were incubated for a further two minutes and cell length was measured again. In some experiments, EPA (50 μ M), noradrenaline (1 μ M), or KCl (60 mM) was added to the PSS bathing the cells for 1 min after the first measurement was taken and a further dose of EPA (50 μ M), NA (1 μ M) or KCl (60 mM) was added for the final minute of the 2 min incubation. The cells were then washed three times using PSS at room temperature and cell length was measured for the final time.

2.2. Calcium influx studies

2.2.1. Cell Preparation

Cells were dispersed and cultured in multiwell plates. After 10-13 days in culture, monolayers of cells were washed three times with PSS at pH 7.4 and at 37°C. The cells were equilibrated for 30 min in PSS at 37°C, under an atmosphere of 5% CO₂ in air.

The PSS used throughout these studies contained BSA (3 mg ml⁻¹) to support cell survival, propranolol (1 μM) to inhibit β-adrenoceptors, ascorbic acid (50 μM) to prevent oxidation of catecholamines and EDTA (10 μM) to chelate heavy metal impurities.

2.2.2. Experimental Methods

2.2.2.1. Time course for calcium influx

After equilibration, the cells were incubated at 37°C for 5, 10, 20, 40 or 60 min with ⁴⁵Ca (2 μCi ml⁻¹) in either PSS or depolarised-PSS (PSS_d). In PSS_d, KCl concentration was raised to 60 mM and the NaCl concentration reduced to 90 mM to maintain osmolarity.

At the end of the incubation, the experiment was terminated by aspiration of the solution and the cells washed four times with lanthanum-containing saline (LSS), which was PSS in which LaCl₃ (10 mM) replaced the CaCl₂ (1 mM). After the final wash in LSS, 0.5 ml of an ice cold trichloroacetic acid (TCA; 10% v/v) solution was added to each well for 30 min. The solution was collected and centrifuged at 12,000 rpm for 5 min, the supernatant added to 10 ml scintillant (Hi safe 3) and the ⁴⁵Ca content determined. Radioactivity was measured as counts per minute (cpm) by liquid scintillation counting (Packard 1900TR) for 5 min.

Blanks, in which tracer was omitted from the extra cellular solution were performed routinely in each experiment and the measurements taken were subtracted from the corresponding measurements made in the presence of tracer. The pellet in the centrifuge tubes and the remaining cell components in the wells were used to determine protein by the method of Lowry *et al* (1951), described in section 2.21. *Standards*, in which ⁴⁵Ca (2 μCi) was added to 10 ml scintillant were prepared in each experiment to allow calculation of calcium influx according to equation 7, shown in appendix 2 and the results expressed as nmol μg⁻¹ protein.

All experimental conditions were performed in duplicate or triplicate and the experiments were repeated using plates containing cells dispersed on different occasions to account for intra- and inter-experimental variation.

2.2.2.2. The effect of KCl concentration on calcium influx.

After equilibration, the cells were incubated in PSS at 37°C for 5 min under an atmosphere of 5% CO₂ in 95% air, containing ⁴⁵Ca (2 μCi ml⁻¹), in the presence of 5, 10, 20, 40 or 60 mM KCl. In experiments in which KCl concentrations were increased, the NaCl concentration was reduced to maintain osmolarity. *Blanks* and *standards* were prepared as described in 2.10.1. and the ⁴⁵Ca content of the samples determined radiochemically.

2.2.2.3. The effect of EPA on calcium influx.

These experiments followed a similar protocol to that described in 2.10.2., however, only a single dose of EPA (50 μM) or ethanol, the EPA vehicle (1% final concentration) was used instead of KCl. In some experiments the incubation period with EPA or ethanol was extended from 5 to 30 min.

2.2.2.4. The effect of nicardipine on KCl-induced calcium influx.

In these experiments either the cells were pre-incubated with PSS containing nicardipine (0.1 μM) at 37°C for 10 min or 35 min and the ^{45}Ca (2 $\mu\text{Ci ml}^{-1}$) with either 5, 10, 20, 40 or 60 mM KCl present was added for the final 5 min or the incubation period was 60 min in which case, ^{45}Ca (2 $\mu\text{Ci ml}^{-1}$) and either 5, 10 or 60 mM KCl were present for the final 30 min.

2.2.2.5. The effect of EPA on KCl-induced calcium influx.

These experiments followed a similar protocol to those described in 2.10.3, however, the incubation time was 40 min and the PSS contained 5, 10 or 60 mM KCl. An increase in KCl concentration was compensated by a decrease in NaCl concentration to maintain osmolarity. In some experiments, cells were pre-incubated for 30 min with nicardipine (0.1 μM) in the culture medium.

The experiment was repeated, however, the incubation time was decreased to 1 min and no pre-incubation was included in order to examine whether EPA mediated an immediate effect on calcium influx.

2.2.2.6. The effect of noradrenaline on calcium influx.

These experiments followed a similar protocol to that described in 2.10.2, however noradrenaline at 10 nM, 0.1 μM , 1 μM , 10 μM or 0.1 mM replaced the use of KCl and the incubation was extended to 30 min.

2.2.2.7. The effect of EPA on noradrenaline-induced calcium influx.

These experiments followed a similar protocol to that described in 2.10.6, except that the cells were incubated under an atmosphere of 5% CO_2 in air in PSS (CON) or PSS containing ethanol (1%, the EPA vehicle) or EPA (50 μM) at 37°C for 60 min. ^{45}Ca (1 $\mu\text{Ci ml}^{-1}$) and either 10^{-8} M, 10^{-7} M or 10^{-6} M noradrenaline were present for the final 30 min.

2.3. Studies on the measurement of intracellular calcium.

2.3.1. Cell preparation

Cells were isolated and cultured in 25 cm³ flasks instead of multiwell plates. After 10-13 days in culture, monolayers of cells were washed three times with calcium-free PSS at pH 7.4 and at 37°C and then incubated in 1 ml calcium-free PSS at 37°C containing trypsin (25 mg ml⁻¹) and EDTA (10 mg ml⁻¹). After 5 min incubation at 37°C with repeated agitation, the solution from a flask was added to 10 ml FBS and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the pellet was re suspended twice in PSS at 37°C. Cell numbers were determined using a haemocytometer slide and cell density was adjusted to 5 x 10⁵ cells ml⁻¹. Cells were exposed to the indicator, fura-2 acetyl methylester (0.5 μM) in PSS at 37°C under an atmosphere of 5% CO₂ in air for 60 min.

The cells were washed twice in PSS and then re suspended in PSS. Cell density was adjusted to 5 x 10⁵ cells ml⁻¹. The cell solution was incubated at 37°C for 30 min to allow complete hydrolysis of the indicator from the acetyl methylester and a sample of the solution (2 ml) was then transferred to a cuvette which was placed into a chamber in a luminescence spectrometer (Perkin Elmer LS50). Excitation wavelengths were set at 340 and 380 nm and recording emission at 510 nm. The solution was stirred and equilibrated at 37°C for 5 min.

2.3.2. Experimental Methods

2.3.2.1. Effects of noradrenaline, KCl or EPA on [Ca²⁺]_i.

In initial experiments, the response of the aortic cells to the non-selective α-adrenoceptor agonist noradrenaline, to KCl or to EPA was determined. After the 5 min equilibration period, a single dose (10 μl) of noradrenaline, KCl, EPA or ethanol (1%; the EPA vehicle) was added to the solution in the cuvette to give the following final concentrations: noradrenaline, 1 μM; KCl, 60 mM; EPA, 50 μM or ethanol, 1%, for a period of up to 10 min. [Ca²⁺]_i was recorded for up to 10 min using an Epson computer with intracellular calcium measurement software (Fluorescent Data Manager).

After each measurement, digitonin (2mM) was added to the solution to determine F_{max}, the fluorescence measured when all the indicator is in the bound form. EGTA (5 mM) was then added to determine F_{min}, the fluorescence measured when all the indicator is in the free form. The resultant [Ca²⁺]_i level was calculated according to the equation reported by Grynkiewicz *et al* (1985; appendix 2) from the ratios of fluorescence at the two excitation maxima.

2.3.2.2. Effects of phentolamine or nicardipine on noradrenaline- or EPA-induced $[Ca^{2+}]_i$.

In these experiments, where the effect of the non-selective α -adrenoceptor antagonist, phentolamine and the L-type calcium channel blocker, nicardipine were examined, the cells were equilibrated for 5 min in the presence of phentolamine (1 μ M) or nicardipine (0.1 μ M) and a single dose of noradrenaline, KCl, EPA or ethanol was then added, as described previously.

2.3.2.3. Effects of calcium removal on noradrenaline- or EPA-induced $[Ca^{2+}]_i$.

In these experiments, the cells were equilibrated for 5 min in calcium-free PSS containing 2 mM EGTA. After the tissues had been exposed to this bathing solution for 2 min, a single dose of noradrenaline, KCl, EPA or ethanol was then added, as described previously.

2.4. Studies of inositol phosphate hydrolysis

2.4.1. Cell Preparation

Cells were dispersed and cultured in multiwell plates. Monolayers of cells were exposed to a labelling solution containing culture medium and 5 μ Ci ml⁻¹ *myo*- $[^3H]$ inositol. The age of the monolayers and the labelling time was determined according to the experiment performed. In some wells 5 μ Ci ml⁻¹ *myo*- $[^3H]$ inositol was absent. These cells were *blanks*, and were performed routinely in each experiment. The measurements taken for the *blanks* were subtracted from the corresponding measurements made in the presence of tracer. After exposure to *myo*- $[^3H]$ inositol, the cells were washed three times with PSS and then equilibrated in PSS at 37°C for 20 min. Following equilibration, the cells were incubated in PSS at 37°C in the presence of LiCl (10 mM) for a further 20 min. During equilibration and the subsequent incubation, cells were under an atmosphere of 5% CO₂ : 95% air.

The PSS used throughout these studies contained BSA (3 mg ml⁻¹) to support cell survival, propranolol (1 μ M) to inhibit β -adrenoceptors, ascorbic acid (50 μ M) to prevent oxidation of catecholamines and EDTA (10 μ M) to chelate heavy metal impurities.

2.4.2. Preparation of the anion-exchange columns

Dowex-1 (X8; formate form) was added to distilled H₂O, mixed and allowed to settle for 30 min. The supernatant was discarded, the pellet re suspended in distilled H₂O and the solution allowed to settle for a further 30 min. This procedure

was repeated twice more. The volume of distilled H₂O in the final re suspension was adjusted to produce a 1:1 (v/v) resin suspension.

A sample (1 ml) of the suspension was loaded into a column and washed with 2 x 4 ml distilled H₂O.

2.4.3. Experimental Methods

2.4.3.1. Determination of the recovery of *myo*-[³H] inositol from the anion-exchange columns.

A solution of 5 μ Ci *myo*-[³H] inositol in 4 ml distilled H₂O was loaded onto the column and the eluent collected, added to 10 ml scintillant and counted for radioactivity. This was compared with controls, in which 5 μ Ci *myo*-[³H] inositol was added to 10 ml scintillant directly and then counted.

2.4.3.2. Investigation of *myo*-[³H] inositol incorporation into aortic smooth muscle cells.

At 24 h intervals, one well from a plate was used for experimentation, the methodology for which followed that described in 2.4.1. However the cells in a well were labelled for 24 h. After equilibration and incubation in the presence of LiCl, the solution was replaced with 1 ml of an ice-cold solution containing trichloroacetic acid (TCA; 20% w/v) and EDTA (2 mM) for 15 min. *Blanks* were also performed. The solution was collected, added to 10 ml scintillant (Hi safe 2) and radioactivity was measured using a ³H program (Packard 1900TR) for 5 min. Incorporation of total *myo*-[³H] inositol into the cells was expressed as counts per minute (cpm).

Cells in a plate, obtained during one dispersal procedure were identified as part of one experiment. In some experiments, cells in one well were incubated with 5 μ Ci *myo*-[³H] inositol after the cells had been desegregated from the aorta and placed into the culture plates 2 h previously.

2.4.3.3. Determination of labelling time.

These experiments followed the previous protocol, however, cells at 7, 10 or 14 days only were used and exposure to the labelling solution was 24, 48 or 72 h.

2.4.3.4. Effect of labelling time on α -adrenoceptor agonist-stimulated inositol phosphate incorporation.

These studies followed the procedure described in the previous section, except that after equilibration, the cells were exposed to the agonist for the final 5 min of the LiCl-containing incubation.

2.4.3.5. Effect of α -adrenoceptor agonists on inositol phosphate formation.

These experiments were performed according to the methodology described in the previous section, however, cells were used between 10 and 13 days as this corresponded to steady state incorporation of the radio label (see figure 6.1). Following dispersal from the aorta cells were labelled with radioactive *myo* [^3H]-inositol for a period of 24 hours, as no greater incorporation of the label was achieved at 48 or 72 h (see figure 6.2). After the agonist was added to the LiCl-containing incubation solution for 5 min, the action of all agents was terminated by aspiration of the solution and addition of 1 ml of an ice-cold solution containing trichloroacetic acid (TCA; 20% w/v) and EDTA (2 mM) was added to the cells for 15 min.

The solution was collected and centrifuged at 12,000 rpm for 5 min. The supernatant was added to a 1:1 mixture of 1,1,2-trichloro-1,2,2-trifluoroethane and tri-*n*-octylamine. A sample (0.5 ml) of the upper phase was removed and analysed for inositol phosphates by anion-exchange chromatography on columns containing 0.5 ml of Dowex-1 (X8; formate form) prepared as described in section 2.4.2.

All columns were washed twice with 4 ml distilled water to elute free inositol and twice with a solution containing 4 ml sodium formate (60 mM) and disodium tetraborate (5 mM), to elute glycerophosphoinositol (deacylated phosphatidyl inositol). Total [^3H]inositol phosphates were eluted with a solution containing 4 ml formic acid (0.1 M) and ammonium formate (1 M) and the eluent was added to 10 ml scintillation fluid. Radioactivity in the eluate was measured by liquid scintillation counting (Packard 1900TR) for 5 min. Inositol phosphate formation was expressed as counts per minute (cpm).

In some experiments, the pellet in the centrifuge tubes and the remaining cell components in the wells were used to determine protein by the method of Lowry *et al* (1951), described in 2.23. Inositol phosphate formation was then expressed as counts per minute (cpm) mg^{-1} protein.

2.4.3.6. Effect of EPA on inositol phosphate formation.

These experiments followed the same methodology as described in the previous section except that a single dose of EPA (50 μM) or ethanol (1% final concentration) replaced the use of the α -adrenoceptor agonist. In some experiments a combination of noradrenaline (1 μM) and a single dose of EPA (50 μM) or ethanol (1% final concentration) was used.

2.4.3.7. Effects of α -adrenoceptor antagonists on agonist- or EPA-induced inositol phosphate formation.

These experiments followed a similar protocol to that described in 2.4.3.5 however, a single dose of antagonist was added to the PSS for the duration of the

LiCl-containing incubation. A single dose of α -adrenoceptor agonist, EPA (50 μM) or ethanol (1% final concentration) was added for the final 5 min of this incubation. In some experiments noradrenaline and EPA or ethanol were added simultaneously.

2.5. Rubidium efflux studies

2.5.1. Cell Preparation

Cells were isolated and cultured in multiwell plates. After 10-13 days in culture monolayers of cells were washed three times with PSS at pH 7.4 and at 37°C. The cells were equilibrated for 30 min in PSS at 37°C, under an atmosphere of 5% CO₂ in air.

2.5.2. Experimental Methods

2.5.2.1. Determination of the ⁸⁶Rb loading period.

Following equilibration, the PSS was replaced with PSS at 37°C containing ⁸⁶Rb (1 $\mu\text{Ci ml}^{-1}$). After 0, 30, 60, 90, 120 or 150 min, the solution was aspirated and discarded. The cells were washed twice with PSS at 37°C and then 0.5 ml of an ice cold solution containing trichloroacetic acid (TCA; 20% w/v) and EDTA (2 mM) was added to the cells for 15 min to lyse the cells and retrieve any residual isotope. The solution was collected and radioactivity measured by γ counting on a Cerenkov program (Packard 1900TR) for 5 min. ⁸⁶Rb influx was expressed as counts per minute (cpm).

Blanks, in which tracer was omitted from the extra cellular solution were performed routinely in each experiment and the measurements taken were subtracted from the corresponding measurements made in the presence of tracer.

2.5.2.2. Determination of basal ⁸⁶Rb efflux.

The experiments described above indicated that maximum ⁸⁶Rb loading was achieved after 90 min incubation with the isotope (see figure 7.1 in results). Therefore, efflux experiments were made following a 90 min loading period. Then, the cells were washed twice with PSS at 37°C and then the efflux of ⁸⁶Rb was monitored at 5 min intervals over a period of 60 min using a series of efflux solutions, each of 1 ml PSS at 37°C, applied to the well. Following exposure to the cells, the efflux solution was collected and transferred to a vial. The subsequent efflux solution was added to the well immediately. After 60 min the cells were lysed with 0.5 ml ice-cold solution containing TCA (20% w/v) and EDTA (2 mM) for 15 min. The solution was collected to determine the remaining ⁸⁶Rb content of the

cells. *Blanks*, in which tracer was omitted from the extra cellular solution were subtracted from these measurements.

Radioactivity in all vials was measured as cpm and rubidium efflux from cells was calculated as the fractional loss of tracer from the cells for the duration of the efflux period and expressed in terms of a rate coefficient $\times 10^{-2} \text{ min}^{-1}$, according to equation 8, described in appendix 3.

2.5.2.3. Effect of cromakalim on ^{86}Rb efflux.

The experiments in this study were performed according to the methodology described in 2.5.2.2 except that the efflux solutions exposed to the cells at 20 and 25 min contained a single dose of cromakalim ($1 \mu\text{M}$) and efflux solutions added at 30, 40 or 50 min remained on the cells for 10 min. After 60 min, the cells were lysed, radioactivity was measured and rubidium efflux determined according to that described previously.

In some experiments, all efflux solutions contained a single dose of cromakalim ($1 \mu\text{M}$). Notwithstanding this modification, the methodology remained the same.

2.5.2.4. Effect of using a single efflux solution on ^{86}Rb efflux.

In these experiments, the methodology remained similar to that described in 2.5.2.3, however, one efflux solution alone was applied to a well for 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 60 min. As a consequence of this modification, commencement of the loading period of the cells with ^{86}Rb was staggered at the corresponding 5 min interval to permit the simultaneous termination of experiments. *Blanks* were included in each experiment. After 60 min, the cells were lysed, radioactivity was measured and rubidium efflux determined according to that described in 2.5.2.2.

In some experiments, the cells were exposed to the efflux solution at 1 min intervals. In a subsequent experiment, the exposure was decreased to 15 sec intervals.

In another study, all efflux solutions contained a single dose of cromakalim ($1 \mu\text{M}$), EPA ($50 \mu\text{M}$) or ethanol (1% final concentration).

2.5.2.5. Effect of glibenclamide or tetraethylammonium on ^{86}Rb efflux.

In these experiments, a single dose of glibenclamide ($1 \mu\text{M}$) or tetraethylammonium (1 mM) was added to the PSS containing ^{86}Rb ($1 \mu\text{Ci ml}^{-1}$) for the final 30 min. The cells were washed twice with PSS at 37°C and then one efflux solution alone was applied to a well for 0, 15, 30, 60, 90, 120, 180, 240 or 300 sec. Commencement of the loading period of the cells with ^{86}Rb was staggered at the corresponding time interval to permit the simultaneous termination of experiments.

Blanks were included in each experiment. After 5 min, the cells were lysed, radioactivity was measured and rubidium efflux determined according to that described in 2.5.2.2.

To investigate the effect of glibenclamide and TEA on cromakalim, EPA or ethanol-induced efflux, the efflux solutions contained a single dose of cromakalim (1 μM), EPA (50 μM) or ethanol (1% final concentration).

2.6. Protein determination studies

2.6.1. Cell Preparation

Following experimentation, 1 ml calcium-free physiological solution at 37°C containing trypsin (25 mg ml⁻¹) and EDTA (10 mg ml⁻¹) was added to each well of cells for 5 min with repeated agitation. The solution was collected and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the pellet was analysed for protein content by the method of Lowry *et al* (1951). The pellet was re suspended in a sample (500 μl) of NaOH (0.05M).

2.6.2. Preparation Of The Reagent

The alkaline copper reagent used throughout the protein determination studies contained 1 ml of CuSO₄ (10 mg ml⁻¹) and 1 ml of K-Na tartrate (20 mg ml⁻¹), which were mixed. 100 ml of 0.1 M NaOH containing 2 g Na₂CO₃ was added.

2.6.3. Experimental Methods

2.6.3.1. Protein assay of cells.

The re suspended pellet was mixed with 5 ml alkaline copper reagent. After 10 min incubation at room temperature, 0.25 ml Folin & Ciocalteau's phenol reagent was added. The solution was mixed and incubated for 30 min at room temperature. A sample (2 ml) was removed and analysed for protein. The absorbance of the sample was read at 750 nm on a spectrophotometer.

2.6.3.2. Protein assay of standards.

A standard curve was prepared in duplicate each time a protein assay was performed on cells. 0.05M NaOH containing bovine serum albumin (BSA; 1 mg ml⁻¹) was used to prepare a stock standard solution. A sample of 0, 5, 10, 25, 50, 100 or 200 μl standard solution was made up to a volume of 500 μl using 0.05M NaOH. 5.0 ml of alkaline copper reagent was added to each solution and mixed. After 10 min incubation at room temperature, 0.25 ml Folin & Ciocalteau's phenol reagent was

added. The solution was mixed and incubated for 30 min at room temperature. A sample (2 ml) was removed and analysed for protein. The absorbance of the sample was read at 750 nm on the spectrophotometer.

2.7. Organ bath studies

2.7.1. Tissue Preparation

Male Wistar rats (200-250 g) were stunned and killed by cervical dislocation. The fur and skin were removed from the chest region and the internal organs displaced to expose the thoracic aorta. This was carefully dissected free from the spine and placed into a dish containing Krebs solution. The aorta was cleaned of fatty debris and connective tissue and sectioned into rings (5 mm). The endothelium was removed mechanically by inserting forceps into the lumen and gently rolling the aortic ring over the surface of the dish. The rings were suspended horizontally between two wire triangles in 10 ml Krebs in an organ bath. One triangle was connected *via* a wire hook to a transducer (Pioden Isometric UF1) whilst the other was attached *via* another wire hook to an aerator which was clamped in place in the organ bath (see figure 2.1). The bath was maintained at 37°C and the solution was gassed with 95% O₂, 5% CO₂. The tissue was placed under a resting tension of 2 g and allowed to equilibrate for 1 h. During this time, the bathing solution was changed every 10 min.

Following equilibration of all tissues used in these experiments, a sensitising response to NA (3 μ M) was elicited to determine tissue viability. At the peak of the sensitising response, acetylcholine (1 μ M) was applied to test for the release of endothelium-derived relaxing factor (Furchgott & Zawadzki, 1980). In the event that a relaxation of 30% or greater of the sensitising response was observed, the tissue was discarded. The determination of the absence of endothelium was followed by a 90 min washout period during which time, the PSS was replaced at 10 min intervals.

Changes in tension were recorded using a Lectromed preamplifier connected to a BBC flatbed recorder.

2.7.2. Experimental Methods

2.7.2.1. Effect of EPA on agonist-induced contractures.

Non-cumulative concentration-response curves to a single agonist were constructed prior to and 30 min after EPA (50 μ M) or ethanol (1% final bath concentration; the EPA vehicle). Each dose of agonist was left in contact with the tissue for a minimum period of 5 min and successive doses of agonist were added

after a minimum period of 15 min had elapsed, during which time the tissue was washed every 5 min. The contact times and washout times varied with the concentration of agonist used to induce contractures, as the higher concentrations often required a longer period of time to induce a maximum response and to wash out.

2.7.2.2. Effect of α -adrenoceptor antagonists on agonist-induced contractures

In these experiments, non-cumulative concentration-response curves were constructed to a single agonist in the absence and presence of an antagonist. Throughout the final 45 min of the washout following the sensitising response to NA, a single dose of the α_1 -antagonist prazosin (5 nM) or the non-selective α_1 - and α_2 -antagonist phentolamine (1 μ M) was added to the bathing PSS. During the time the antagonist was incubated on the tissues, the bathing PSS was changed at 10 min intervals. The agonist was added to the tissues in the same manner described in the previous section and all washes were made with the antagonist present in the PSS.

2.7.2.3. Effect of EPA and α -adrenoceptor antagonists on agonist-induced contractures.

These experiments followed a similar protocol to that described in the previous section, however, during the final 30 min incubation period with the antagonist, a single dose of EPA (50 μ M) or ethanol (1%; the EPA vehicle) was also added and remained in contact with the tissue throughout the rest of the experiment.

2.7.2.4. Effect of calcium removal on agonist-induced contractures.

Following washout of the sensitising dose, Krebs was replaced with calcium-free Krebs containing 2 mM EGTA. After the tissues had been exposed to this calcium-free solution for 2 min a single dose of agonist was added for a minimum period of 10 min.

2.7.2.5. Effect of EPA on the EGTA-resistant response to noradrenaline.

In experiments in which the effect of EPA and calcium removal on agonist-induced contractures of rat aorta was investigated, the tissues were pre-treated with EPA (50 μ M) for 30 min before replacement of Krebs with calcium-free Krebs containing 2 mM EGTA and EPA (50 μ M).

2.7.2.6. Effect of EPA and α_1 --adrenoceptor antagonists on the EGTA-resistant response to noradrenaline.

These experiments followed a similar protocol to that described in the previous section, however, the antagonist was pre-incubated on the tissues for 15 min prior to the addition of EPA or ethanol.

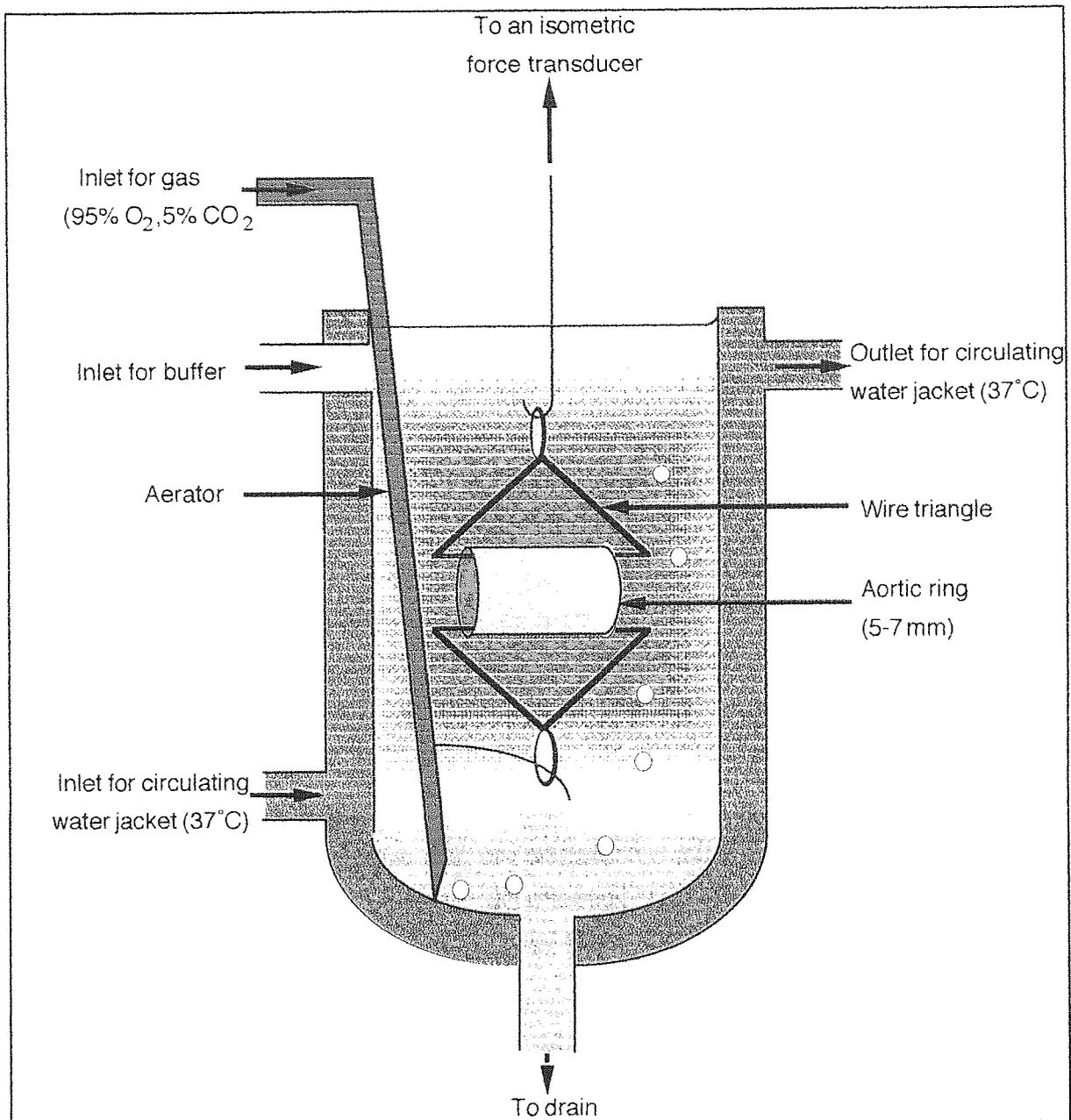


Figure 2.1 Diagrammatic representation of the organ bath preparation.

2.7.2.7. Effect of EPA on the response to $[Ca^{2+}]_e$ under depolarised conditions.

These experiments measured contractile responses induced by extracellular calcium in a nominally calcium-free high potassium solution (KPSS). KPSS was prepared as calcium-free, EGTA-containing PSS as for the experiments reported in the previous section, except that there was equimolar substitution of 60 mM KCl for NaCl. Also, a sensitising response was elicited to 60 mM KCl instead of 3 μ M noradrenaline. After 30 min of washout, PSS was replaced with KPSS which remained exposed to the cells for a further 60 min. EPA (50 μ M) or ethanol (ETH, 1 %; the EPA vehicle) was added for the final 30 min. Cumulative concentration-response curves to calcium were then constructed.

2.8 Data analysis

All data are presented as the means of n observations \pm the standard error of the mean (s.e. mean). Statistical comparisons were made by Student's two-tailed t test for paired or unpaired data as appropriate, or in the case of multiple comparisons, analysis of variance (ANOVA) followed by Scheffe's multiple range test. Significance was identified at $P < 0.05$.

2.8.1. Change in cell size studies

Cell length and width after exposure to KCl, noradrenaline or EPA was assessed as the measured percentage reduction in cell length and width from before exposure. All responses are given as the mean \pm s.e. mean for n cells. Differences were determined using Student's t test.

2.8.2. Calcium influx studies

Calcium influx into cells was calculated by conversion of counts per minute (cpm) using the specific activity of ^{45}Ca in the incubation medium. Values were normalised for cell protein and influx was expressed as nmol mg^{-1} protein. Differences between values were determined using Student's t test.

2.8.3. Inositol phosphate studies

Total inositol phosphates was normalised for cell protein and expressed as cpm mg^{-1} protein. Differences were determined using either Student's t test or ANOVA followed by Scheffe's multiple range test.

2.8.4. Rubidium efflux studies

Rubidium efflux from cells was calculated according to the fractional loss of tracer from the cells for the duration of the efflux period and efflux was expressed in terms of a rate coefficient. The ^{86}Rb efflux curves were obtained by plotting the efflux rate coefficients against time. Differences between rate coefficients were determined using Student's t test or ANOVA followed by Scheffe's multiple range test.

2.8.5. Organ bath studies

The sensitivity of the aorta preparations to noradrenaline was assessed as the negative logarithm of the concentration required to cause 50 % of the maximum response (pD_2). Differences between responses were determined using either Student's t test or ANOVA followed by Scheffe's multiple range test.

2.9. Drugs and solutions

2.9.1. Drugs

Drugs and chemicals used in the study, and their suppliers are listed below:

<i>Drugs and chemicals</i>	<i>Supplier</i>
Acetylcholine chloride	Sigma
Activated charcoal	Sigma
[8- ³ H] adenosine 3', 5'-cyclic phosphate, ammonium salt	Amersham
Ammonium formate	Fisons
Amphotericin B	Gibco
L-Ascorbic acid	BDH
Bovine serum albumin	Sigma
⁴⁵ Calcium chloride	Amersham
Calcium chloride (aqueous)	BDH
cAMP binding protein (protein kinase A)	Sigma
Charcoal, activated	Sigma
Collagenase (Type XI)	Sigma
Copper sulphate	BDH
Cromakalim	Sigma
Digitonin	BDH
DL-Dithiothreitol	Sigma
Dowex-1 resin (X8; formate form)	Sigma
cis-5,8,11,14,17-Eicosapentaenoic acid (free acid; 99%)	Sigma
Ethanol	BDH
Ethylenediamine tetra-acetic acid (EDTA)	BDH
Ethylene glycol tetra-acetic acid (EGTA)	Sigma
Foetal bovine serum	Gibco
Folin & Ciocalteu's phenol reagent	Sigma
Formic acid (99%)	Sigma
Fura-2, acetyl methylester	Calbiochem
Gentamicin	Gibco
Glibenclamide	Sigma
D-Glucose	BDH
L-Glutamine	Gibco
Hydroxymethylpiperazine-N-2-ethansulphonic acid (HEPES)	Sigma
Idazoxan hydrochloride	Sigma
myo-[²⁻³ H]-Inositol with PT6-271	Amersham
3-Isobutyl-1-methyl-xanthine (IBMX)	Sigma

Lanthanum chloride	BDH
Lithium chloride	BDH
Magnesium chloride (aqueous)	Sigma
Magnesium sulphate heptahydrate	BDH
Minimum essential medium-D valine	Gibco
(-)-Noradrenaline bitartrate	Sigma
Optiphase scintillant	Fisons
Papain	Sigma
-Phentolamine hydrochloride	Sigma
L-Phenylephrine hydrochloride	Sigma
Phosphate buffer tablets	Sigma
Potassium chloride	BDH
Potassium dihydrogen orthophosphate	BDH
Potassium, sodium tartrate	BDH
Prazosin hydrochloride	Sigma
DL-Propranolol hydrochloride	Sigma
⁸⁶ Rubidium chloride	Amersham
Sodium carbonate	Sigma
Sodium chloride	BDH
Sodium formate	BDH
Sodium hydroxide	BDH
Sodium hydrogen carbonate	BDH
di-Sodium tetraborate	BDH
Tetraethylammonium chloride	Sigma
Trichloroacetic acid	Fisons
1,1,2-trichloro-1,2,2-trifluoroethane	Aldrich
tri- <i>n</i> -octylamine	Aldrich
Trizma base (TRIS)	Sigma
Trypsin	Sigma
Trypsin inhibitor II-S	Sigma

Chemicals used were of Analar grade and obtained as pure powders unless otherwise specified.

The following stock solutions were prepared in distilled water and were frozen until required; ACh (10 mM), ⁴⁵Ca (10 μ Ci ml⁻¹), glutamine (200 mM), HEPES (1 M), idazoxan (10 mM), KCl (3 M), noradrenaline (10 mM) containing 100 μ M ascorbic acid to reduce drug oxidation, phentolamine (10 mM), phenylephrine (10 mM) and prazosin (10 mM).

Nicardipine (10 mM) was prepared in absolute ethanol with dilution's made in distilled H₂O and EPA (5 mM) was also prepared in absolute ethanol and stored under vacuum. Forskolin (10 mM) and glibenclamide were prepared in DMSO, with dilution's made in distilled H₂O.

Dilutions of all drugs were made on the day of the experiment in the solution used in the particular experiment (in distilled water in the organ bath studies). All drugs were kept on ice throughout the course of the experiment (and discarded at the end of the day).

2.9.2. Solutions

2.9.2.1. Preparation of the physiological solution.

The physiological solution (PSS) was prepared as the following composition (mM); NaCl 145, KCl 5, MgCl₂ 1, CaCl₂ 1, glucose 10, HEPES 5 at pH 7.4 and adjusted to pH 7.4 with NaOH.

Krebs' solution was used throughout the organ bath studies. This was prepared as the following composition (mM); NaCl 118.3, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 251.2, NaCOH₃ 25, MgSO₄.7H₂O 1.2 and glucose 11.7 and adjusted to pH 7.4 using NaOH. Propranolol (1 μM) to inhibit β-adrenoceptors, EDTA (10 μM) to chelate heavy metal impurities and ascorbic acid (50 μM) to prevent oxidation of the catecholamine used in the studies were added.

2.9.2.2. Preparation of calcium-free physiological solution.

Calcium-free physiological solution was prepared as the following composition (mM); NaCl 120, KCl 6, MgCl₂ 1.2, HEPES 5 and glucose 11.4, and adjusted to pH 7.4 with NaOH.

2.9.2.3 Preparation of phosphate buffered solution.

This solution was used in the immunohistochemical cell staining studies, as it was the required solution for use with the staining kit. Commercially available phosphate buffered solution tablets were dissolved in distilled H₂O to give the following composition; 0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl at pH 7.4.

Chapter 3

The Development Of A Viable Smooth Muscle System

3.1. Introduction

Most studies of mammalian vascular smooth muscle mechanics (Murphy, 1980) and pharmacology (Bolton, 1979) have relied upon data obtained in whole artery preparations. Whilst these studies have been instrumental in the characterisation of basic smooth muscle cell function, extrapolating individual cellular pharmacological or mechanical responses from whole tissue data is complicated by the large muscle cell population within the artery which is embedded in a dense connective tissue matrix. For example, exogenous agonist concentration at cellular sites within the artery is influenced by diffusional barriers associated with the extra cellular matrix. In addition, other cellular components within the wall (*e.g.* nerves and endothelial cells; Furchgott & Zawadski, 1980) can modulate smooth muscle cell contractile responses. To avoid the interpretative complexities associated with multicellular preparations, it is desirable to isolate single vascular smooth muscle cells from intact blood vessels, thus allowing direct assessment of cellular mechanics and pharmacology.

In the present study, a cell culture was used to avoid the problems outlined above. It was recognised that, whilst any observations made should be compared against the behaviour of cells in their natural environment *in vivo*, this isolated cell system permits an examination of cellular changes in smooth muscle cells. This chapter describes the development of a method for the isolation and growth of a suitable cell culture. The studies presented have focused upon post-receptor events by measuring second messenger levels, or determining the intracellular flux of particular ions across the cell membrane. These types of investigations lend themselves particularly to tissue culture (Freshney, 1994).

Cells isolated from a tissue, grown *in vitro* but which are not subcultured are regarded as a primary cell culture (Freshney, 1994). It has been reported that some aspects of specialised function are expressed more strongly in primary cell culture, particularly when the culture becomes confluent; at this stage the culture will show its closest morphological resemblance to the parent tissue and retain some diversity in cell type (Freshney, 1994).

A primary cell culture is derived either by outgrowth of migrating cells from a fragment of tissue or by dispersing the cells of a tissue mechanically or enzymatically and then allowing the cells to grow (Chamley-Campbell *et al*, 1979). Mechanical and enzymatic disaggregation avoids problems of selection by

migration, but more importantly, has generally been shown to yield cells which are more representative of the whole tissue (Freshney, 1994). However, it should be noted that dispersal techniques themselves select cells which are resistant to the method of disaggregation and which remain capable of attachment to the substrate.

It is widely recognised that embryonic tissue disperses more readily and gives a higher yield of proliferating cells than new-born or the adult. The increasing difficulty in obtaining viable proliferating cells with increasing age is likely due to factors including the onset of differentiation, the increase in fibrous connective tissue and extra cellular matrix and a reduction of the undifferentiated proliferating cell pool. However, the use of embryonic tissue was rejected in favour of tissue from older animals because of the limited cell yield from such tissues and also because there was no intention to subculture the cells. The aorta, although not necessarily a good model for the study of mechanisms of blood pressure reduction, was selected for use because contractile mechanisms in this vessel have been extensively studied, it exemplifies contractile mechanisms linked to both calcium influx and the mobilisation of intracellular calcium and because a comparable model with published functional studies was required. Furthermore, there is extensive experience with this vessel in this laboratory.

Therefore, in initial investigations in this study the layer of smooth muscle cells in the aorta from male Wistar rats aged approximately 4 weeks old, was identified under a light microscope (Zeiss) and mechanically separated from the *intima* and *adventitia*. The aorta was enzymatically desegregated and a comparison of the yield of cells obtained using various enzyme solutions was made. The next aim was to examine the growth of the aortic cells following cell disaggregation.

Since the first reports of smooth muscle in culture by Champy in 1913 many studies have attempted to improve the techniques used to culture smooth muscle cells (Campbell *et al.*, 1974) with subsequent studies made on the culture of vascular smooth muscle (Campbell *et al.*, 1971; Chamley-Campbell *et al.*, 1974; 1979). The enzymes used most frequently are crude preparations of trypsin, although collagenase, elastase, papain, pronase or various combinations have been used successfully (Paul, 1975).

The development of cell culture over the past decade has led to attempts to provide more defined media to sustain continuous cell growth. Basal media of Eagle (Eagle, 1959) and the more complex Media 199 of Morgan (Morgan *et al.*, 1950) are popular choices of defined media and they are usually supplemented with 5 to 20% serum. Serum contains major components including albumin and transferrin which are well characterised and a wide range of minor components, (amino acids, nucleosides, sugars, peptide growth factors, hormones, minerals, lipids) the action of which have not been fully determined. However, it is well established that the

major functions of serum are to provide growth factors, attachment and spreading factors and transport proteins. Foetal bovine serum was selected for use in the cell culture techniques described in this thesis primarily due to its widespread availability and also because controls are routinely updated by the suppliers to standardise any inherent variability in this serum product. It was used at a concentration of 10 % (v/v) which I discovered to be the optimum concentration and which has been reported previously (Ross, 1971; Chamley-Campbell *et al.*, 1979).

Basal media of Eagle is well suited for use with primary or diploid mammalian cell cultures. Minimum Essential Medium (MEM; GIBCO Life Sciences) were patterned after basal media of Eagle and is well suited for the growth of a broad spectrum of mammalian cell lines. Furthermore, use of basal media of Eagle requires a gas phase of 5% CO₂ (Freshney, 1994), a condition available in this laboratory. Therefore this synthetic culture medium was selected for use in the methodology described throughout this thesis. MEM (GIBCO) contains 46.0 mg l⁻¹ L-valine. Preliminary investigations in this study identified that use of MEM D-valine (GIBCO) which contains 92.0 mg l⁻¹ D-valine successfully supported the growth of smooth muscle cells, whilst inhibiting growth of contaminating fibroblast cells. Hence MEM D-valine was chosen as the culture medium for use.

In order to confirm that the cultured cells were aortic smooth muscle cells an immunohistochemical stain was applied to the cells. A commercially available kit, utilising the avidin-biotin technique was obtained. This technique is reported to virtually eliminate background staining whilst amplifying the specific reaction (Naritoku & Taylor, 1982). It involved applying a primary antibody, the monoclonal anti-alpha smooth muscle actin, (produced using the NH₂ terminal synthetic decapeptide of α -smooth muscle actin as the immunogen; Skali *et al.*, 1986) to the cells. A secondary, biotinylated antibody was then applied to detect the primary antibody. Upon the addition of a peroxidase reagent, an avidin-biotin complex is formed with the bound secondary antibody. A chromogen is then applied. The bound peroxidase reagent catalyses the oxidation of the chromogen. The resultant colour change, at the antigen sites, is identified under a light microscope (Zeiss), attached to a camera (Olympus PM-6).

The final aim of this section was to determine the functional viability of the cells. It was hoped that a contractile response of the aortic cells could be demonstrated by observing changes in cell shape in the presence of noradrenaline (Ito *et al.*, 1993) and KCl (Kitajima *et al.*, 1996) which induce cell contraction by increasing the intracellular calcium ion concentration ($[Ca^{2+}]_i$), (refer to General Introduction).

3.2. Results

3.2.1. Effect of the primary explant technique and mechanical disaggregation on viable cell yield.

The outgrowth of cells from the primary explanted aorta was investigated. The primary explant technique was the original method developed by Harrison (1907), Carrel (1912) and others for initiating a tissue culture. Cells were desegregated by the procedure described in section 2.3.1. After 14 days in culture, the explanted tissue pieces were removed and of the cells remaining, viable cell numbers were determined by trypan blue exclusion (Tennant, 1964) using a haemocytometer. In six separate experiments, the number of viable cells obtained was consistently below 1×10^3 cells per aorta. Therefore, this method of cell disaggregation was considered uneconomical for obtaining the cell yields needed for experimentation, as cells would have to be plated into at least one multiwell plate (24 wells x 1 ml volume) at a density of 2×10^4 cells ml⁻¹, in order that confluent monolayers of cells could be obtained and used for experimentation. Consequently, the alternative method of enzymatic tissue disaggregation was examined.

3.2.2. Effect of enzyme solutions on viable cell yield.

In these experiments, five different enzyme solutions were prepared in duplicate, in 10 ml Ca²⁺-free physiological solution at 25°C as described in 2.3.3.1. The thoracic aorta from one rat (male, Wistar, 200-250 g) was dissected out, cleaned and chopped (refer to section 2). The pieces were incubated in one of the enzyme solutions at 37°C. After 30 or 60 min incubation with repeated agitation, the action of the enzymes was terminated; FBS (10 ml) was added to the partially digested tissue which was centrifuged at 1000 rpm for 5 min. The pellet was re suspended in another enzyme solution at 37°C. After 30 min incubation with repeated agitation the action of the enzymes was terminated, as before. In some cases, the second incubation was omitted.

The pellet was re suspended twice in 2 ml culture medium. Single cells were obtained by trituration of the tissue through a narrow bore pipette. Mean viable cell yields, determined by trypan blue exclusion (3% v/v) are given in table 3.1.

It can be seen that the cell yields obtained using trypsin (0.01% w/v) for 30 min or collagenase (5.4 units ml⁻¹) for 60 min were 10 or 20 times greater respectively than was achieved using the primary explant technique. When enzymatic disaggregation proceeded from a simple dispersal to a more complex combination of the use of two successive dispersals involving collagenase (5.4 units ml⁻¹) for 60 min followed by trypsin (0.01% w/v) for 30 min a significantly greater

cell yield compared with either enzyme solution alone was obtained. A further increase in cell yield was observed when the collagenase mix, containing collagenase ($5.4 \text{ units ml}^{-1}$), trypsin inhibitor (0.75 mg ml^{-1}) and bovine serum albumin (BSA; 2 mg ml^{-1}) was used. The greatest yield, however, was measured following incubation with the papain enzyme mix, containing papain (7 units ml^{-1}), dithiothreitol (2.5 mM) and BSA (2 mg ml^{-1}).

Table 3.1. Cell yield following incubation in enzyme solutions. Following dispersal of aortic smooth muscle cells, using either one or two enzyme solutions, cell yield was determined using a haemocytometer counting method.

Incubation 1		Incubation 2		Cell yield ($\times 10^4$)
Enzyme solution	Time (min)	Enzyme solution	Time (min)	
trypsin	30	—	—	1.20 ± 1.03
collagenase	60	—	—	2.13 ± 1.31
collagenase	60	trypsin	30	5.53 ± 2.03
collagenase mix	60	—	—	7.90 ± 1.97
collagenase mix	60	elastase	30	6.17 ± 1.76
papain mix	30	—	—	10.10 ± 1.74

Values are mean cell yields \pm s.dev. for 3 experiments ($n=3$).

3.2.3. Effect of papain and collagenase on viable cell yield.

In these investigations, the two enzyme solutions which yielded the greatest number of cells were used in succession. The methodology is described in 2.1.3.3. and was the same as that outlined in the previous section, except that the first incubation was in an enzyme solution containing the papain mix at 37°C followed by the second incubation in an enzyme solution containing the collagenase mix at 37°C . The effect of incubation time was examined. Viable cell yields were measured by trypan blue exclusion (3% v/v).

The greatest cell yield was $2.09 \pm 0.77 \times 10^5$ cells, determined after incubation in the papain mix for 20 min, followed by incubation in the collagenase mix for 15 min, shown in Table 3.2.

Table 3.2. Effect of papain and collagenase on cell yields. Cells were dispersed using two successive enzyme solutions; the first contained papain and the second contained collagenase. The effect of the duration of incubation in either solution on cell yield was determined.

Incubation time (min)		Cell yield ($\times 10^5$)
Papain mix	Collagenase mix	
10	10	0.92 ± 0.34
10	20	1.18 ± 0.37
10	30	1.45 ± 0.43
20	10	1.66 ± 0.39
20	15	2.09 ± 0.77
20	20	1.77 ± 0.41
20	30	1.38 ± 0.58
30	10	1.46 ± 0.50
30	20	1.08 ± 0.57
30	30	0.87 ± 0.40

Values are mean cell numbers \pm s.dev. for 3 experiments ($n=3$).

The combination of successive disaggregation of the aortic smooth muscle tissue using the enzyme solutions containing the papain mix and the collagenase mix for 20 and 15 min respectively was therefore used in all subsequent dispersals.

3.2.4. Growth curve for aortic smooth muscle cells in culture.

The thoracic aorta from four rats (male, Wistar, 200-250 g) were dissected out, cleaned and chopped, according to the methodology described in 2.1.1. The pieces were combined and the cells desegregated using the papain mix and collagenase mix, described in the previous section. Then, a growth curve for the AOCs was determined, according to the methodology described in 2.1.5. Briefly, the cells were transferred to 24 well plates in culture medium at a density of 2×10^4 cells ml^{-1} and were maintained at 37°C in a humidified atmosphere of 5% CO_2 :95%air. At 24 h intervals, culture medium from one well was removed and the cells were trypsinised (described in 2.5) and counted to allow construction of a cell growth curve (Figure 3.1). The curve was used to determine the lag period, doubling time and saturation density (density at which a *plateau* is achieved) of the cells. It can be seen that the lag phase occurred between 2 and 48 h, the logarithmic growth phase occurred between 96 and 240 h and a steady plateau was achieved at 264 h.

Therefore, the time for the cell population to double (T) can be determined from the logarithmic phase of the growth curve, using

$$\log N = \log N_0 + kt \log 2 \quad \text{Equation 1}$$

where $T = 1/k \quad \text{Equation 2}$

and N_0 refers to initial inoculum cell number, N is cell number at time t , k is a regression constant and the factor kt is the generation number (refer to Paul, 1975).

therefore, rearranging Equation 1, at 168 h

$$k = (\log 9.2 \times 10^4) - (\log 2 \times 10^4) / 168 \log 2$$

$$k = 0.01$$

substituting for k in Equation 2,

$$T = 1 / 0.01$$

$$T = 100 \text{ h}$$

Therefore the time for the population of aortic smooth muscle cells to double was 100h.

Cells from primary cultures have been reported to multiply more slowly than cells from primary cell lines (subcultured primary culture) which are reported to double the population in 1.5 to 3 days. This is turn is slow, compared with established cell lines (subcultured at least 70 times) which are reported to have short doubling times, of the order of 12-20 h (Paul, 1975). Therefore, it was inferred that in this investigation, the cells exhibited an acceptable growth rate, and were in the log phase of growth for 7 days.

3.2.5. Immunohistochemical identification of cells

Aortic smooth muscle cells were cultured on three glass slides, in culture medium for 7 days at 37°C under an atmosphere of 5% CO₂, 95% air and then prepared for immunohistochemical staining, described in 2.6, using a commercially available kit (Sigma, IMM-H-2). The procedure was repeated using cells dispersed on two separate occasions.

A photograph of a typical cell is shown in figure 3.2a. The resultant red-brown staining was throughout the cytoplasm of smooth muscle cells only. The nuclei of smooth muscle cells, fibroblasts and endothelial cells stained blue.

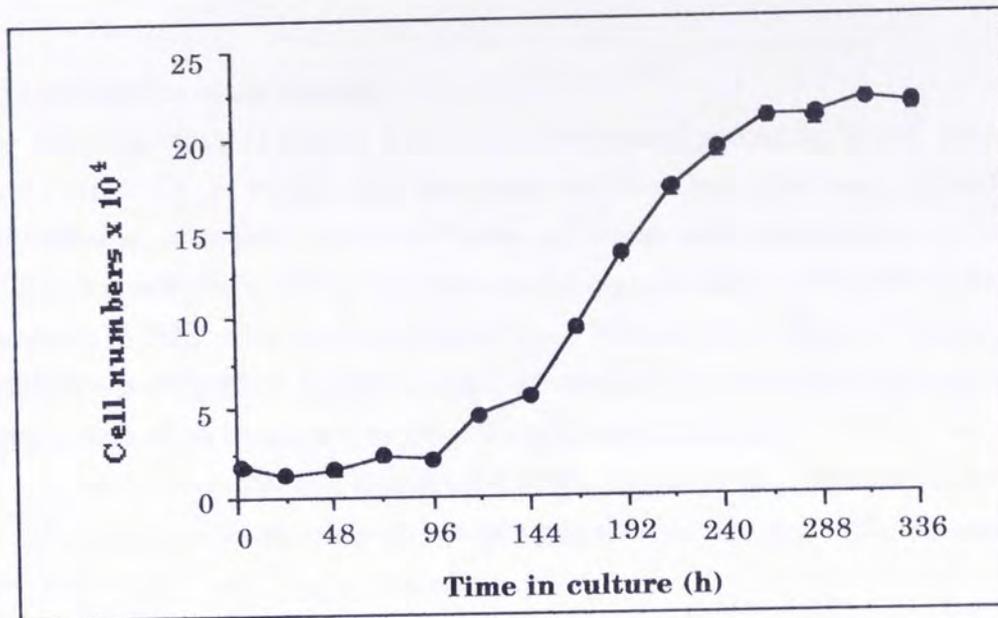


Figure 3.1. Growth curve of aortic smooth muscle cells in culture.

Cells from 4 aorta were isolated using enzymatic disaggregation in a papain mix (7 units ml⁻¹ papain) for 20 min, at 37°C and then a collagenase mix (5.4 units ml⁻¹ collagenase) for 15 min at 37°C. 2 x 10⁴ cells ml⁻¹ were cultured in 24xwell plates for 0 to 336 h. At 24 h intervals, the cells in 3 wells were subjected to trypsinisation. The number of cells per well were counted. Each value represents the mean cell number well⁻¹ ± s.e.mean for 3 experiments (n=3).

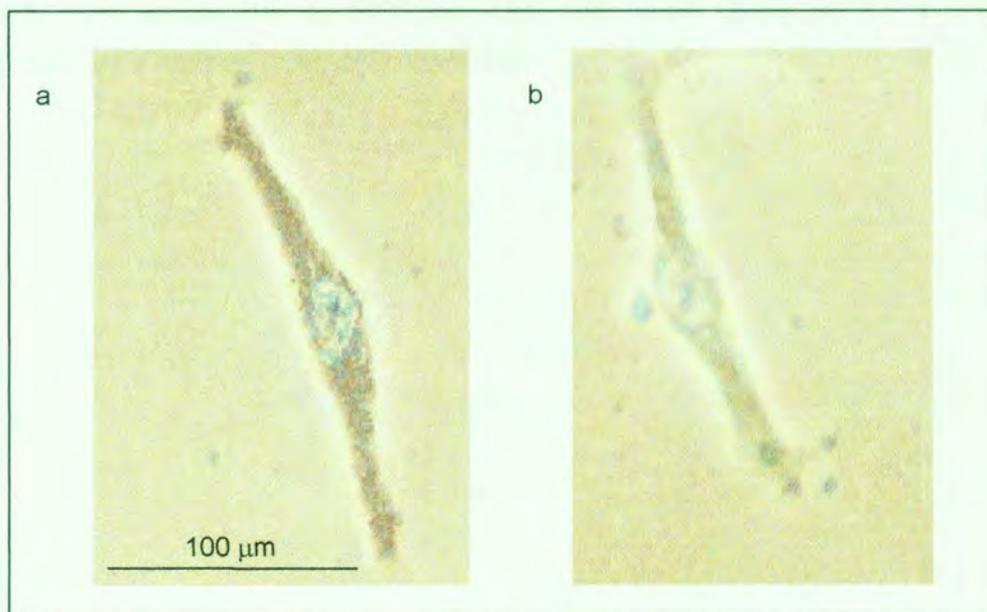


Figure 3.2. Representative photograph of aortic smooth muscle cells. Cells, grown in culture, were stained with monoclonal anti-alpha smooth muscle actin. An avidin-biotin technique was used to identify the smooth muscle cells. The cytoplasm of (a) positive cells was red-brown, whilst control cells (b) did not stain.

3.2.6. Investigation of cell viability

The experiments in this study were performed according to the methodology described in 2.1.7, in which cells desegregated from two aorta were transferred to petri dishes at a density of 2×10^4 cells ml^{-1} and were maintained at 37°C in a humidified atmosphere of $5\%\text{CO}_2$, 95% air for up to 21 days. The cells were washed three times in PSS at 25°C and bathed in 1 ml PSS at 25°C . Single cells, attached to the dish were identified under a light microscope. Preliminary studies involved determination of an average size for cells cultured (Table 3.3).

At 24 h intervals, cell length and width (taken as the widest part of the cell) of 10 cells was measured using an eye piece graticule. By day 8 after dispersal, cell length and width reached a maximum.

The size of cells obtained from separate dispersals was compared, to determine whether inter-dispersal variation existed. At 24 h intervals, cell length and width of 10 different cells was measured. There was no significant inter-dispersal variation measured, except at day 5 of dispersal ($P < 0.05$; $n = 30$), shown in figure 3.3. Therefore, in subsequent experiments, cells were selected for use after 9 days in culture.

To ascertain that the cells were functionally viable, known vasoconstrictor agents were added to the PSS and the cells were observed for a change size, identified as a change in length or width.

Table 3.3. Effect of time in culture on cell length and width. Cells cultured in a petri dish were washed three times in PSS at 25°C and bathed in 1 ml PSS at 25°C. The cells were observed for a change in size, identified as a change in length or width (taken as the widest part of the cell).

<i>Time in culture (days)</i>	<i>Length (μm)</i>	<i>Width (μm)</i>
0 (2 h)	21.5 \pm 7.6	20.0 \pm 8.20
1	29.0 \pm 11.0	25.0 \pm 8.20
2	41.0 \pm 18.5	28.0 \pm 10.2
3	60.0 \pm 30.0	26.5 \pm 10.2
4	83.5 \pm 37.3	31.0 \pm 11.8
5	93.5 \pm 41.4	34.5 \pm 13.7
6	109 \pm 39.0	42.0 \pm 16.0
7	121 \pm 40.0	42.5 \pm 16.3
8	134 \pm 32.3	43.0 \pm 16.1
9	138 \pm 33.8	44.0 \pm 15.2
10	145 \pm 30.6	44.5 \pm 16.0
11	145 \pm 29.3	45.5 \pm 17.8
12	149 \pm 31.4	43.5 \pm 16.9
13	148 \pm 28.6	43.5 \pm 15.4
14	149 \pm 32.1	42.5 \pm 16.8
15	149 \pm 24.3	45.0 \pm 15.3
16	147 \pm 32.6	47.0 \pm 16.1
17	144 \pm 23.9	52.5 \pm 15.1
18	144 \pm 27.2	46.5 \pm 16.4
19	141 \pm 31.2	43.5 \pm 15.4
20	134 \pm 31.1	45.0 \pm 16.2

Values are mean \pm s.e.mean for 10 cells from each of 3 experiments ($n=30$).

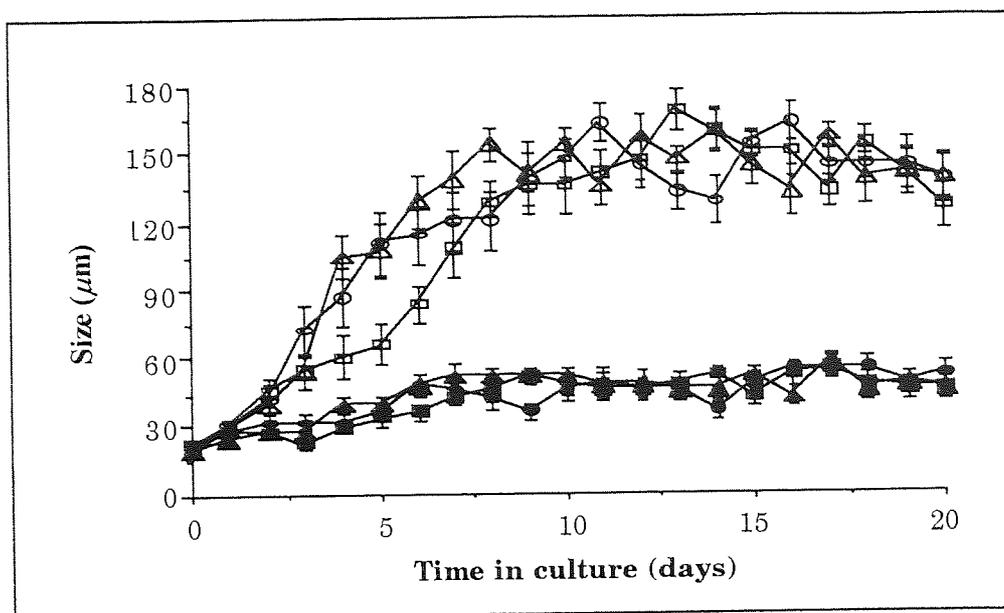


Figure 3.3. Effect of time in culture on size of cells. Aortic smooth muscle cells (AOCs), cultured in a petri dish were washed three times in PSS at 25°C and bathed in 1 ml PSS at 25°C. Cell length (open symbols in upper trace) or width (closed symbols in lower trace) was measured for 10 cells from three separate isolations. Values are mean \pm s.e.mean for \circ isolation 1, \square isolation 2, \triangle isolation 3 ($n=10$).

In cells cultured for 10 days, the effect of a depolarising solution containing KCl (60 mM) was examined on cell length and width to determine whether the cells responded to an influx of extra cellular calcium. Initial experiments were performed to investigate the time course of the contraction of cells in response to 60 mM KCl. Measurements of length were made on six cells taken from each of 4 separate isolations. In each case, cells were exposed to KCl for 300 sec.

The measured percentage changes in cell length and width are shown in Figure 3.4. It can be seen that following an initial reduction in cell length and width at 20 sec, to $87.67 \pm 1.88\%$ and $90.5 \pm 3.07\%$ of the length measured prior to the addition of KCl (60 mM) respectively, there was no further reduction. Therefore, in subsequent experiments, which examined the effect of KCl on cell length and width, the cells were exposed to KCl-containing saline for 60 s.

A primary consequence of these experiments was that the cells contracted, thereby demonstrating their functional viability. Furthermore, the contractile response to KCl indicates the cells were polarised and responded to an influx of extra cellular calcium, presumably evidence of voltage-gated channels.

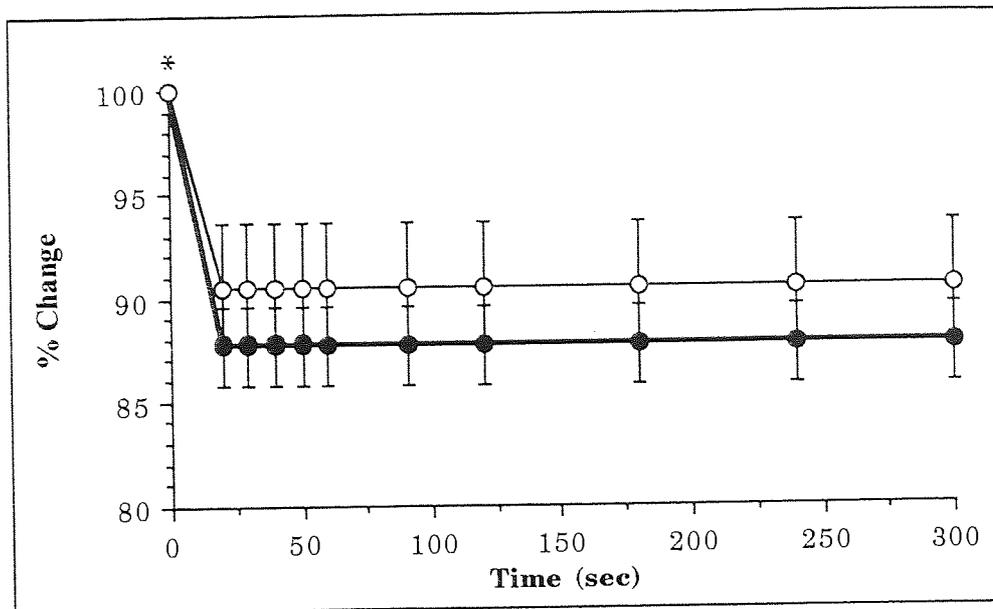


Figure 3.4. Effect of KCl on change of cell size. AOCs in culture for 10 days were bathed in PSS at 25°C containing 60 mM KCl. Changes in cell length (lower trace) and width (upper trace) were measured and expressed as a percentage of length measured before addition of KCl. * indicates significant difference from values measured after KCl. Values are mean \pm s.e. mean of 6 cells from each of 4 isolation procedures ($n=24$).

The next study was designed (i) to identify whether the effect of KCl (60 mM) on the cells was reversible and (ii) determine whether the response by the cells was repeatable. Cells were used between 0 and 20 days in culture. Measurements were taken for 3 different cells at each time point. This was repeated for cells taken from each of 3 dispersals. For cells which remained in culture for longer than 1 day prior to experimentation, the mean length of the cells in the presence of KCl (60 mM) was reduced. The reduction was reversed following washout. Reduction in cell length was repeatable in the presence of a subsequent application of KCl (60 mM) to the PSS (figure 3.5).

Changes in the width of cells when exposed to KCl were less clear. No change was observed in the presence of KCl (60 mM) in cells used between 0-5 days in culture. Width was reduced in cells used between 6-20 days but recovery of width during the washout period was not apparent in all cells examined. Further shortening in width in the presence of a subsequent application of KCl to the PSS was not consistently observed.

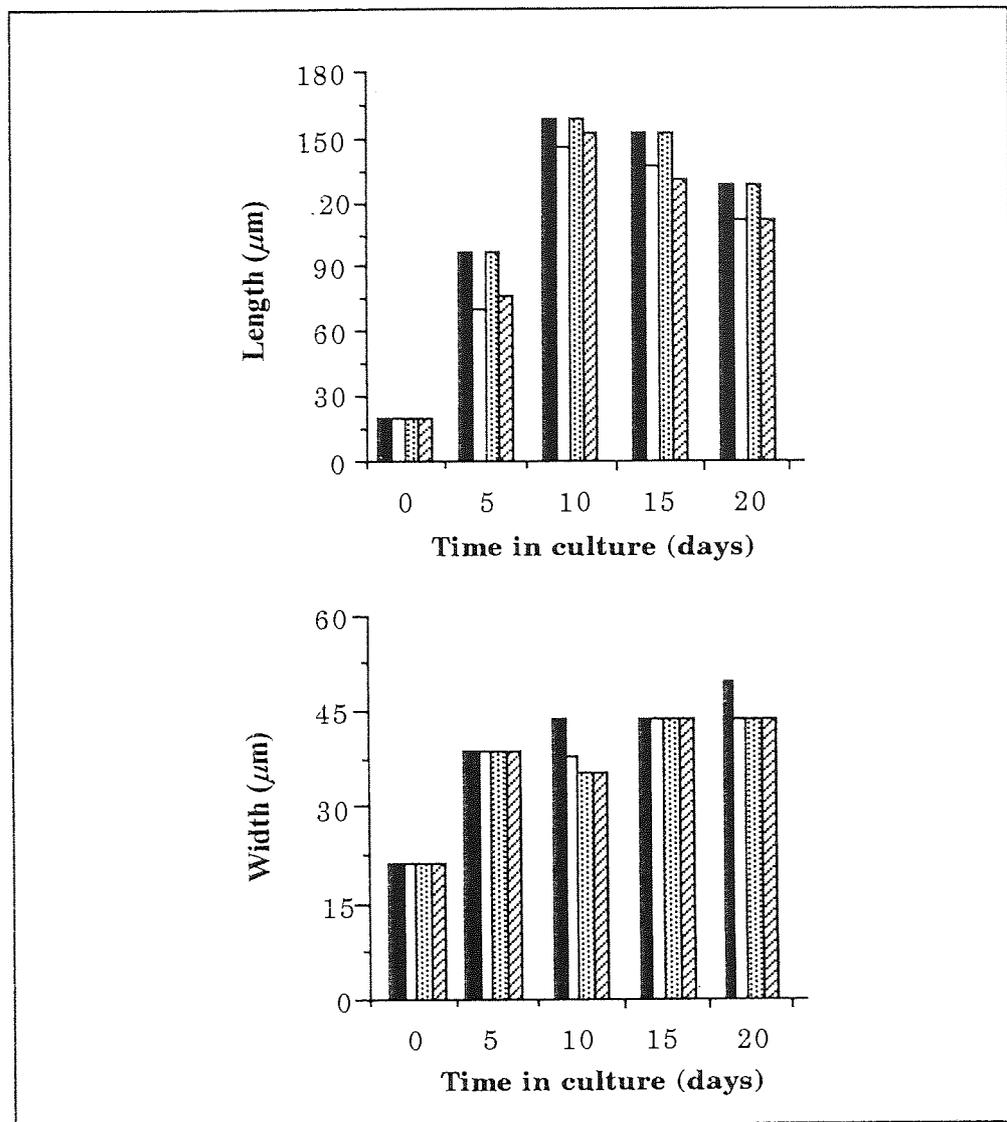


Figure 3.5. *The effect of KCl (60 mM) upon cell length (upper graph) and width (lower graph) of aortic cells cultured for up to 20 days. At 24 h intervals, a sample of which is shown above, cells were bathed in PSS at 25°C and cell length measured after 60 sec ■. KCl (60 mM) was added to the PSS and cell length measured after 60 sec □. Cells were then washed three times in PSS at 25°C and cell length measured ▤. KCl (60 mM) was reintroduced into the PSS and cell length was measured after 60 sec ▨ (n=9).*

The next study examined the effect of noradrenaline (NA) on cell length and width to determine whether alpha-adrenoceptor-mediated contraction could be demonstrated in the cell preparation. Cells at 10 days in culture were exposed to NA ($1 \mu\text{M}$) for between 0 to 300 sec. The measured percentage changes in cell length and width are shown in Figure 3.6. It can be seen that following an initial reduction in cell length and width at 20 sec, to $80.2 \pm 3.37\%$ and $89.2 \pm 0.50\%$ of the length and width measured prior to the addition of NA ($1 \mu\text{M}$) respectively, there was no further reduction. Therefore, the results suggested that the cells have functional α -adrenoceptors.

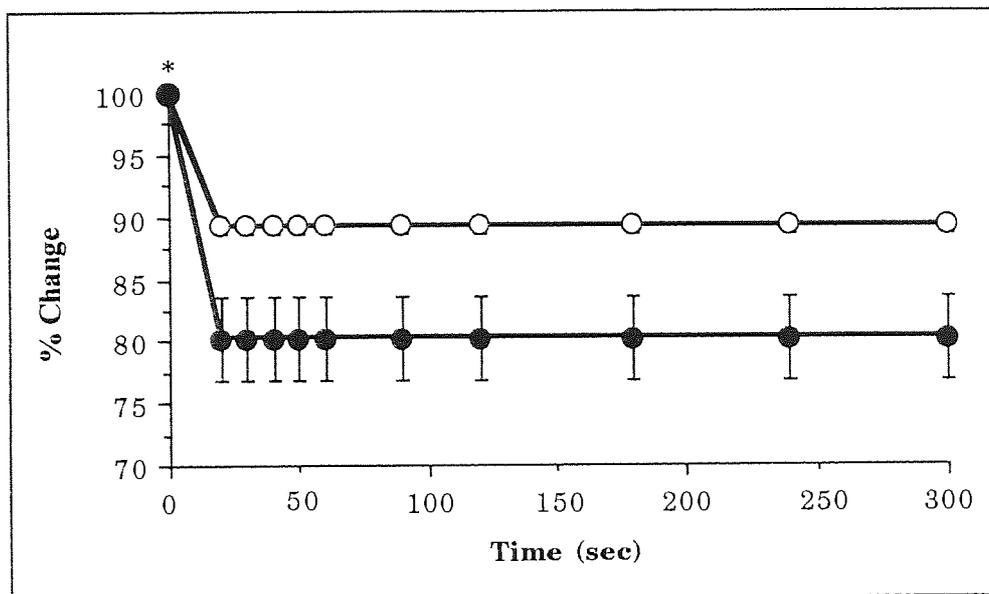


Figure 3.6. *Effect of noradrenaline on change of cell size.* AOCs in culture for 10 days were bathed in PSS at 25°C in the presence of noradrenaline ($1 \mu\text{M}$). Changes in cell length (lower trace) and width (upper trace) were measured and expressed as a percentage of length measured before addition of KCl. * indicates significant difference from values measured after noradrenaline. Values are mean \pm s.e.mean of 6 cells from each of 4 isolation procedures ($n=24$). Cell length (upper trace) and width (lower trace) were measured.

3.3 Discussion

To understand the physiology of smooth muscle, attempts were made in the 1960s and early 1970s to study the properties of the ionic channels of the smooth muscle cell membrane by means of voltage clamping applied to small strips of smooth muscles. However, this posed a number of problems including the interpretation of results for multi-cellular preparations, the presence of a large series resistance that arises from the narrow extra cellular cleft between the elongated cells, the diffusional barriers associated with the extra cellular matrix and the accumulation of intracellular ions, all leading to an uncertainty about the ionic currents recorded. Therefore, to avoid the complexities associated with strip preparations, it became desirable to isolate single vascular smooth muscle cells (Bolton *et al*, 1985) which would allow direct assessment of cellular mechanics and pharmacology (Yamada *et al*, 1992). However, there are also disadvantages associated with cell culture, attributed largely to the isolation and culture techniques used. Culture techniques must be carried out under strict aseptic conditions to prevent contamination from bacteria, mould or yeast which may grow more quickly than animal cells. Furthermore, provision has to be made to overcome problems encountered in obtaining the desired isolated cell population and skill is required in selecting and providing the requirements of the cell system. As a consequence of these difficulties, relatively little tissue is often produced at the expenditure of much effort and materials.

Once in culture, cell modulation may occur (Kocher *et al*, 1984). For example, the daily cell replication rate of normal, quiescent (non-growing) cells in vascular smooth muscle is 0.01% compared to 5 or even 10% *in vitro* (Jackson & Schwartz, 1992). Moreover, in obtaining isolated cells, it should be remembered that there will inevitably remain differences between single cells and the tissue present *in vivo*. One important property is the ability of the cells to contract. It has been reported that smooth muscle cells which have migrated from explants or are in subculture are not capable of contraction in response to neurotransmitters such as angiotensin II or noradrenaline (see review by Chamley-Campbell *et al*, 1979). Alternatively, the same review also reported evidence that noradrenaline was able to cause non-spontaneously active smooth muscle cells from enzymatically dispersed rat aorta to contract. Therefore it would appear that the cell isolation technique is an important factor in successfully producing viable cells. Variation in functional viability was examined by Groeschel-Stewart *et al*, (1975) who reported that the ability of enzymatically dispersed smooth muscle cells to contract varied with length of time in culture. They showed that the cells retained their contractile state for between 1-4 days following dispersal but by 3-5 days, modification of the phenotype

was commencing so that by 6-8 days in culture, the ability of the cells to contract was absent. They reported that by day 8, the proliferation rate of the cells was immense and at 9-16 days, the cells re-differentiated and resumed the contractile state observed at 1-5 days. The reported inability of cells to contract between 6-9 days is consistent with a report by Chamley *et al*, (1977) who documented that at 7-9 days in culture, isolated smooth muscle cells had lost most of their myosin, their capacity to contract and had assumed a morphology similar to that of fibroblasts. This, they termed de-differentiation.

Based on such reports therefore, it would appear that primary cultures, at a confluent level in culture between 9-16 days could be a useful experimental model for the study of excitation-coupling in vascular smooth muscle at the cellular level. Furthermore, it has been reported that the proportion of non-responsive cells to agonists increases with the age after dispersal, of the primary culture (Bodin *et al*, 1991). In support of this, Pavenstadt *et al*, (1991), used aortic cells which were obtained by primary culture, but were passaged and subcultured a number of times so that the cells used for experiments were from long term cultures. It was found that whilst they were able to measure reasonable membrane potentials of -40mV to -60 mV and also a dominating potassium conductance from the cells, they were only able to identify large, non-selective cation channels when using the cells in patch-clamp studies. Hence they were prompted to use cells from primary cultures which had not undergone subculture (Pavenstadt *et al*, 1991).

The use of a cell line was considered but not adopted for the present study as there are reports that after the early stages of culture, when the cells remain euploid (i.e. have the correct diploid complement of chromosomes), the cells later become aneuploid (Chamley-Campbell *et al*, 1979). Furthermore, continuous cell lines have a higher rate of mycoplasma infection than primary cultures (Freshney, 1994).

There is evidence that low serum presented to smooth muscle cells in culture promotes the contractile status of the cells (Li *et al*, 1994). This was confirmed by Jackson & Schwartz (1992) who reported the existence of a relationship between the growth state of the cells and their ability to bind dihydropyridine calcium antagonists to L-type calcium channels. As a consequence, in the present study the cultured cells were exposed to 10% v/v foetal bovine serum in the culture medium, but not exposed to serum during the experimental procedures.

In embryonic or young animals, vascular smooth muscle cells exist largely in the proliferative state during development. As the animals mature, the cells convert to the contractile phenotype (Li *et al*, 1994). This change is described as differentiation, which generally can be defined as the change in morphology and function that occurs during development and involves the cell altering from an

initial multipotential state to the specialised form of the adult (Chamley-Campbell *et al*, 1979).

Enzymatically desegregated smooth muscle cells have been reported to maintain their differentiated morphology and contractile state in primary culture if the dish is seeded sufficiently densely (Chamley-Campbell *et al*, 1979). Therefore, in the present study, cells were seeded at 2×10^4 cells ml^{-1} to ensure the cells reached confluency by the time they were to be used for experimentation, which was between 7-14 days after disaggregation. Based upon the report by Gröeschel-Stewart *et al* (1975), it was inferred that the cells would have re-differentiated and resumed the contractile state at this time. Following subculture, a process involving trypsinising the cells, the cells appear ultra structurally modulated; they have the morphological structure similar to that of fibroblasts (Chamley-Campbell *et al*, 1979). In the event that a culture is so sparsely seeded that it takes more than 3 weeks for the cells to achieve confluency, the initial contractile phenotype is never regained (Chamley-Campbell *et al*, 1979).

Different substrates have been used to support the growth of isolated cells. These have included Matrigel, a solubilised basement membrane matrix extracted from Englebreth-Holm-Swarm mouse sarcoma, and which has laminin as its main component (Li *et al*, 1994). Matrigel has been used to maintain highly differentiated phenotypes in culture for a number of cells, including smooth muscle cells, as it is reported that it promotes the constrictor state of the cells (Li *et al*, 1994). An alternative substrate was used by Tosseli *et al* (1984) in which collagen-HEMA (2-hydroxyethyl methacrylate) hydrogels were incorporated into successful studies on cell-to-cell substrate contact formation. However, a substrate used to coat cell culture flasks or multiwell plates in order to ensure the growth of aortic smooth muscle cells was not incorporated into the culture procedures used in the studies in this thesis.

In attempting to isolate aortic smooth muscle cells, the initial difficulty to overcome was the identification of the aortic layer of smooth muscle cells to be separated from the overlying *adventitia*. Preliminary investigations involved the removal of thin layers of aorta from the outer side which were then examined under a light microscope and the cells obtained were compared with the literature on the subject. With time and practice, distinguishing the different layers was possible.

Media are based on a balanced salt solution which is important in supplying essential ions to the cells and maintaining osmotic balance in the cells. It is important to maintain the correct pH of growth media between 7.2-7.5 (refer to Adams, 1980) at 37°C and this is generally achieved in the culture media presently in use today by the sodium ion and phosphate concentration in the salt solution. Basal media of Eagle, based on Earle's basal salt solution, contains sodium chloride

to maintain the osmotic balance of the cells and sodium bicarbonate and phosphate to achieve a pH of around 7.4 in the presence of 5% CO₂, provided in the incubation chamber. Minimum Essential Medium (MEM; GIBCO Life Sciences) was patterned after basal media of Eagle. It is apparently well suited for the growth of a broad spectrum of mammalian cell lines. Furthermore, the modified, MEM D-val culture medium, has the amino acid D-valine substituted for L-valine. This is because D-valine supports the growth of smooth muscle cells, whilst inhibiting growth of contaminating fibroblast cells (GIBCO). Therefore, based on such considerations, preliminary investigations were performed in this study, which confirmed the choice of MEM D-val (GIBCO) culture medium to be optimal for the primary culture of vascular smooth muscle cells.

Calcium and magnesium ions are essential for the attachment of cells to a glass or plastic surface and therefore were omitted from the physiological salt solutions used to desegregate the aorta and to disperse cell monolayers. Also included in the composition of these salt solutions was 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (HEPES) which is an organic buffer used instead of bicarbonate as an extra cellular buffer to maintain pH in the range 7.2-7.8 (Freshney, 1994) and which is non-toxic to cells.

The primary explant technique was the original method developed by Harrison (1907), Carrel (1912) and others for initiating a tissue culture. In their investigations, a fragment of tissue was embedded in blood plasma or lymph, mixed with embryo extract and serum and placed on a slide or coverslip. The plasma clotted and held the tissue in place. The embryo extract plus serum both supplied nutrients and stimulated migration out of the explant across the solid substrate. This technique is particularly useful for small amounts of tissue where there is a risk of losing cells during enzymatic disaggregation. However, in this study, outgrowth from the explants was observed to be a relatively slow process and repeatedly yielded too few viable cells ($< 1 \times 10^3$) for use in experiments. Cells were required in larger yields to be used in adequate numbers in assays, so enzymatic disaggregation was performed.

To achieve effective disaggregation of a tissue, the exposure time of the disaggregating enzyme solution to the cells may vary between tissues (Freshney 1994). The aim in the present study was to ensure that any technique adopted was readily repeatable and consistent. To this end, set incubation times were determined and maintained whether or not there was incomplete disaggregation of the tissue. Efforts were made to optimise the yield of cells in the knowledge that procedures of greater severity which would be required to completely desegregate the tissue may destroy the more fragile components of the tissue. Despite the widespread use of enzymes in the procedure of cell isolation and dispersal, there is

immense variation in the composition of the enzyme solution, the length of time of the incubations, the number of incubations performed and the choice of enzymes selected. It was noted from the literature that the use of papain has been used more frequently when dispersing veins compared with arterial preparations. For example, Beech & Bolton (1989) used papain during the dispersal of portal mesenteric vein whilst Byrne & Large (1988) used it during dispersal of hepatic portal vein. In the dispersal of arterial preparations, however, various combinations of collagenase and elastase have frequently been used (Worley *et al*, 1986, Aaronson *et al*, 1988, Renterghem *et al.*, 1988; Yamada *et al*, 1992). However, the quantities of enzymes used have also varied considerably, as have the incubation parameters. When attempting to determine the most appropriate conditions for use in the present study, it was difficult to compare the conditions used by different researchers as they often quoted quantities of enzymes in units ml⁻¹, mg ml⁻¹, units mg⁻¹ or even as mg alone, the latter without referring to the volume in which the enzyme was placed. Furthermore, incubation times varied widely, ranging from one 30 min incubation at 37°C in the case of Yamada *et al* (1991) to a 30 min incubation at 37°C after which time the adventitia and intima were removed, prior to the remaining smooth muscle layer being incubated overnight, under a CO₂ atmosphere, followed by a further incubation in collagenase for 60-90 min (Bodin *et al*, 1991). What became apparent from these and other reports was that there was little consistency in the technique applied, probably arising out of the fact that the isolated cells were subsequently used for a diverse range of experiments.

Therefore, an initial aim of the present study was to determine the optimum procedure for the disaggregation of aortic smooth muscle cells. Two sequential digestion's in a papain-containing solution for 20 min at 37°C, followed by incubation in a collagenase-containing solution for 15 min, also at 37°C was identified as the optimum enzymatic disaggregation technique for the primary culture of the cells.

Following disaggregation of the tissue, a growth curve for aortic smooth muscle cells was constructed. It has been reported previously (Chamley-Campbell *et al*, 1979) that smooth muscle cells reached a stationary growth phase by 6 weeks in culture. However, in the present study, the rate of growth did not increase after 10 days. This may have been a consequence of the growth conditions presented to the cells, in that the cells were not subcultured and had reached confluency by this time.

An important characteristic of contractile vascular smooth muscle cells is their low growth rate compared with cells in the proliferative phenotype (Li *et al*, 1994). In the present investigation, the doubling time of the cell population was 100

h, despite previous reports of doubling times at a rate of almost twice this (Li *et al*, 1994). However, in the latter report, the cells were shown to be in the proliferative and not the contractile state, shown by [³H] thymidine uptake experiments. Therefore, the growth rate for the cell population in the present investigation was considered acceptable for vascular smooth muscle cells grown in culture, but it was noticed that the lag period of growth of 5 days extended beyond the expected 48 h for cells. This is considered a long lag (Freshney, 1994) and implies that the culture is having to adapt. However, it has been documented that optimum lag periods vary with cell type (Chamley-Campbell *et al*, 1979) and that characteristically longer lag times are not necessarily deleterious to the viability of the cells.

The *media* of mammalian blood vessels consists entirely of smooth muscle (Chamley-Campbell *et al*, 1979). It follows therefore, that when the *adventitia* and *intima* are mechanically removed, pure smooth muscle cell cultures could be obtained. Risk of contamination cannot be completely eliminated, however, therefore the ability to distinguish smooth muscle cells from fibroblasts and endothelial cells is important.

To this end, cellular purity can be measured using antibodies against known cellular components (Chamley-Campbell *et al*, 1977). Many antibodies prepared against proteins from a variety of sources allow immunological cross reaction. However, subtle but important differences in the primary structure of contractile proteins of muscle and non-muscle cells must exist as their properties can vary considerably. Such antibodies against smooth muscle myosin give an intense reaction with differentiated smooth muscle cells, but not de-differentiated smooth muscle cells nor fibroblasts (Chamley-Campbell *et al*, 1977). Therefore this antibody cannot distinguish between the latter two. However, muscle and non-muscle actin differ biochemically and therefore are not identical. Therefore, antibodies against smooth muscle actin are specific and give an intense reaction to differentiated and de-differentiated cells, but not fibroblasts or endothelial cells (Chamley-Campbell *et al*, 1977). Monoclonal anti smooth muscle actin is a homogenous population of antibody molecules which may be used for immunocytochemical localisation of the alpha smooth muscle actin by means of immunoperoxidase, immunofluorescence, ELISA and immunoblot techniques (Skalli *et al*, 1986). In the present study, the immunoperoxidase technique was used on the isolated smooth muscle cells, which reacted intensely when treated with antibodies against smooth muscle actin. Cultures did not stain endothelial or fibroblast cells. Thus, a clear distinction was made between the cells, making it a useful tool to identify that the cultures in the present study were >95% pure.

To establish the viability of the desegregated cells, it was considered appropriate to identify an effect of vasoactive agents on the contractile response of

the cells. Techniques previously used to measure this response have varied. Bitar & Makhlof (1982) measured contractile response in smooth muscle of the guinea pig stomach. The technique adopted by these workers involved exposing the cells to the test agent and measuring cell length after the cells had been fixed. Similarly, Warshaw *et al.*, (1986) determined cell contractile responses by measuring cell length after fixation as did Fay & Singer (1977) who exposed cell suspensions of freshly dispersed tissue to a vasoactive agent, fixed cells and then pulse amplitude histograms were generated to assess the contractile state of the cells. An alternative methodology was described by Bodin *et al.*, (1991) in which cell contractions were measured using the surface area of cells generated by microscope images, before and after the test agent was applied.

In the present study, neither of the techniques involving cell fixation, or measurement of cell surface area were selected for use. It was considered that fixation might prevent the identification of recovery and reproducibility of the contractile state in the same cell when exposed to a vasoactive agent. Also, it was considered imperative that in taking surface area measurements generated by microscope images, account should be taken of the changed three-dimensional configuration observed following exposure of cells attached to a substrate to vasoactive stimuli.

It was concluded, therefore, that due to the limitations imposed by equipment availability, the contractile response in the present investigation was taken as the decrease in the average length of a population of aortic smooth muscle cells exposed to KCl (60 mM) or noradrenaline (1 μ M). Average cell width (taken as the widest part of the cell) was also measured, to take into account the effect of the test agent upon cell attachment to the substrate.

It has been reported that variability in cell size occurs following dispersal (Warshaw *et al.*, 1986) as does the reproducibility of measuring the contractile responses in smooth muscle cells (Bodin *et al.*, 1991). In the present study, it was shown that generally, there was no significant effect of dispersal on cell length measured. Furthermore, the effect of length of time the cells were in culture was examined in the present study and it was found that at 8-20 days in culture, there was no significant difference in cell length measured. This is consistent with a report by Bodin *et al.*, (1991) who found that there was no significant differences in the size of cells at two, three, four or five weeks old. Bodin *et al.*, (1991) also examined the reproducibility of measuring the contractile responses in rat aortic smooth muscle myocytes using three primary cultures. Values were not significantly different in the preparations used.

In this study, it was demonstrated that exposure of the cells to KCl (60 mM) or noradrenaline (1 μ M) produced contraction of the cells. This confirmed the

viability of the cultured cells. Maximal reduction in length occurred following the application of KCl (60mM) for a period of 30 s. The cells remained in this contractile state for at least 5 min. Previous reports have shown a similar but not identical response to KCl: in the presence of 40 mM KCl, only 50% of the maximal constrictor effect of aortic smooth muscle cells was reached after 30 s (Bodin *et al.*, 1991). Furthermore, these workers reported that the cells returned to their original length after 3-5 min exposure to KCl (40 mM). Another report demonstrated a peak in the contractile response of smooth muscle cells to 75 mM KCl after only 15 secs (Fay & Singer, 1977) and they also showed that the value was maintained at >80% peak for the duration of the experiment which was 60 s but reported that the cells remained contracted for over 4 min.

Likewise with the results to noradrenaline. In the present study, the cells achieved maximal reduction in length following exposure to noradrenaline (1 μ M) for 20 s. The cells remained in this contractile state for at least 5 min. Bodin *et al.* (1991) reported however, that noradrenaline (10 μ M) took 3-4 min to reach 50% peak response and from the studies performed using serotonin (0.1 mM), angiotensin II (0.1 μ M) or vasopressin (1 μ M), thereby concluded that responses to agonists were always slower than that of KCl.

It should be noted, however, that the contractile response to vasoactive agents has been reported to vary according to the cell type used and differences have even been observed according to the topological origin in the aorta (Bodin *et al.*, 1991).

It was also shown in this study that cell width (taken as the widest part of the cell) was reduced following exposure to the vasoactive agents, although less clearly than that of cell length. The contractile filaments in smooth muscle cells are aligned with the long axis of the cell (Alberts *et al.*, (1983) resulting in an overall observed reduction in length and increase in height in response to a vasoactive stimulus. However, in the cell culture system used in the present investigation, all cells were adherent to the culture dish surface at the commencement of experimentation. Upon exposure of the cells to KCl or NA, the outer part of the cell would detach from the surface of the dish during the contractile response. This resulted in an observed reduction in cell width in these cells.

In the present study, it was also demonstrated that the effect of vasoactive agents on the aortic smooth muscle cells in culture were reversible. The contractile response to KCl (60 mM) was eliminated when the cells were exposed to a physiological saline in the absence of KCl. When KCl was subsequently re-exposed to the cells, a contractile response was observed. This served to confirm that the enzymatically desegregated cells were functionally viable.

A number of workers have reported that in contractile studies performed in isolated smooth muscle cells, there was a change in the dose-response relationship for vasoactive agents, with reduced (Warshaw *et al*, 1986), greater (Bitar & Makhlouf, 1982) or equal (Morgan, 1987) sensitivity compared to that observed in intact tissue preparations. In the present investigation, the time course of the peak contractile response to KCl (60 mM) was similar to that obtained in the intact aorta preparation whereas the peak response to noradrenaline (1 μ M) was achieved more quickly than the intact tissue (chapter 7). The selective enhancement of sensitivity to noradrenaline may imply an increased sensitivity to agonist stimulation.

In conclusion, therefore, it is reasonable to suggest that when cells *in vitro* express their normal functions, an attempt can be made to relate them to their tissue of origin. In this chapter it was shown that primary cultured aortic smooth muscle cells (AOCs) express their normal functions; the cells retained their ability to respond to the vasoactive agents KCl and noradrenaline, shown by a change of the shape of the cells. Furthermore, by allowing the culture to reach confluency prior to experimentation, the closest morphological resemblance to the parent tissue was achieved. Subject to certain limitations, therefore, evidence has been presented that the cells are a viable experimental system in which to further examine the effect of EPA upon the contractile mechanisms linked to both calcium influx and the mobilisation of intracellular calcium in vascular smooth muscle. The main limitation of the system is that there may be differences in the sensitivity of the responses achieved in the AOCs compared with the aortic tissue *in vivo*. Nevertheless, the system has the potential to allow direct assessment of cellular mechanics and pharmacology and avoids the interpretative complexities associated with multicellular preparations.

Chapter 4

The Determination Of EPA-Induced Vasorelaxation In Primary Cultured Aortic Smooth Muscle Cells.

4.1. Introduction

Dietary supplementation with fish oil has been shown to reduce the incidence of coronary heart disease and atherosclerosis (Bang *et al*, 1976; Dyerberg *et al.*, 1978; Kromann & Green, 1980) and exert an antihypertensive effect (Knapp & Fitzgerald, 1989; Bonaa *et al*, 1990). These effects have been attributed to n-3 fatty acids, the biologically active components of fish oil. Investigations designed to explain the antihypertensive effect have demonstrated that EPA, an n-3 fatty acid found in fish oil, mediates a vasorelaxatory response in isolated vascular preparations including coronary arteries (Shimokawa & Vanhoutte, 1989), the perfused rabbit ear artery (Juan & Sametz, 1986), the rat and monkey mesenteric vascular bed (Mano *et al*, 1995) and also the thoracic aorta (Lockette *et al*, 1982; Smith *et al*, 1992; Engler *et al.*, 1992a; 1992b; 1994; Engler, 1990, 1994). The vasorelaxatory response has been attributed to a number of endothelium-dependent or endothelium-independent mechanisms. Endothelium-dependent relaxations and a reduction in endothelium-dependent contractions in normal, atherosclerotic and hypercholesterolaemic arteries has been reported (Harris-Hooker *et al.*, 1983; Shimokawa & Vanhoutte, 1989) and it has been proposed that n-3 fatty acids increase endothelial nitric oxide levels (Shimokawa & Vanhoutte, 1989; Shimokawa *et al*, 1987; Schini *et al*, 1993; Joly *et al*, 1995), leading to activation of smooth muscle guanylate cyclase, thereby inhibiting vascular tone (Furchgott & Vanhoutte, 1989). Alternatively, an endothelium-independent vasorelaxant action has been shown (Juan *et al*, 1987; Engler *et al*, 1992a; Engler, 1990; Bretherton *et al*, 1995; 1996a; 1996b; Hallaq *et al*, 1992; Pepe *et al*, 1994; Bregestovski *et al*, 1989; Smith *et al*, 1992, Asano *et al*, 1997; Gardener *et al*, 1998). Also, n-3 fatty acid incorporation into the phospholipids of cell membranes with subsequent modification of membrane-bound protein function including receptors (Locher *et al*, 1989), ion channels (Ordway *et al*, 1989; Bregestovski *et al*, 1989; Asano *et al*, 1997) and enzymes (de Jonge *et al*, 1996), or alteration of the balance of prostaglandins involved in constrictor and relaxatory actions (Fischer & Weber, 1984) have been reported.

Therefore, the aim of the present chapter was to examine the effect of EPA on the contraction of vascular tissue, devoid of endothelium. Furthermore, EPA has been shown to exert effects on single cells (Asano *et al*, 1997, 1998), so the method developed for isolating and culturing large numbers of viable single aortic smooth muscle cells (AOCs) described in the previous chapter was used to obtain the vascular tissue for use in this study.

The concentration of PUFAs used in studies on membrane signal transduction range from as low as 1.4 μM to 400 μM . However, it has been documented that concentrations of 50-100 μM of arachidonic acid are within the physiological range (Sumida *et al*, 1993) and furthermore, that the normal plasma concentration of EPA is 120 μM (Asano *et al*, 1998). Previous investigations into the vasorelaxatory mechanism of action of the n-3 PUFAs, performed by colleagues working in the same laboratory as the author have used EPA and DHA at 50 μM , therefore, the concentration of EPA used throughout all investigations in this thesis was 50 μM , which was considered to be within the physiological range.

The method to obtain AOCs, growing in culture on culture plates, involved removing the endothelium from the aortic tissue, followed by a two-stage, enzymatic disaggregation technique, whereby the tissue was exposed firstly to collagenase and then to papain. When exposed to the vasoactive agents noradrenaline (1 μM) or KCl (60 mM), the resultant AOCs contracted, measured in terms of the length and width of the cells. It was also shown that the response was reversible following removal of the vasoactive agent and was repeatable if the cells were subsequently exposed to the agent, which indicated that the AOCs were a viable system in which to perform this investigation.

The objectives of the chapter were to determine whether pre-contracted AOCs would relax when exposed to EPA and whether pre-treatment of the AOCs with EPA would prevent contraction following exposure of the cells to KCl or noradrenaline. In addition, the effect of EPA was compared with that of nifedipine, a dihydropyridine calcium channel blocker, widely recognised to inhibit the response to KCl on vascular smooth muscle and phentolamine, a reversible competitive antagonist of α -adrenoceptors which causes vasodilation and is a recognised blocker of the contractural response to noradrenaline in vascular smooth muscle.

In performing these studies, the methodology described in 2.8 was followed. In summary, the methodology involved culturing cells for 10 days, then after 2 min equilibration in PSS at room temperature (as no perfusion system to maintain a temperature of 37°C was available), cell length and width (taken as the widest part of the cell) were measured using an eye piece graticule. KCl (60 mM) or noradrenaline (1 μM) was then added to the PSS, which contained propranolol (1

μM) to inhibit β -adrenoceptors, ascorbic acid ($50 \mu\text{M}$) to prevent oxidation of catecholamines and EDTA ($10 \mu\text{M}$) to chelate heavy metal impurities, where all concentrations given are final concentrations. After 1 min, cell length and width were measured again. The effect of EPA upon the KCl or noradrenaline response was examined by adding a single dose of EPA ($50 \mu\text{M}$) to the PSS after 1 min of the equilibration. In some experiments, nicardipine ($0.1 \mu\text{M}$) or phentolamine ($1 \mu\text{M}$) was used instead of EPA to confirm that the block of the vasoconstrictor response of the AOCs was mediated by reducing intracellular calcium ion concentrations $[\text{Ca}^{2+}]_i$.

Cell length and width after exposure to KCl, noradrenaline or EPA was assessed as the percentage reduction in cell length and width measured prior to exposure. All responses are given as the mean \pm s.e. mean for n cells. Statistical comparisons were made by Student's two-tailed t test for paired or unpaired data as appropriate and significance was identified at $P < 0.05$.

4.2. Results

4.2.1. Effect of EPA on the KCl-induced response.

AOCs, were grown on coverslips, in culture for 10 days as described in 2.4. One coverslip was removed from the petri dish and transferred to another dish containing 5 ml PSS at 25°C. After 2 min equilibration, cell length and width were measured. KCl (60 mM) was then added to the PSS (depolarising PSS; PSS_d). After 1 min, based on the results in the previous chapter (see figure 3.4), cell length and width were measured again. The effect of EPA upon the KCl response was examined by adding a single dose of EPA (50 μ M) to the PSS after 1 min of the equilibration. A diagram to outline the drug additions is summarised in figure 4.1.

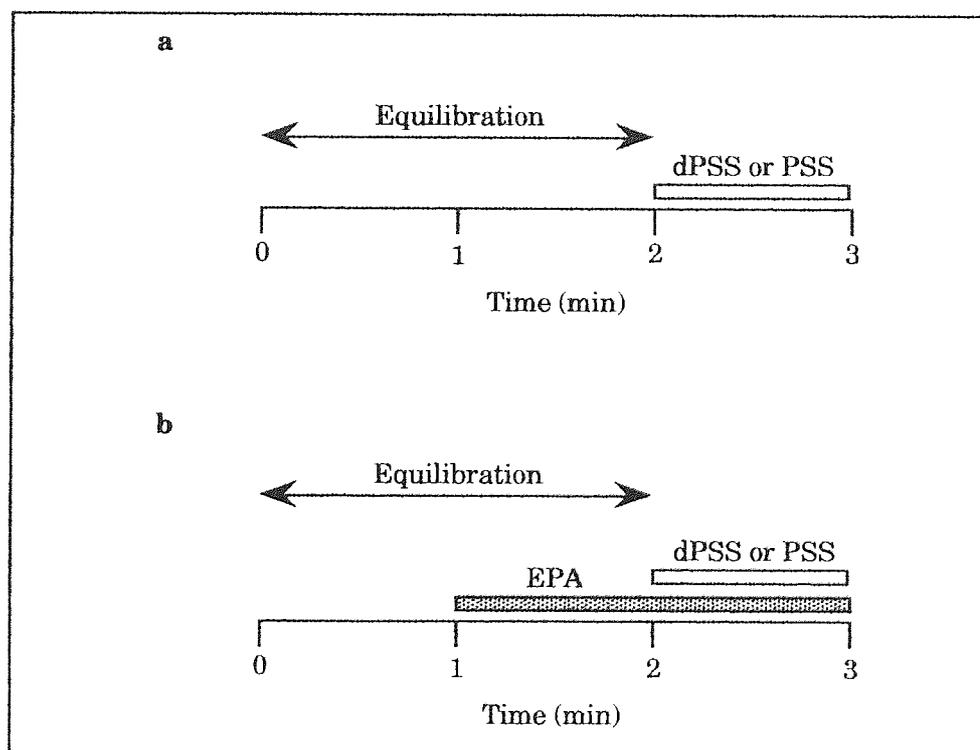


Figure 4.1. Diagram to outline the experimental procedure used to investigate the effect of EPA upon the KCl-induced response of AOCs. *a* Effect of KCl. *b* Effect of EPA on the KCl response. In some experiments, the addition of EPA (50 μ M) and KCl (60 mM) to the bathing solution were reversed.

Exposure of the cells to PSS_d (60 mM KCl) for 1 min significantly ($P < 0.05$) reduced cell length from 162 ± 6.6 ($n=9$) to 138 ± 5.7 μm ($n=9$) with no change in width observed, shown in Figure 4.2.

Cell length and width after EPA alone (158 ± 7.2 ; $n=9$ and 39 ± 4.4 ; $n=9$ μm respectively) was not significantly different from control (156 ± 7.2 ; $n=9$ and 39 ± 4.4 ; $n=9$ μm for length and width respectively), as shown in figure 4.3.

The effect of EPA on the KCl response was then examined. After 1 min exposure to EPA (50 μM) cell length and width were 150 ± 6.9 ($n=9$) and 39 ± 4.5 ($n=9$) μm . KCl (60 mM) was then also added and 1 min later, no significant reduction in cell length or width was measured (144 ± 6.6 and 42 ± 3.6 μm respectively) shown in figure 4.4.

The experiment was repeated, but the order of addition of EPA and KCl to the bathing medium was reversed. After 1 min PSS_d (60 mM KCl), cell length and width were 138 ± 5.7 ($n=9$) and 39 ± 4.5 ($n=9$) μm respectively. EPA was then added and 1 min later no significant difference in cell length or width was measured (147 ± 6.9 ; $n=9$ and 42 ± 4.2 ; $n=9$ μm ; respectively), shown in figure 4.5.

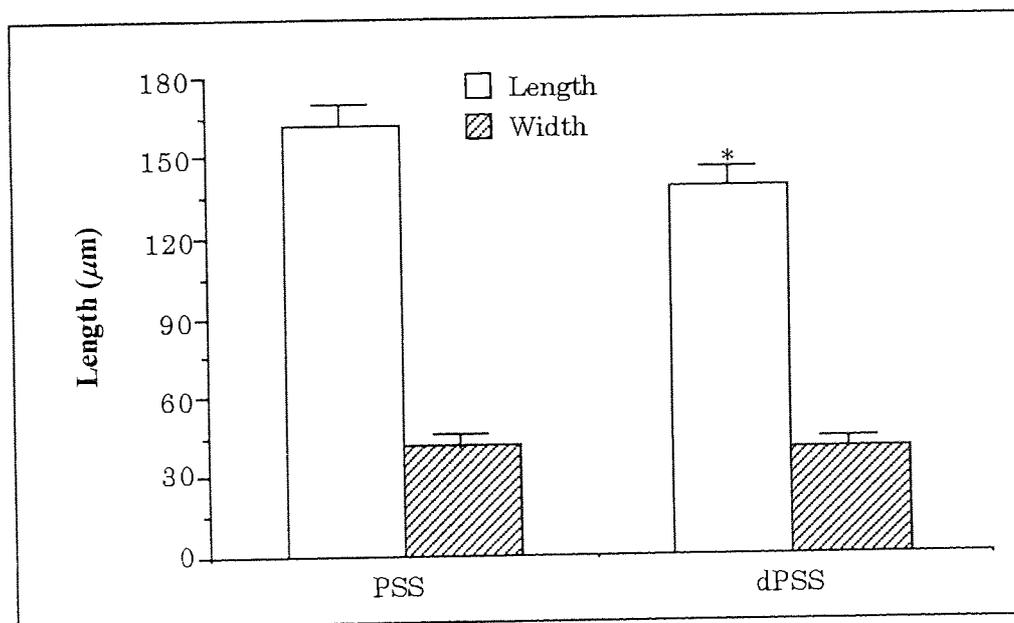


Figure 4.2. Effect of KCl on size of AOCs. Three cells, at 10 days of culture were bathed in PSS at 25°C for 2 min then measurements were taken. KCl (60 mM) was then added for 1 min and further measurements were taken (PSS_d). Values are mean \pm s.e.mean for three separate cell dispersal procedures. * indicates a significant difference from corresponding control value where $P < 0.05$ ($n=9$).

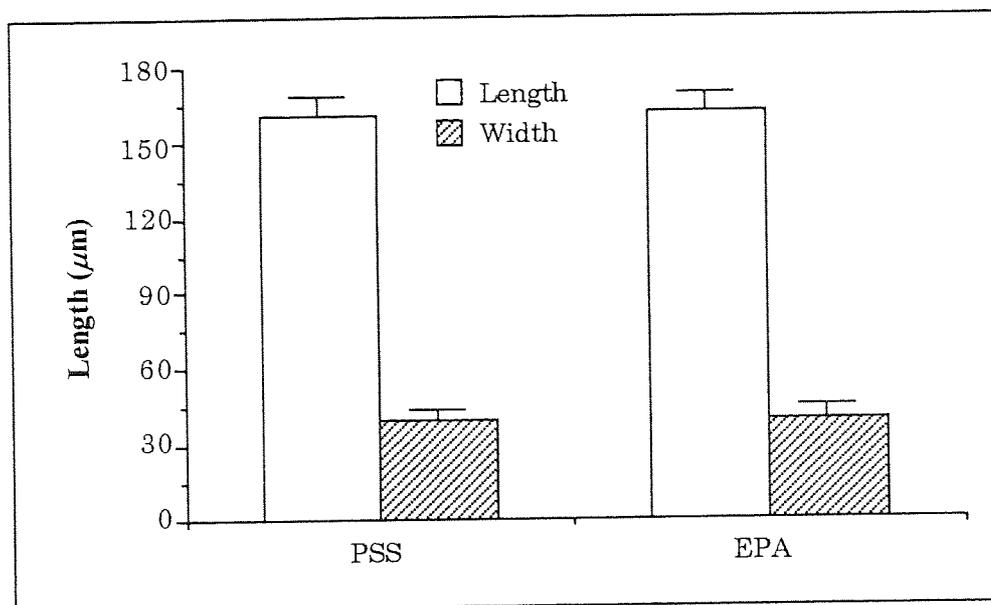


Figure 4.3. Effect of EPA on size of AOCs. Three cells, at 10 days of culture were bathed in PSS at 25°C for 2 min, then measurements were taken. EPA (50 μM) was then added for 1 min and further measurements were taken. Values are mean ± s.e.mean for three separate cell dispersal procedures (n=9).

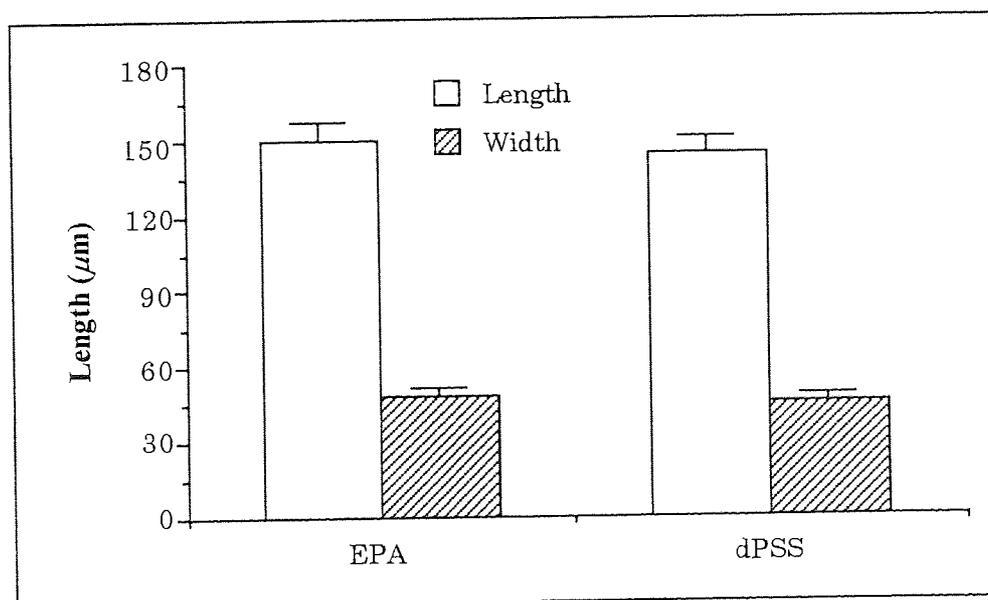


Figure 4.4. Effect of KCl on size of EPA-pretreated AOCs. Three cells, at 10 days of culture were bathed in PSS at 25°C for 1 min. EPA (50 μM) was added for 1 min, then measurements were taken. KCl (60 mM) was then also added for a further 1 min and measurements were taken again (PSS_d). Values are mean ± s.e.mean for three separate cell dispersal procedures (n=9).

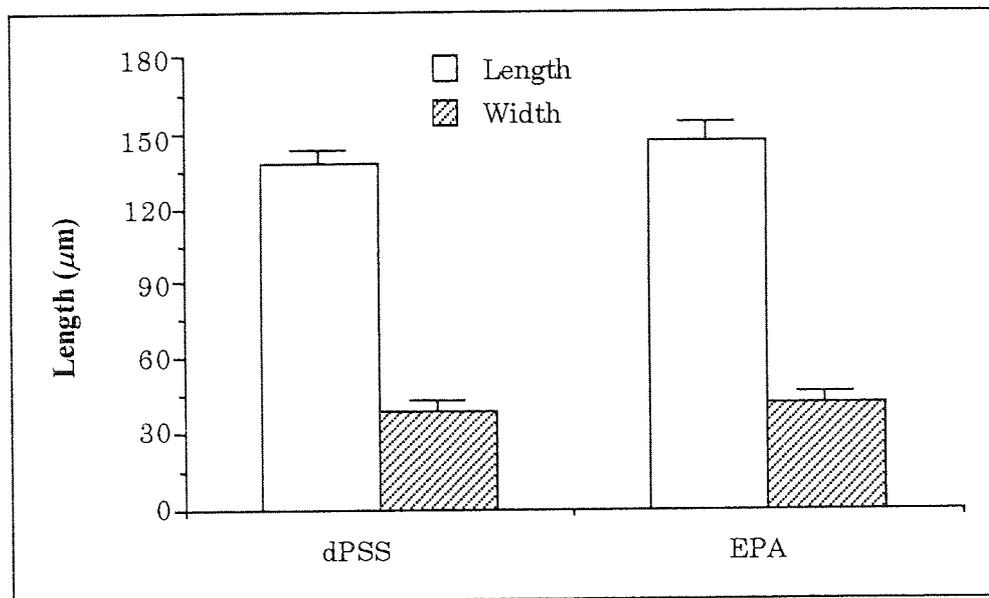


Figure 4.5 Effect of EPA on size of KCl-pre contracted AOCs. Three cells, at 10 days of culture were bathed in PSS at 25°C for 1 min. KCl (60 mM) was added for 1 min, then measurements were taken (PSS_d). EPA (50 μM) was then also added for a further 1 min and measurements were taken again. Values are mean ± s.e.mean for three separate cell dispersal procedures (n=9).

4.2.2. Effect of EPA on the noradrenaline-induced response.

The experiments were repeated, with noradrenaline (1 μM) replacing the use of KCl (refer to figure 4.1). After 2 min equilibration, cell length and width were measured. Noradrenaline (1 μM) was then added to the PSS and after 1 min, cell length and width were measured again. Noradrenaline significantly reduced cell length ($P < 0.05$) from 156 ± 7.2 (n=9) to 138 ± 6.0 (n=9) μm. Cell width did not change, shown in figure 4.6.

The control experiments for EPA, which were run on different occasions and were run in addition to the control experiments shown in figure 4.3 showed that cell length and width after EPA was not different from before EPA (153 ± 6.3 ; n=9 and 45 ± 4.2 ; n=9 μm), shown in figure 4.7.

The effect of EPA on the noradrenaline response was then examined. After 1 min exposure to EPA (50 μM) cell length and width were 150 ± 6.0 (n=9) and 39 ± 3.6 (n=9) μm respectively. Noradrenaline (1 μM) was then also added and 1 min later, a non-significant ($P < 0.05$) reduction to 141 ± 6.0 (n=9) and 30 ± 2.2 (n=9) μm for cell length and width was measured, shown in figure 4.8.

The experiment was repeated, but the order of addition of EPA and noradrenaline to the PSS were reversed. After 1 min noradrenaline (1 μM), cell length and width were 138 ± 6.0 (n=9) and 39 ± 4.2 (n=9) μm respectively. EPA (50 μM) was then also added and 1 min later, there was no significant ($P < 0.05$) change

in cell length and width (144 ± 7.5 ; $n=9$ and 45 ± 3.6 ; $n=9 \mu\text{m}$ respectively), shown in figure 4.9.

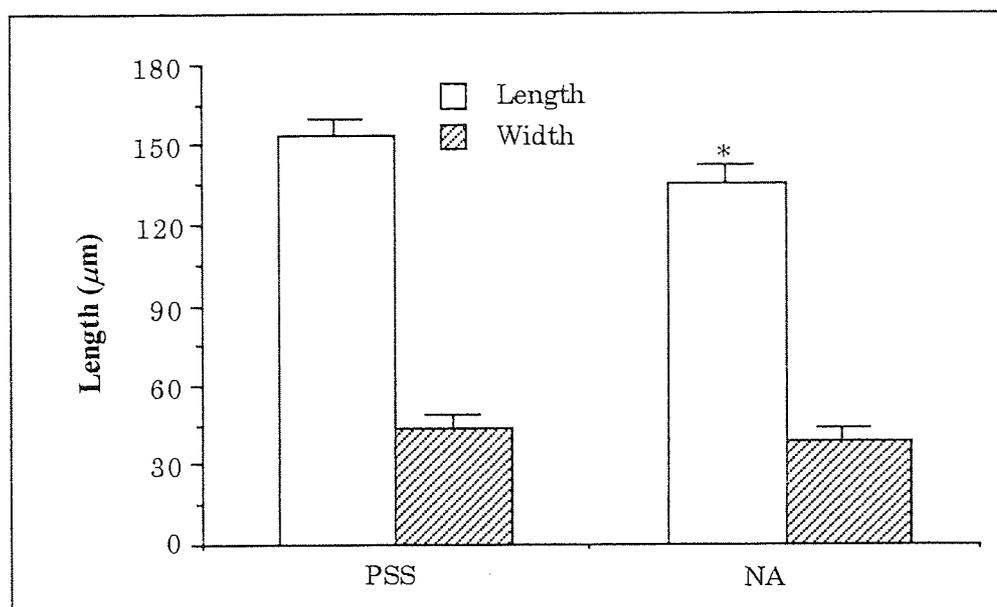


Figure 4.6. Effect of noradrenaline on size of AOCs. Three cells, at 10 days of culture were bathed in PSS at 25°C for 2 min then measurements were taken. Noradrenaline (NA; $1 \mu\text{M}$) was then added for 1 min and further measurements were taken. Values are mean \pm s.e.mean for three separate cell dispersal procedures. * indicates a significant difference from corresponding control value where $P < 0.05$ ($n=9$).

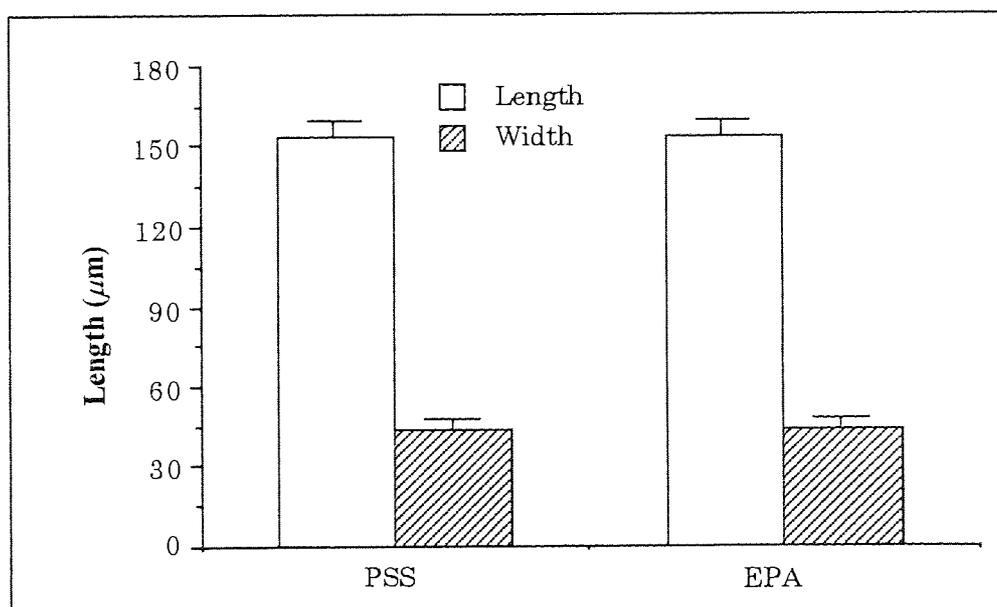


Figure 4.7. Effect of EPA on size of AOCs. Three cells, at 10 days of culture were bathed in PSS at 25°C for 2 min then measurements were taken. EPA ($50 \mu\text{M}$) was then added for 1 min and further measurements were taken. Values are mean \pm s.e.mean for three separate cell dispersal procedures ($n=9$).

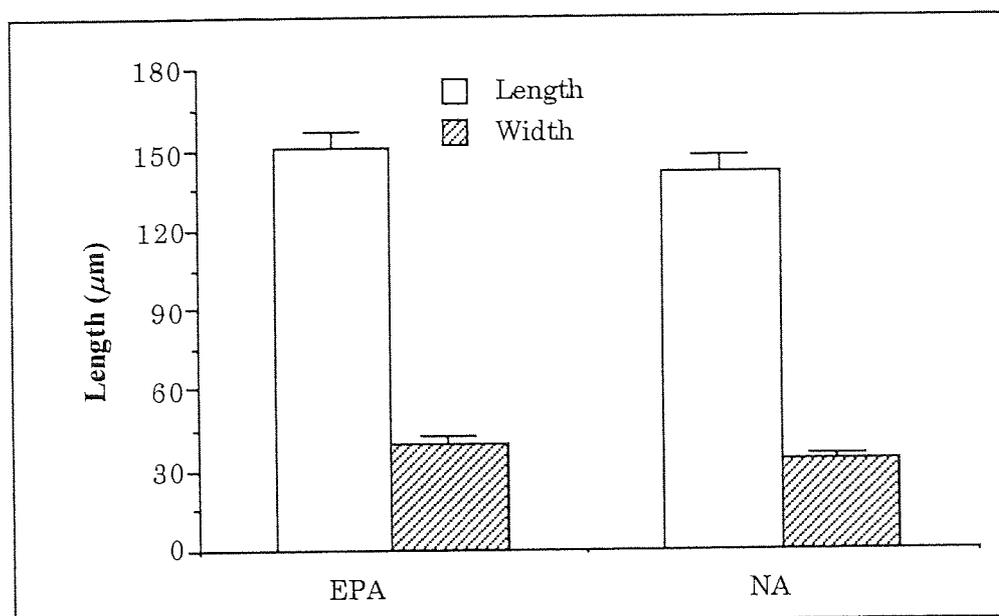


Figure 4.8. Effect of noradrenaline on size of EPA-pretreated AOCs. Three cells, at 10 days of culture were bathed in PSS at 25°C for 1 min. EPA (50 μM) was added for 1 min, then measurements were taken. Noradrenaline (NA; 1 μM) was then also added for a further 1 min and measurements were taken again. Values are mean ± s.e.mean for three separate cell dispersal procedures (n=9).

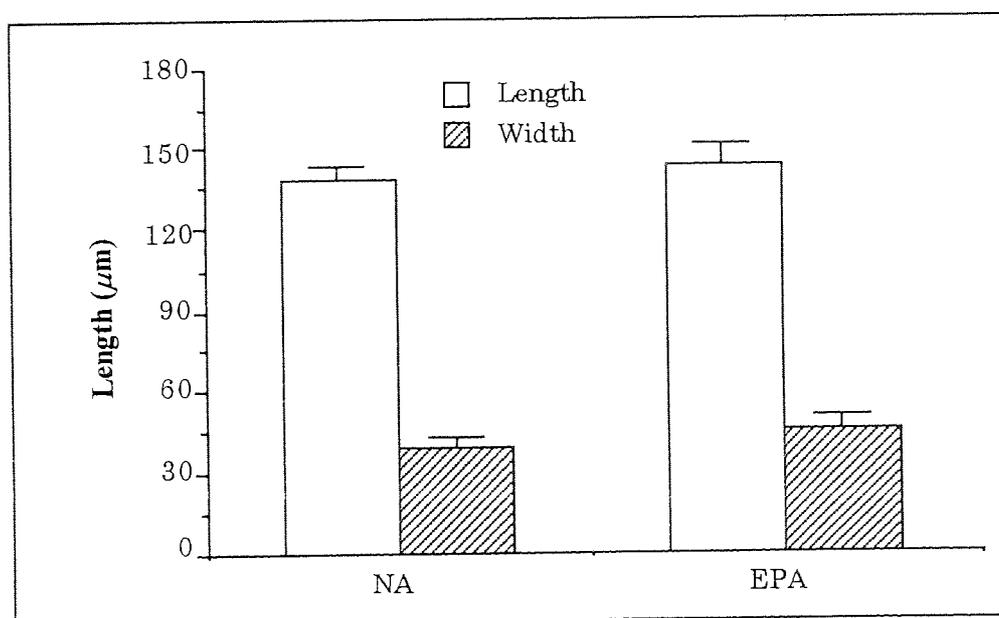


Figure 4.9. Effect of EPA on size of noradrenaline-pre contracted AOCs. Three cells, at 10 days of culture were bathed in PSS at 25°C for 1 min. Noradrenaline (NA; 1 μM) was added for 1 min, then measurements were taken. EPA (50 μM) was then also added for a further 1 min and measurements were taken again. Values are mean ± s.e.mean for three separate cell dispersal procedures (n=9).

4.2.3. Determination of the reversibility of the responses.

This study involved equilibrating the cells grown on a coverslip in 5 ml PSS at room temperature for 1 min, then measuring cell length. The cells were incubated for a further two minutes and cell length was measured again. In some experiments, EPA (50 μ M), noradrenaline (1 μ M), or KCl (60 mM) was added to the PSS bathing the cells for 1 min after the first measurement was taken and a further dose of EPA (50 μ M), NA (1 μ M) or KCl (60 mM) was added for the final minute of the 2 min incubation. The cells were then washed three times using PSS at room temperature and cell length was measured for the final time.

The results showed that all responses to KCl, noradrenaline or EPA alone or in combinations, were reversible, as shown in table 4.1.

Table 4.1. *The determination of the reversibility of the effect of EPA, noradrenaline or KCl. Three cells, at 10 days of culture were bathed in PSS at 25°C for 1 min. Cell measurements were made before and following two successive 1 min incubations in which EPA (50 μ M), noradrenaline (1 μ M; NA), KCl (60 mM) or PSS (10 μ l) was added to the PSS. The cells were then washed three times with PSS at 25°C and cell length was measured again.*

Incubation 1	Incubation 2	Cell length (μ m)		
		Before incubation	After incubation	After washout
		1	2	
PSS	KCl	153 \pm 6.3	135 \pm 6.0*	150 \pm 7.2
KCl	EPA	156 \pm 6.6	147 \pm 6.9	153 \pm 6.3
EPA	KCl	150 \pm 6.9	144 \pm 6.6	150 \pm 6.9
PSS	NA	156 \pm 6.6	138 \pm 5.7*	153 \pm 6.9
NA	EPA	153 \pm 8.1	144 \pm 7.5	150 \pm 7.2
EPA	NA	150 \pm 6.0	141 \pm 6.0	150 \pm 6.0

* indicates significant difference ($P < 0.05$) from value obtained before incubation 1, determined using ANOVA, followed by Scheffe's test. Each value represents the mean \pm s.e.mean for three separate dispersals ($n=9$).

4.2.4. Effect of nicardipine on the KCl-induced response and the effect of phentolamine on the noradrenaline-induced response.

It is well established that the KCl-induced contraction of vascular smooth muscle is mediated *via* calcium movement through L-type calcium channels (Van Breeman, 1989), whereas noradrenaline-induced contraction is mediated by a release of intracellular calcium and calcium entry (refer to General Introduction). Therefore, the next series of experiments in this study were designed to examine whether it was possible to block KCl- or noradrenaline-induced responses with known antagonists.

Throughout this study the cultured aortic cells were used at 10 days in culture and bathed in PSS at 25°C for 1 min. Nicardipine (0.1 μM) or phentolamine (1 μM) was added for 1 min prior to the addition of KCl (60 mM) or noradrenaline (1 μM).

It was shown that after 2 min incubation in PSS, followed by 1 min incubation in PSS_d (60 mM KCl), cell length significantly decreased (table 4.1). However, after 1 min KCl (60 mM) in the presence of nicardipine (0.1 μM), cell length and width were 153 ± 5.7 ($n=9$) and 36 ± 2.4 ($n=9$) μm respectively, which was not significantly different from the values measured prior to the addition of KCl (156 ± 5.1 ; $n=9$ and 42 ± 2.4 ; $n=9$ μm), shown in figure 4.10. Therefore, nicardipine blocked the vasoconstrictor effect of KCl.

Similarly, it was shown that after 2 min incubation in PSS, followed by 1 min incubation in PSS containing noradrenaline (1 μM), cell length significantly decreased (table 4.1). However, after 1 min noradrenaline (1 μM) in the presence of phentolamine (1 μM), cell length and width were 156 ± 3.6 ($n=9$) and 30 ± 3.0 ($n=9$) μm respectively, which was not significantly different from the values measured prior to the addition of noradrenaline (159 ± 5.7 ; $n=9$ and 36 ± 2.4 ; $n=9$ μm), shown in figure 4.11. Therefore, phentolamine blocked the vasoconstrictor effect of noradrenaline.

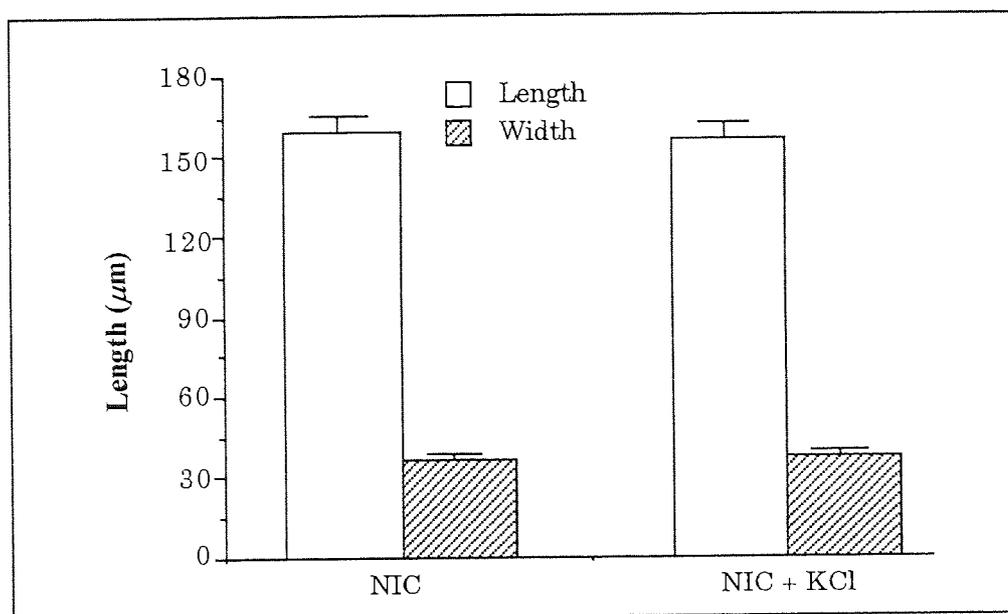


Figure 4.10. Effect of nicardipine on the KCl-induced size response of AOCs. Three cells, at 10 days of culture were bathed in PSS at 25°C for 1 min. Nicardipine (0.1 μM) was added for 1 min, then measurements were taken (NIC). KCl (60 mM) was then also added for a further 1 min and measurements were taken again (NIC + KCl). Values are mean ± s.e.mean for three separate cell dispersal procedures (n=9).

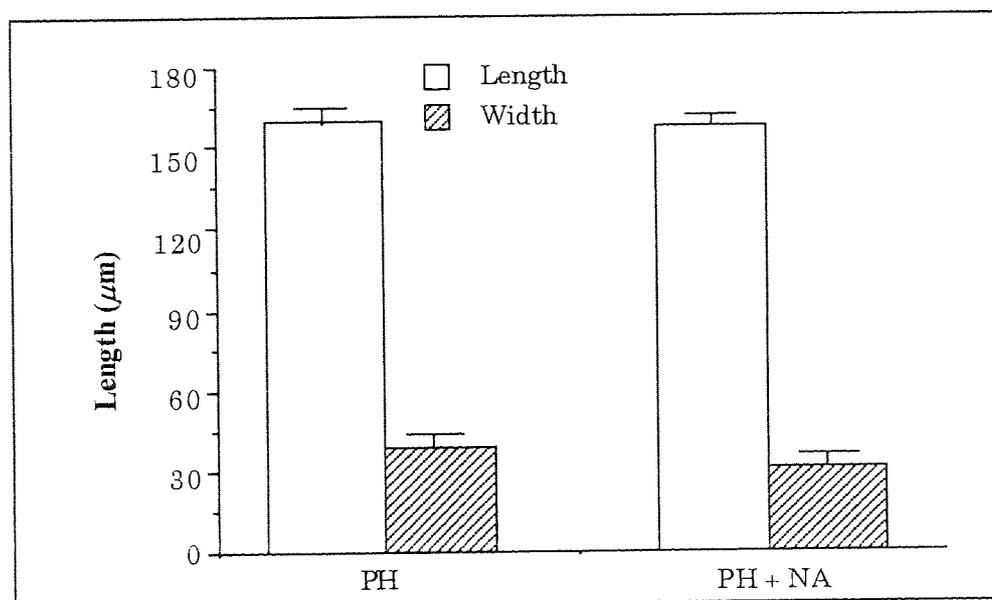


Figure 4.11. Effect of noradrenaline in the presence of phentolamine on size of AOCs. Three cells, at 10 days of culture were bathed in PSS at 25°C for 1 min. Phentolamine (PH; 1 μM) was added for 1 min, then measurements were taken (PH). Noradrenaline (1 μM) was then also added for a further 1 min and measurements were taken again (PH + NA). Values are mean ± s.e.mean for three separate cell dispersal procedures (n=9).

4.3. Discussion

Dietary supplementation with fish oil has been shown to have beneficial effects on the cardiovascular system, by reducing the incidence of coronary heart disease and atherosclerosis (Bang & Dyerberg, 1972; Dyerberg *et al.*, 1978; Kromann & Green, 1980; Lervang *et al.*, 1993; McLennan, 1993a; McLennan *et al.*, 1993b) and exerting an antihypertensive effect (Knapp & Fitzgerald, 1989; Bonaa *et al.*, 1990). Eicosapentaenoic acid (EPA), an n-3 fatty acid, is present in large amounts in fish oil and appears to be the biologically active component to which the effects are attributed. The mechanisms by which EPA reduces coronary heart disease are the regulation of the automaticity of cardiac contraction and the termination of lethal tachyarrhythmias (Kang & Leaf, 1994; 1995). Its antiatherosclerotic effects include the inhibition of platelet aggregation, the decrease in platelet and blood lipid levels (Leaf & Weber, 1988) and the inhibition of platelet, endothelial cell or macrophage thrombogenicity (Goodnight, 1991). Investigations designed to explain the antihypertensive effect of EPA have demonstrated that modulation of vascular tone may contribute to the hypotensive effects (Chin *et al.*, 1993). Indeed, EPA mediates a vasorelaxatory response in isolated vascular preparations including coronary arteries (Shimokawa & Vanhoutte, 1989), the perfused rabbit ear artery (Juan & Sametz, 1986), the rat and monkey mesenteric vascular bed (Mano *et al.*, 1995) and also the thoracic aorta (Lockette *et al.*, 1982; Smith *et al.*, 1992; Engler, 1994; 1992a; 1992b; 1990). The vasorelaxatory response has been attributed to a number of endothelium-dependent mechanisms, including the facilitation of endothelium-dependent relaxations, possibly *via* the attenuated release of nitric oxide from the endothelium (Shimokawa & Vanhoutte, 1989; Shimokawa *et al.*, 1987; Schini *et al.*, 1993) or by enhancing the production of nitric oxide synthase (Joly *et al.*, 1995). However, Mano *et al.* (1995) concluded from their studies on the n-3 fatty acids, that the mechanism of action was unrelated to endothelial cell-mediated relaxation and in support of this, other reports have demonstrated that the vasorelaxatory effect of the n-3 fatty acids is apparent in tissues which are devoid of endothelium (Engler, 1990; 1992a; 1992b). Furthermore, it has been suggested that the PUFAs mediate an action on the vascular smooth muscle cells following n-3 fatty acid incorporation into the phospholipids of cell membranes, which has been shown to induce alterations in the balance of prostaglandins involved in constrictor and relaxatory actions (Fischer & Weber, 1984; Dyerberg *et al.*, 1980; Lorenz *et al.*, 1984) or to modify membrane-bound protein function including receptors (Locher *et al.*, 1989), ion channels (Ordway *et al.*, 1989; Bregestovski *et al.*, 1989; Asano *et al.*, 1997) and enzymes (de Jonge *et al.*, 1996)

It is therefore apparent from such reports that the antihypertensive mechanism of action of the n-3 fatty acids on the vasculature has not been clearly defined, although it has been suggested that the PUFAs mediate an action on the vascular smooth muscle cells (Bretherton *et al.*, 1995; 1996a; 1996b; Asano *et al.*, 1997; 1998). The aim of the studies reported in this chapter, therefore, was to determine whether EPA mediated a relaxatory effect on vascular smooth muscle.

In the previous chapter a method for isolating and culturing large numbers of viable single aortic smooth muscle cells (AOCs) was developed, using a two-stage, enzymatic disaggregation technique. It was shown that the isolated aortic smooth muscle cells (AOCs) changed shape in response to vasoactive agents. Due to limitations imposed by equipment availability, the shape change was taken as the decrease in the average length of a population of AOCs whilst the average width of the cells (taken at the widest part of the cell) was also taken, to take into account any affect on cell attachment to the substrate. In the presence of noradrenaline (1 μM) or KCl (60 mM) the length significantly decreased, whilst the width either decreased or remained unchanged, therefore demonstrating that the AOCs were physiologically responsive to vasoactive agents. It was also shown that the response was reversible following removal of the vasoactive agent and was repeatable if the cells were subsequently exposed to the agent. These results were taken to indicate that the AOCs were a viable system in which to examine the effect of EPA on the contraction-relaxation mechanism.

Previous studies have reported the existence of a vasorelaxatory action of EPA in vascular smooth muscle (Juan & Sametz, 1986; Engler, 1989; Yin *et al.*, 1991; Lervang *et al.*, 1993). Therefore, the objectives of the study reported in this chapter were to determine whether pre-contracted AOCs would relax when exposed to EPA and whether pre-treatment of the AOCs with EPA would prevent contraction following exposure of the cells to KCl or noradrenaline.

Pre-treatment of the AOCs with EPA (50 μM) prevented a change in the shape of the cells to KCl (60 mM) or NA (1 μM). This is consistent with a previous report by Juan *et al.*, (1987), in which pre-treatment of rings of thoracic aorta with EPA (30 $\mu\text{g ml}^{-1}$) significantly decreased subsequent contractions to noradrenaline (10ng ml⁻¹ to 30 $\mu\text{g ml}^{-1}$). However, in the report by Juan *et al.*, (1987), the response to 1 $\mu\text{g ml}^{-1}$ was still approximately 40 % of the response obtained in the absence of EPA. The results obtained in the present study also demonstrated an incomplete abolition of the responses by EPA (50 μM) to the vasoactive agents, which is consistent with the report by Juan *et al.* (1987), although it should be noted, that in the previous chapter, it was explained that there may be selective enhancement of sensitivity to noradrenaline in single cell preparations.

In the present study, the single dose of EPA was not able to reverse KCl or noradrenaline-induced pre-contraction. In a previous report by Engler (1992), EPA (31 μM or 255 μM) was shown to significantly relax aortic tissue pre-contracted with phenylephrine, an α_1 -adrenoceptor agonist. The significant result was obtained by comparison with the phenylephrine-induced response to n-9 fatty acids, which Engler reported did not mediate any relaxation. Conversely, in the present study, the effect to EPA was compared to the cell shape measured prior to the addition of EPA. This factor and that of noradrenaline use instead of phenylephrine in the present study should not affect interpretation of the results, but it should be noted that there may be selective enhancement of sensitivity to noradrenaline in the present study; a number of workers have reported that in contractile studies performed in isolated smooth muscle cells, there is a change in the dose-response relationship for vasoactive agents compared to that observed in intact tissue preparations. Therefore, there may be selective enhancement of sensitivity to noradrenaline in the present study. Furthermore, it should also be noted that the contractile response to vasoactive agents has been reported to vary according to the species from which the tissue is derived and differences have even been observed according to the topological origin in the aorta (Bodin *et al*, 1991). The aorta used by Engler was taken from a different strain of rat to that used in the present study.

Taken together, however, the results in the present chapter and those of Engler demonstrate that the effect of EPA was dose-related and that there was an anti-vasoconstrictor effect of EPA in AOCs which was not endothelium-dependent. This confirmed previous suggestions that n-3 fatty acids may exert an antihypertensive effect by modulating vascular tone. Since the vasorelaxant effects of EPA in the present study and DHA in previous studies in the rat aorta are independent of the endothelium and not affected by the use of lipoxygenase or cyclooxygenase inhibitors (Engler, 1989, Engler *et al*, 1990) nor is the attenuation of noradrenaline and angiotensin II contractions by EPA in the rabbit aorta and isolated perfused rabbit ear dependent on the endothelium or eicosanoid production (Juan *et al*, 1987; Juan & Samtez, 1986), it is suggested that a calcium-mediated mechanism of n-3 fatty acid induced relaxation is likely.

Calcium plays a critical role in intracellular signalling and is the primary regulator of the smooth muscle contraction-relaxation cycle (Morgan, 1987). There are two sources of activator calcium in the rat aorta (Godfraind & Kaba, 1969); extracellular calcium entering the cell cytosol (Benham & Tsien, 1987) or released calcium into the cytosol, from intracellular storage compartments (Berridge & Irvine, 1989). Calcium entry from the extra cellular solution occurs through voltage-dependent or receptor-operated channels (Van Breemen, 1989) and is under the control of the membrane potential and agonists such as hormones, autocooids

and neurotransmitters. Other mechanisms, such as sodium/calcium exchange also operate. KCl induces calcium entry through voltage-dependent calcium channels and it is well established that the KCl-induced contractile response of vascular smooth muscle cells is mediated *via* L-type calcium channels.

Calcium entry into the cytosol from the sarcoplasmic reticulum is under the control of second messengers (Van Breeman, 1989 and review by Gurney & Clapp, 1994), including inositol 1,4,5-trisphosphate, which is produced following stimulation of the plasma membrane-associated α -adrenoceptor. The phasic part of the noradrenaline-induced contracture in vascular smooth muscle cells mediates an increase in $[Ca^{2+}]_i$ by an effect associated with the activation of membrane-bound α -adrenoceptors which then causes mobilisation of Ca^{2+} from intracellular storage sites. The sustained component of the noradrenaline response induces calcium entry through receptor-operated calcium channels.

Calcium is removed from the cytosol by sequestration into the sarcoplasmic reticulum or by the action of the calcium activated ATP-ase associated with the plasma membrane (Berridge & Irvine, 1989).

Therefore, in addition to the initial objectives set in this chapter, the effect of EPA was compared with a calcium channel blocker and an α -adrenoceptor antagonist.

The calcium channel blockers are a chemically and pharmacologically diverse group of drugs. They are utilised clinically, mostly to treat conditions confined to the cardiovascular system, although they have been shown to affect many different physiological processes. This is due to the widespread involvement of intracellular calcium as a regulator of cell function, including muscle contraction, hormone secretion, neurotransmitter release and platelet function. However, due to the heterogeneity of calcium channels, the calcium channel blockers do not cause a collection of physiological disasters, as the blockers have structural specificity for particular calcium channels. This specificity was considered in selecting a calcium channel blocker for use in the present study.

There are three main types of channel blocker, identified on the basis of the site to which they bind. These are the L-channel selective agents, the agents interacting with other voltage-dependent calcium channels and the non-selective channel modulators (Spedding & Paoletti, 1992). Of the six voltage-dependent channels which are now recognised, the T and L channels are found in cardiac and vascular smooth muscle so a blocker of these channels was sought. L-type channels are highly sensitive to three groups of agents, identified on the basis of which subunit of the L-type channel they bind to; phenylalkylamines, benzothiazepines and dihydropyridines. The most commonly used phenylalkylamine is verapamil, which acts mainly on the heart. Of the benzothiazepines, diltiazem is most

commonly used and this acts on the heart and smooth muscle. There are many dihydropyridines, which act preferentially on vascular smooth muscle, so this type of agent was selected. Furthermore, the T-channel is also blocked by some of the dihydropyridine agents. nifedipine and nicardipine are L-type dihydropyridine blockers, which are widely recognised to inhibit the response to KCl on vascular smooth muscle. However, nicardipine is more potent than nifedipine on T-type channels (Spedding & Paoletti, 1992) therefore nicardipine was used.

It was shown that nicardipine (0.1 μM) blocked the KCl response in the AOCs, suggesting the presence of L and/or T-type channels on the AOCs. At the dose selected, the EPA-mediated block of cell contraction was less than that of nicardipine (0.1 μM), but this was consistent with previous studies performed in the laboratory (unpublished data) which compared the effect of EPA (50 μM) and nicardipine (0.1 μM) on the contractural-response curves of endothelium-denuded aortic tissue to KCl. Furthermore, the results in the present study suggested that EPA might be associated with a calcium-mediated mechanism of relaxation.

The classification of adrenoceptors into α - and β - subtypes (Ahlquist, 1948) and later into α_1 - and α_2 - and β_1 - and β_2 -adrenoceptors (Lands *et al*, 1967) was made on the basis of the rank orders of potency of a series of structurally related catecholamines in organ bath experiments. Currently, there is overwhelming evidence from receptor binding, functional and second messenger level measurements that multiple subtypes of these receptors exist (for review, see Minneman, 1988). However, α_1 -adrenoceptors are cell-surface receptors, linked to phosphoinositide-specific phospholipase C by a G-protein, probably G_q (Smrcka *et al*, 1991), described in the general introduction, leading to increased intracellular inositol 1,4,5-trisphosphate and consequently, $[\text{Ca}^{2+}]_i$. α_2 -adrenoceptors are also cell-surface receptors, but are coupled by G_i to adenylate cyclase or alternatively to ion channels, so alter cellular activity either by reducing intracellular levels of cyclic AMP or by directly modifying activity of ion channels, such as calcium or potassium channels.

The main groups of α -adrenoceptor antagonists are ergot derivatives, specific α_1 - or α_2 -adrenoceptor antagonists or are non-selective α -adrenoceptor antagonists. Ergot derivatives, including ergotamine were considered unsuitable for use in this study because, besides blocking receptors and thus antagonising the actions of full agonists, they also have weak agonist effects of their own, *ie.* they have the characteristics of partial agonists. Specific α_1 -adrenoceptor antagonists were considered for use, particularly prazosin which is used clinically to cause vasodilation, but was not available in the laboratory at the time. Conversely, specific α_2 -adrenoceptor antagonists were not considered for use, as the presence of α_2 -adrenoceptors has not been reported on rat aortic smooth muscle cells. Non-

selective α -adrenoceptor antagonists are divided into the haloalkylamine group and the imidazoline group. The haloalkylamines bind covalently to the α_1 - or α_2 -receptor, leading to very slow (>24 hours) dissociation, so were considered unsuitable for making a comparison to EPA which has acute, reversible effects. Agents in the imidazoline group act as reversible competitive antagonists, and their action is short-lasting. Phentolamine is an imidazoline, which causes vasodilation and is a recognised blocker of the contractural response to noradrenaline in vascular smooth muscle. Therefore, phentolamine was selected for use in the present investigation and it was shown that phentolamine (1 μM) blocked the noradrenaline response in AOCs. However, the EPA-mediated block of cell contraction was less than that of phentolamine, suggesting that at the dose selected, EPA might be associated with an effect to decrease intracellular calcium mobilisation, but its effect could not be solely attributed to this mechanism of action.

This is contrary to a suggestion made in a previous report by Engler (1992b) who examined whether the reduction of noradrenaline-induced contractions in rat aortic tissues by DHA or EPA was related to the inhibition of intracellular calcium mobilisation. Engler demonstrated that DHA or EPA was able to reduce the noradrenaline-induced contractile response in calcium-free, EGTA-containing buffer, thus leading to the suggestion of a role of n-3 fatty acids in intracellular calcium regulation. Furthermore, at a higher concentration, EPA (214 μM) was previously shown to decrease the α_1 -stimulated phospholipase C β activity in rat cardiac myocytes using phenylephrine (5 μM), which also suggests that n-3 fatty acids may mediate an action on membrane-bound receptor-signalling pathways associated with intracellular calcium mobilisation (de Jonge *et al*, 1996). Taken together, however, the results demonstrated that the effect of EPA was dose-related and that there was a relaxatory effect of EPA in AOCs which was likely associated with regulation of intracellular calcium concentrations.

In conclusion, therefore, the results in this chapter show that pre-treatment of a homogeneous population of AOCs with EPA (50 μM) reduced but did not completely block the change in the shape of the cells after KCl (60 mM) or NA (1 μM), demonstrating an anti-vasoconstrictor effect of EPA which was endothelium-independent. The effect of KCl (60 mM) or NA (1 μM) on the AOCs was reversible, however, the single dose of EPA (50 μM) did not reverse KCl or NA-induced pre-contraction. The results also demonstrate that the response to noradrenaline was blocked by the α -adrenoceptor antagonist phentolamine (1 μM) and the effect to KCl was blocked by the calcium channel blocker, nifedipine (0.1 μM). Although these effects were greater than the effect of EPA (50 μM) at the concentration selected, the results demonstrated that the AOCs expressed functional α -adrenoceptors and L-type calcium channels. Moreover, the results demonstrated that the AOCs were a

physiologically viable system in which to commence the investigations in this thesis on the effect of EPA upon smooth muscle contraction. Furthermore, the results suggested that the action of EPA may be related to an effect on relaxation associated with the regulation of intracellular calcium, however, the precise mechanism remained to be elucidated.

Chapter 5

The Effect Of EPA On The Influx Of Ca^{2+}

5.1 Introduction

In chapter 4, the partial inhibition of the KCl - or noradrenaline-induced response by EPA in the aortic smooth muscle cells (AOCs) indicated that EPA mediated an anti-vasoconstrictor effect which was endothelium-independent. The results suggested that the action of EPA may be related to regulation of $[\text{Ca}^{2+}]_i$. $[\text{Ca}^{2+}]_i$ is modulated by agents which regulate calcium influx into the cells and/or by agents which alter the release of calcium from intracellular storage sites. Taking the first of these two regulatory mechanisms, the aim of this chapter was to determine whether the observed anti-vasoconstrictor effect of EPA in chapter 4, was associated with block of calcium influx into the AOCs and furthermore, whether the effect on influx altered the concentration of intracellular calcium.

The influx of calcium occurs *via* voltage-dependent or receptor-operated calcium channels in the plasma membrane. Voltage-dependent channels allow an inward calcium current following activation during depolarisation. Depolarisation may be induced experimentally by the application of a solution to the cellular surface, containing potassium ions at concentrations in excess of 20 mM (Iesaki *et al*, 1996; Greenwood & Weston, 1993; Bodin *et al*, 1991; Droogmans *et al*, 1977). Calcium entry through the voltage-dependent calcium channels may be inhibited either directly, for example by calcium antagonists which act to prevent the opening of calcium channels, or indirectly, for example by potassium channel activators which induce the efflux of potassium ions and hence a hyperpolarisation of the plasma membrane, thereby moving the membrane potential farther from the activation range for calcium channels.

In vascular smooth muscle, membrane depolarisation is involved in the activation of the L-type channel (Renterghem *et al*, 1988). The opening of the L-type calcium channel is modulated by most known calcium blockers (Triggle, 1991), including the organic, dihydropyridine, calcium entry blockers which bind selectively to the calcium channel and thereby favour the non-opening state of the channel. The direct effect of EPA on calcium entry in aortic smooth muscle cells has not previously been investigated, but it has been suggested that the enrichment of myocardial membrane phospholipids with DHA may exert a functional influence on the calcium channels in the plasma membrane. Pepe *et al* (1994) showed that DHA blocked the effects of nitrendipine and the calcium

agonists BAY K 8644 in cardiac myocytes. These results have led to suggestions that n-3 fatty acids may acutely bind to a specific subunit of the L-type channel which is associated with the dihydropyridine binding site (Pepe *et al*, 1994) and consistently, Hallaq *et al* (1992) demonstrated that EPA and DHA inhibited the specific binding of [³H]-nitrendipine to cardiac myocytes. This suggests that EPA might mediate an action at the calcium-ion channel which is similar to that of a channel blocker.

Therefore, in the present study, the first objective was to determine the effect of EPA on calcium influx in AOCs under depolarised and non-depolarised conditions after an initial preliminary study established that calcium influx could be measured in the AOCs. A procedure reported by Yamada *et al* (1992) which was based on a previously published method by Sperti & Colucci (1987) was adapted and then used to investigate the effect of depolarisation on calcium influx, described in 2.2.2.2. In summary, monolayers of cells were used after 10-13 days in culture. Either PSS which contained 5 mM KCl or depolarised-PSS (PSS_d) which contained 60 mM KCl, was exposed to the cells for 5, 10, 20, 40 or 60 min with tracer concentrations of the radioactive calcium chloride isotope, ⁴⁵Ca also present in order to enable measurement of the calcium entering the cells. The PSS used throughout these studies was also supplemented with the following compounds to limit variability in the results; BSA (3 mg ml⁻¹) to support cell survival, propranolol (1 μM) to inhibit activity at β-adrenoceptors, ascorbic acid (50 μM) to prevent oxidation of catecholamines and EDTA (10 μM) to chelate heavy metal impurities.

At the end of the incubation, the cells were washed with lanthanum-containing saline (LSS) to prevent further calcium influx and also loss of calcium from the cells (Weir & Weston, 1988) and the ⁴⁵Ca content of the cells was determined by liquid scintillation counting after lysis of the cells.

The next objective was to determine whether the action of EPA at the calcium ion channel was similar to the action of a channel blocker, in particular, that of an L-type calcium channel blocker. In these experiments, the methodology was the same as that described above, except that either nicardipine, a dihydropyridine calcium channel blocker, selected for the reasons described in the previous chapter, or EPA (50 μM) was included in the incubation solution or in a pre-incubation solution.

The influx of calcium into vascular smooth muscle cells also occurs *via* receptor-operated calcium channels in the plasma membrane. In vascular smooth muscle tissue, exposure to noradrenaline results in contraction which has an initial phasic phase followed by a sustained, tonic phase. The latter phase is associated with the influx of extra cellular calcium through receptor-operated

channels. It has been reported that agonists at the receptor-operated channels induce tonic contractions in fully depolarised smooth muscle (van Breeman & Saida, 1989), although evidence to support the existence of such channels remains controversial (Gurney & Clapp, 1994). However, EPA has been shown to reduce contractures induced by noradrenaline (Engler, 1992a; 1992b; Bretherton *et al*, 1995; 1996a; Juan *et al*, 1987; Mano *et al*, 1995; Reithman *et al*, 1996). Therefore, the third objective was to measure the effect of EPA on noradrenaline-induced calcium influx into AOCs to determine whether EPA was associated with decreased calcium influx associated with entry through ROCs. The methodology for these experiments was the same as that for the previous experiments using KCl, except that noradrenaline replaced the use of KCl.

Once the effect of EPA on calcium influx was established, the next objective was to determine whether the change in influx was associated with a change in $[Ca^{2+}]_i$ directly. A number of methods are available for measuring $[Ca^{2+}]_i$ directly including calcium-selective micro electrodes and nuclear magnetic resonance, but the most widely used methods employ optical probes which monitor calcium as a change in absorbance, luminescence or fluorescence (McCormack & Allen, 1991). Fluorescent calcium-sensing indicators are popular mainly because they are apparently incorporated easily into cells and they produce signals which can be monitored with fairly simple and widely available instrumentation.

Ca^{2+} indicators must bind with calcium to detect it. Grynkiewicz *et al* (1985) derived that if one indicator molecule is assumed to bind to one calcium ion, the calcium which binds to the indicator (Ca^{2+}_{bound}) depends on the dissociation constant K_d according to

$$[Ca^{2+}_{bound}] = [Ca^{2+}_{free}] [I_{tot}] / (K_d + [Ca^{2+}]) \quad \text{Equation 3}$$

where I_{tot} is the sum of free and bound indicator. Since it is actually the calcium bound to the indicator which is measured, the proportion of calcium bound to the indicator relates to the free calcium according to

$$[Ca^{2+}_{bound}] / [I_{tot}] = [Ca^{2+}_{free}] / (K_d + [Ca^{2+}]) \quad \text{Equation 4}$$

To avoid disturbing the equilibrium the fraction bound to the indicator is minimised by employing an indicator with low affinity for calcium at a low concentration. To make this possible, the calcium signal must be large.

The most widely used fluorescent indicators are modelled on the calcium-chelator BAPTA (1,2-bis[2-aminophenoxy] ethane N,N,N',N'-tetracetic acid) which binds calcium ions with high selectivity over magnesium ions and hydrogen ions (Tsien, 1980). Its calcium affinity is unaffected by pH in the physiological range

and it binds and releases calcium rapidly. The first fluorescent derivative to gain widespread use was quin-2 (Tsien *et al*, 1982). It has largely been superseded by fura-2 (Grynkiewicz *et al*, 1985) and indo-1 (Grynkiewicz *et al*, 1985). Additions to the group include fluo-3 and rhod-2 (Minta *et al*, 1989).

Fura-2 has two excitation maxima, one at 362 nm in the presence of zero calcium and the other at 335 nm in the presence of high calcium (Grynkiewicz *et al*, 1985). These are well separated from the corresponding emission maxima (512 nm and 505 nm respectively) which prevents re-excitation by light emitted from nearby molecules.

Fluorescence is proportional to indicator concentration. However, a large concentration of indicator would strongly absorb the light passing through the cells and thereby reduce the intensity of the light. The quantum yield and extinction coefficient of fura-2 is sufficient that a signal above background is given at a low concentration (below 100 μM) (Gurney, 1989). A signal at a low concentration also alleviates the problem of calcium buffering experienced with the use of some optical indicators (Gurney, 1989).

As for BAPTA, the kinetics of calcium binding to fura-2 is fast (Quast *et al*, 1984), therefore, it responds more rapidly to the changes in calcium than the change to be measured. In the presence of calcium, there is an increase in the fluorescence intensity of fluorescent indicators. With fura-2, this relates to a shift to shorter wavelengths of its excitation spectrum. As a consequence, the ratio of fluorescence at the two excitation wavelength maxima relates to $[\text{Ca}^{2+}]_i$ according to

$$[\text{Ca}^{2+}] = K_d \frac{(R - R_{\min})}{(R_{\max} - R)} S \quad \text{Equation 5}$$

where R_{\min} and R_{\max} are the ratios at 340/380 nm in calcium-free and calcium-saturated solutions respectively. S defines the ratio of fluorescence for the calcium-free and calcium-bound indicator at the longer (380 nm) wavelength (Grynkiewicz *et al*, 1985).

Incorporation of the fluorescent indicators into cells is assisted by the use of membrane permeant acetoxymethyl esters. Fura-2 may be bound to the ester, which is then cleaved by intracellular enzymes to leave the indicator trapped in the cell. Prolonged exposure time to the ester should be avoided as this may allow accumulation of the bound indicator/acetoxymethyl ester compound into subcellular compartments and thereby prevent complete hydrolysis of the indicator.

Methods for measuring intracellular calcium must be highly selective and able to discriminate against other cations, particularly magnesium, which lowers

the apparent affinity of the indicator for calcium (Tsien, 1980). Methods must also be sensitive. To maximise sensitivity an indicator with a K_d value in the same range as the calcium concentration to be measured is employed. With fura-2 therefore, a K_d value of 224, which also takes account of the magnesium ions present is used (Gurney, 1989).

Calibration of the signal with fura-2 is straight forward and requires few assumptions. Auto fluorescence at 340 and 380 nm is subtracted before calculating fluorescence ratios and is measured at the end of the experiment by quenching indicator fluorescence by promoting calcium (and magnesium) entry into the cells. Fluorescence ratios are calibrated by exposing the indicator to known $[Ca^{2+}]_i$. The ratio of fluorescence at 340/380 nm is determined when all the indicator is in the calcium-bound form (R_{max}) and then when all the indicator is in the calcium-free form (R_{min}).

Therefore, in the experiments reported in this chapter, KCl or noradrenaline were used to increase $[Ca^{2+}]_i$ and phentolamine or nicardipine were used as controls to investigate whether the increase in $[Ca^{2+}]_i$ was *via* α -adrenoceptors or calcium ion channels. The methodology used in these studies is described in 2.11. In summary, after 10-13 days in culture, cells were exposed to the indicator, fura-2 acetyl methylester (0.5 μ M) in PSS at 37°C for 60 min. After washing, complete hydrolysis of the indicator from the acetyl methylester was allowed to ensure the indicator favoured calcium binding. A sample of the solution was placed into a chamber in a luminescence spectrometer and excitation wavelengths were set at 340 and 380 nm with recording emission set at 510 nm. Noradrenaline, KCl or EPA was added to the chamber and $[Ca^{2+}]_i$ was measured for up to 10 min. After each measurement, digitonin (2mM) was added to the solution to lyse the cells and release all the intracellular Ca^{2+} in order to determine F_{max} . EGTA (5 mM), a calcium chelator, was then added to dissociate the Ca^{2+} from the indicator and thereby determine F_{min} . The resultant $[Ca^{2+}]_i$ level was calculated according to the equation reported by Grynkiewicz *et al* (equation 3) from the ratios of fluorescence at the two excitation maxima. However, the $[Ca^{2+}]_i$ levels measured were variable. Therefore, the responses to nicardipine, phentolamine or EPA were expressed as a percentage of the response obtained to noradrenaline or KCl.

5.2. Results

5.2.1. Time course for ^{45}Ca influx

After 10-13 days in culture, monolayers of AOCs were washed in physiological solution (PSS) at 37°C (see 2.9.2.1 for composition of PSS). Then, following the methodology described in detail in 2.2.2.1., cells were incubated at 37°C for 5, 10, 20, 40 or 60 min with ^{45}Ca ($2\ \mu\text{Ci ml}^{-1}$) in either PSS or depolarised-PSS (PSS_d). In PSS_d, KCl and NaCl concentrations were adjusted to final concentrations of 60 and 90 mM respectively to maintain osmolarity. Unfortunately, experimental constraints associated with the calcium influx study did not permit measurement of influx after 1 min cell stimulation like in the cell contraction studies performed in chapter 3. However the contraction studies in chapter 3 did show that the cells remained contracted for a minimum 5 min period (figure 3.4), hence the initial 5 min time point in the influx studies. *Blanks*, in which tracer was omitted from the extra-cellular solution were performed routinely in each experiment to determine background radioactive levels, and the measurements taken were subtracted from the corresponding measurements made in the presence of tracer. Standard quantities of ^{45}Ca and cellular protein content were also measured to allow calculation of calcium influx (shown in appendix 2) and normalised expression of the results as $\text{nmol}\ \mu\text{g}^{-1}$ protein. All experimental conditions were performed in duplicate or triplicate and the experiments were repeated using plates containing cells dispersed on different occasions to account for intra- and inter-experimental variation. All values are given as the mean \pm s.e. mean for n experiments and statistical comparisons were made by Student's two-tailed t test for unpaired data. Significance was identified at $P < 0.05$.

In PSS, calcium influx rose rapidly during the first 5 minutes of incubation from 6.90 ± 2.35 ($n=6$) to 14.65 ± 5.73 $\text{nmol}\ \mu\text{g}^{-1}$ protein ($n=6$) and then slowed over the next 55 min reaching a value of 21.90 ± 5.10 $\text{nmol}\ \mu\text{g}^{-1}$ protein ($n=6$) at 60 min (figure 5.1).

In PSS_d there was a similar rapid influx of calcium in the first 5 min of incubation, which plateaued over the period 5 to 60 min, with a final value of 28.6 ± 2.27 $\text{nmol}\ \mu\text{g}^{-1}$ protein ($n=6$) at 60 min (see figure 5.1). Influx was significantly greater ($P < 0.05$) in cells exposed to PSS_d at the 20 and 40 min incubation time points. The results suggested that the cells were responsive to a depolarising solution.

However, in chapter 3, it was shown that the AOCs responded to depolarising conditions after 1 min and the cells remained contracted for a

minimum 5 min period (figure 3.4). The inconsistency between these results was considered to be a result of the variation in the data measured at the 5 and 10 min time points in the calcium influx study, rather than an effect of the cells. Therefore, the calcium influx study was repeated, but the effect of depolarisation was examined by the construction of a KCl concentration-response curve, after 5 or 30 min.

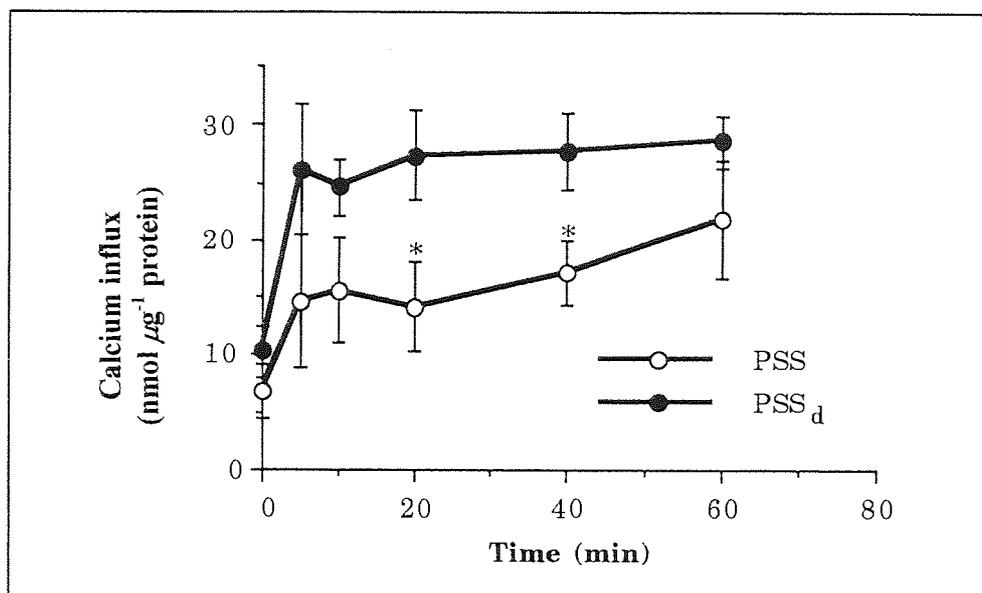


Figure 5.1. Time course for calcium influx. Cells were incubated for 5, 10, 20, 40 or 60 min at 37°C with ^{45}Ca ($2\mu\text{Ci ml}^{-1}$) in PSS or PSS_d containing 60 mM KCl. Following removal of the solution, the cells were lysed and radioactivity measured. Values are expressed as $\text{nmol } \mu\text{g}^{-1} \text{ protein} \pm \text{s.e. mean}$ for 6 experiments ($n=6$). * indicates significant difference from corresponding PSS_d value.

5.2.2. Effect of KCl concentration on calcium influx.

The same methodology as that outlined in the previous section was followed, except that the cells were incubated with ^{45}Ca ($2\mu\text{Ci ml}^{-1}$) in PSS at 37°C or in PSS with final KCl concentrations of 10, 20, 40 or 60 mM KCl. The NaCl concentration was adjusted in each solution as appropriate to maintain osmolarity (described fully in the methods section in 2.2.2.2). All values are given as the mean \pm s.e. mean for n experiments and statistical comparisons were made by analysis of variance (ANOVA) followed by Scheffe's multiple range test. Significance was identified at $P<0.05$. The results, shown in figure 5.2, indicated that after 5 min, KCl caused a concentration-dependent increase in calcium influx, from $15.37 \pm 0.64 \text{ nmol } \mu\text{g}^{-1} \text{ protein}$ ($n=9$) in PSS to 16.77 ± 0.91 ($n=9$) and to $20.55 \pm 1.82 \text{ nmol } \mu\text{g}^{-1} \text{ protein}$ ($n=9$) in PSS containing 10 and 60 mM KCl respectively.

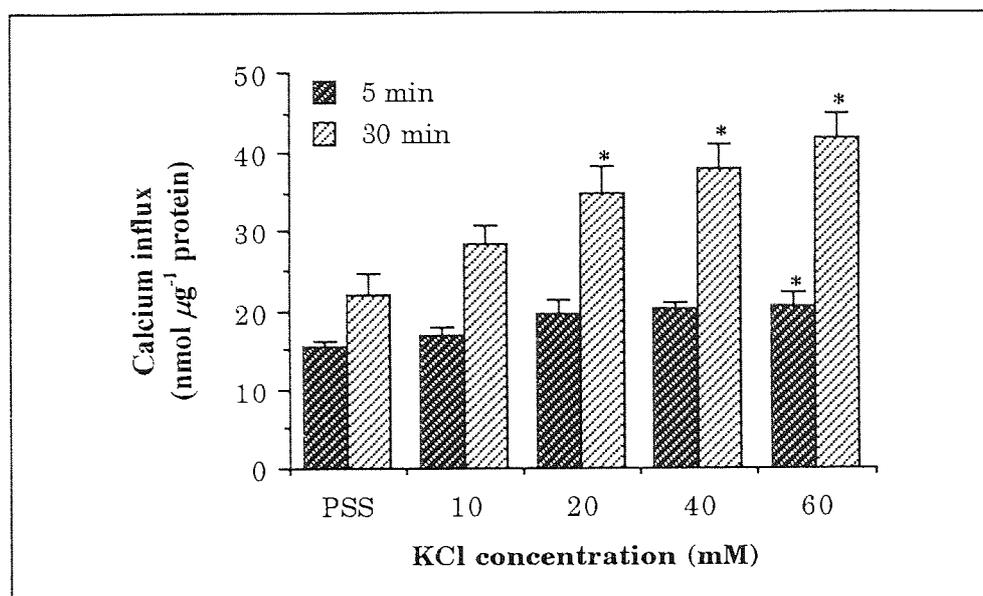


Figure 5.2. *The effect of a 5 or a 30 min incubation on calcium influx.* Cells were incubated for 5 or 30 min in PSS at 37°C, containing ^{45}Ca ($2\mu\text{Ci ml}^{-1}$) and either 10, 20, 40 or 60 mM KCl. Following removal of the solution, the cells were lysed and radioactivity measured. Values are expressed as $\text{nmol } \mu\text{g}^{-1} \text{ protein} \pm \text{s.e. mean}$ from 9 experiments. * indicates significant difference from corresponding PSS value where $P < 0.05$ ($n=9$).

The response to 60 mM KCl was significantly greater than the effect in PSS (figure 5.2), consistent with the cell contraction studies performed in chapter 3. Likewise, after 30 min, KCl caused a concentration-dependent increase in calcium influx, from $21.80 \pm 2.77 \text{ nmol } \mu\text{g}^{-1} \text{ protein}$ ($n=9$) in PSS to 28.38 ± 2.52 ($n=9$) and then to $41.72 \pm 3.30 \text{ nmol } \mu\text{g}^{-1} \text{ protein}$ ($n=9$) in PSS containing 10 and 60 mM KCl respectively.

The results therefore confirmed the results obtained in the cell contraction studies reported in chapter 3 (see figure 3.4) which demonstrated that the cells were responsive to depolarising solution, however, the results showed the effect of depolarisation was more clearly observed after 30 min incubation in the calcium influx experiments. In view of this, the experiment was repeated, measuring calcium influx again after 5 or 30 min, but this time, the effect of EPA on influx was examined. The response to EPA was compared with the vehicle control (1 % ethanol).

5.2.3. Effect of EPA upon calcium influx.

The experiments were a direct repeat of the previous experiments, except that EPA ($50 \mu\text{M}$) or 1% ethanol, the EPA vehicle was also present in the ^{45}Ca -containing PSS, which was exposed to the cells for 5 or 30 min. The complete methodology is described in 2.2.2.3.

It can be seen in figure 5.3 that after 5 min, EPA (50 μM) significantly decreased calcium influx measured in PSS or 10 mM KCl from 15.16 ± 3.51 ($n=9$) and 16.37 ± 1.80 $\text{nmol } \mu\text{g}^{-1}$ protein ($n=9$) for ETH to 4.98 ± 0.62 and 9.64 ± 1.62 $\text{nmol } \mu\text{g}^{-1}$ protein ($n=9$) respectively but failed to reduce influx induced by 20, 40 or 60 mM KCl. Similarly for the data after 30 min, shown in figure 5.4. EPA (50 μM) significantly decreased calcium influx measured in PSS or 10 mM KCl from 10.63 ± 1.50 ($n=9$) and 9.96 ± 0.10 $\text{nmol } \mu\text{g}^{-1}$ protein ($n=9$) for ETH to 3.87 ± 0.46 ($n=9$) and 5.79 ± 0.90 $\text{nmol } \mu\text{g}^{-1}$ protein ($n=9$) respectively but failed to reduce influx induced by 20, 40 or 60 mM KCl. Therefore, the results demonstrated that the relative effect of EPA upon influx was similar after 5 or 30 min, but that measurements of calcium influx showed less variability after 30 min.

As EPA had failed to show any block of influx at the higher, depolarising, concentrations of KCl, the results suggested that EPA may not be acting at the L-type channel. However, it was considered necessary to examine the effect of nicardipine before any conclusion could be drawn. Therefore, the experiments were repeated, but with the L-type calcium entry blocker nicardipine replacing EPA.

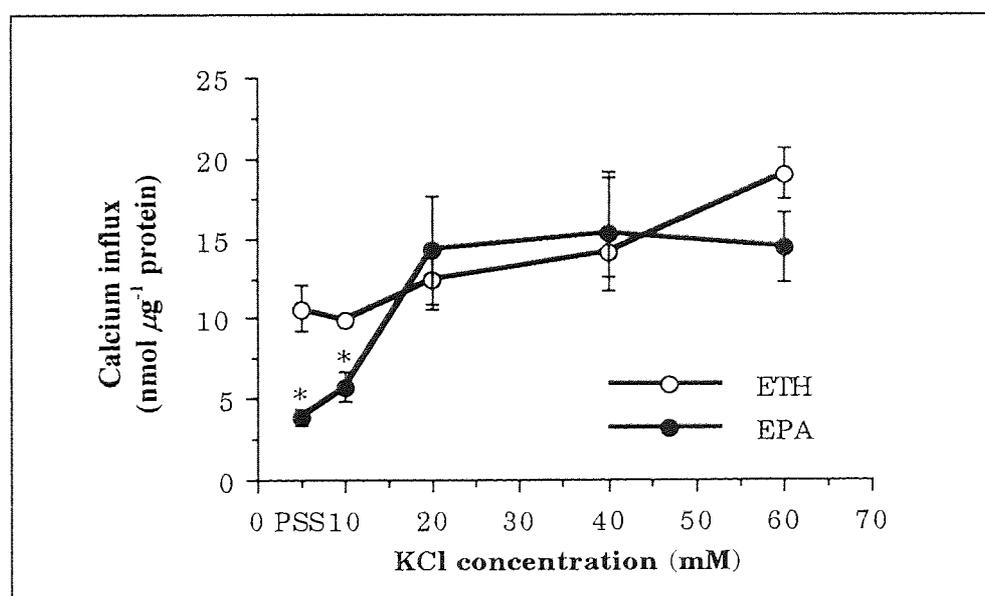


Figure 5.3. Effect of a 5 min incubation with EPA on calcium influx. Cells were incubated for 30 min at 37°C with ^{45}Ca ($2\mu\text{Ci ml}^{-1}$) in PSS (5 mM KCl) or in PSS with 10, 20, 40 or 60 mM KCl. EPA (50 μM) or ethanol (1%; ETH, the EPA vehicle) was also present. Following removal of the solution, the cells were lysed and radioactivity measured. Values are expressed as $\text{nmol } \mu\text{g}^{-1}$ protein \pm s.e.mean from 9 experiments. * indicates significant difference from corresponding ETH value where $P < 0.05$ ($n=9$).

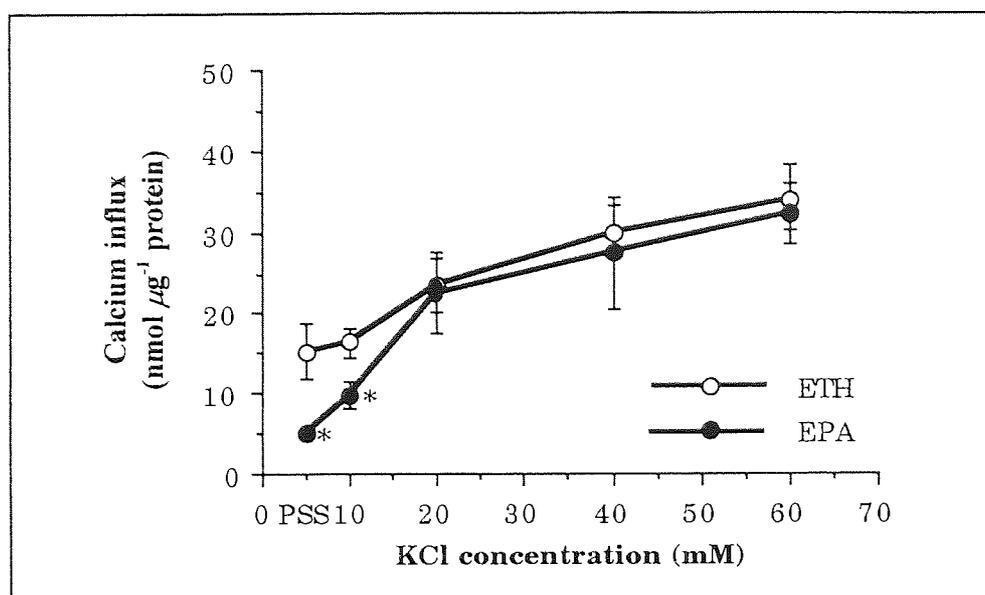


Figure 5.4. Effect of a 30 min incubation with EPA on calcium influx. Cells were incubated for 30 min at 37°C with ^{45}Ca ($2\mu\text{Ci ml}^{-1}$) in PSS (5 mM KCl) or in PSS with 10, 20, 40 or 60 mM KCl. EPA ($50\mu\text{M}$) or ethanol (1%; ETH, the EPA vehicle) was also present. Following removal of the solution, the cells were lysed and radioactivity measured. Values are expressed as $\text{nmol } \mu\text{g}^{-1} \text{ protein} \pm \text{s.e. mean}$ from 9 experiments. * indicates significant difference from corresponding ETH value where $P < 0.05$ ($n=9$).

5.2.4. Effect of nicardipine on KCl-induced calcium influx.

These experiments followed the same methodology as the experiments on EPA, outlined above in 5.2.3. except that nicardipine ($0.1\mu\text{M}$; NIC) was present in the ^{45}Ca -containing PSS instead of EPA. The complete methodology is described in the methods chapter, section 2.2.2.4.

It can be seen in figure 5.5 that after 5 min incubation, nicardipine ($0.1\mu\text{M}$) failed to block calcium influx. These results were in contrast to the results obtained in the cell contraction studies reported in chapter 4 (shown in figure 4.10) which demonstrated that nicardipine ($0.1\mu\text{M}$) blocked the contractile response of the cells to a depolarising solution containing 60 mM KCl. However, it should be noted that in figure 5.2, it can be seen that the effect of depolarisation in the AOCs in the calcium influx experiments was more clearly observed after 30 min incubation; as dihydropyridine blockers have high affinity for the inactivated state of the calcium channel, a state predominant in smooth muscle in response to depolarisation, (Spedding & Paoletti, 1992), it was considered that the effect of nicardipine on the AOCs might be more clearly apparent after 30 min depolarisation.

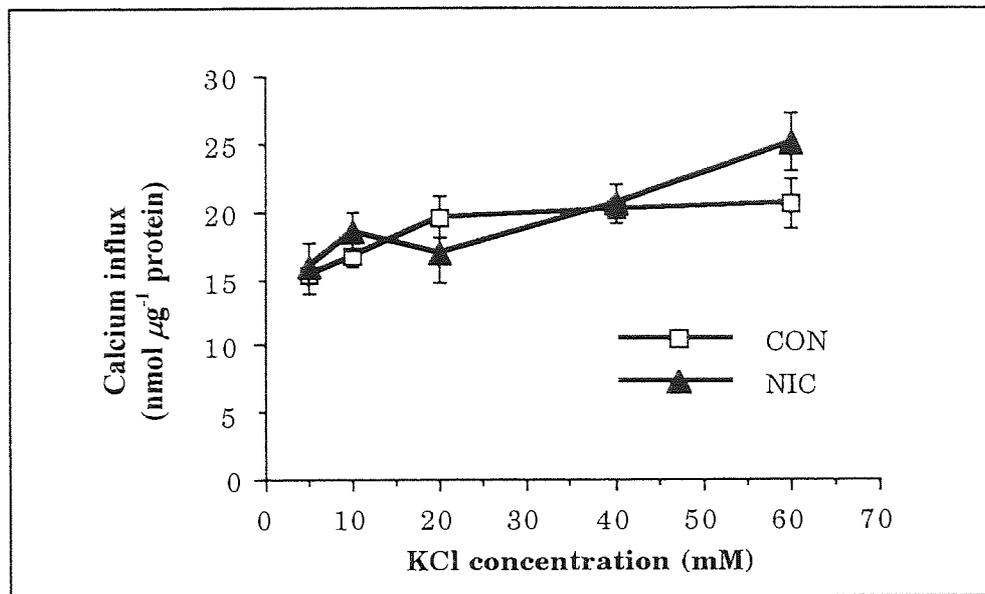


Figure 5.5 Effect of a 5 min incubation with nicardipine on calcium influx. Cells were incubated for 5 min in PSS at 37°C, containing ^{45}Ca ($2\mu\text{Ci ml}^{-1}$) and either 5, 10, 20, 40 or 60 mM KCl. Nicardipine ($0.1\mu\text{M}$; NIC) or PSS ($10\mu\text{l}$; CON) was also present. Following removal of the solution, the cells were lysed and radioactivity measured. Values are expressed as $\text{nmol } \mu\text{g}^{-1} \text{ protein} \pm \text{s.e. mean}$ from 4-9 experiments ($n \geq 4$).

It can be seen in figure 5.6 that after 30 min incubation, nicardipine ($0.1\mu\text{M}$) blocked calcium influx induced by all concentrations of KCl. However, nicardipine ($0.1\mu\text{M}$) unexpectedly blocked calcium influx measured in PSS, suggesting either that the AOCs were depolarised even in PSS, or that the methodology of the experiments resulted in a time dependent increase in calcium influx even in PSS. To determine whether the AOCs were depolarised even in PSS, voltage-clamping experiments were required. These would measure the membrane potential of the AOCs from which it would be possible to conclude whether the membrane potential of the AOCs in PSS was less negative than the resting value, however this technique was not available for use during the course of the investigations. However, what was certain, was that the effect of nicardipine was greater than the effect measured with EPA, shown in figure 5.4.

Therefore, a final experiment was performed to compare the effect of EPA and nicardipine directly, with emphasis upon exposing the cells to PSS and 10 mM KCl in which EPA had previously been shown to decrease influx and also to 60 mM KCl, which represented a depolarising condition (Ieseki *et al*, 1996).

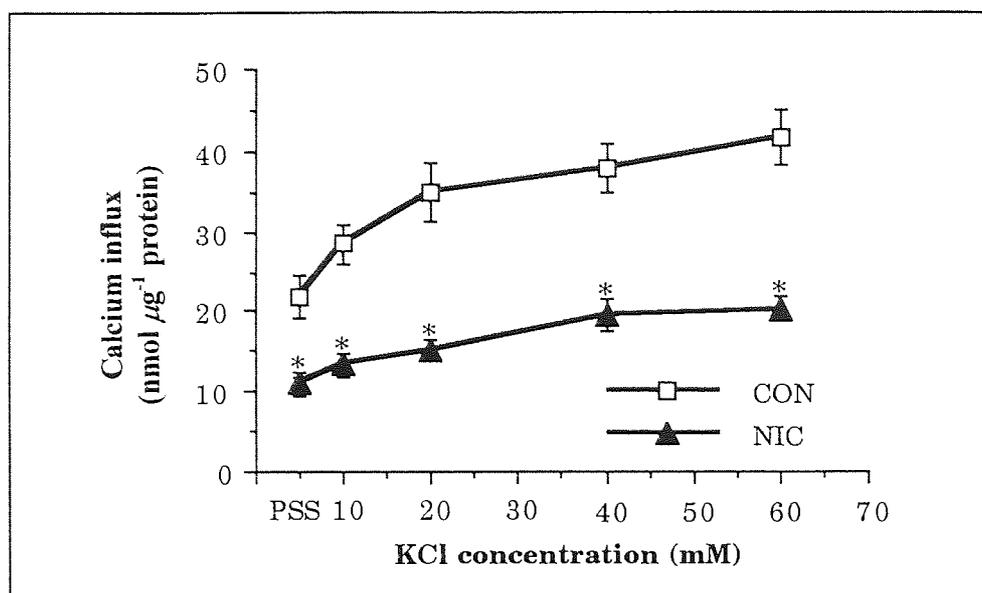


Figure 5.6 Effect of a 30 min incubation with nicardipine on calcium influx. Cells were incubated for 30 min in PSS at 37°C, containing ^{45}Ca ($2\mu\text{Ci ml}^{-1}$) and either 10, 20, 40 or 60 mM KCl. Nicardipine ($0.1\mu\text{M}$; NIC) or PSS ($10\mu\text{l}$; CON) was also present. Following removal of the solution, the cells were lysed and radioactivity measured. Values are expressed as $\text{nmol }\mu\text{g}^{-1}$ protein \pm s.e. mean. * indicates significant difference from corresponding CON value where $P < 0.05$ from 9 experiments ($n=9$).

5.2.5. Effect of EPA on KCl-induced calcium influx.

The experimental procedure was similar to that already outlined in the previous sections, but is described fully in 2.2.2.5. Monolayers of cells were incubated with ^{45}Ca ($1\mu\text{Ci ml}^{-1}$) for 40 min in the presence of 5, 10 or 60 mM KCl. The effect of adding EPA ($50\mu\text{M}$), ETH (1%), PSS ($10\mu\text{l}$; CON) or nicardipine ($0.1\mu\text{M}$) was examined. In the latter experiment a pre-incubation of 30 min with nicardipine ($0.1\mu\text{M}$) added to the culture medium was also included. All values are given as the mean \pm s.e. mean for n experiments and statistical comparisons were made by analysis of variance (ANOVA) followed by Scheffe's multiple range test. Significance was identified at $P < 0.05$. The results are shown in table 5.1 and figures 5.7 and 5.8.

It can be seen in figures 5.7 that calcium influx in PSS, or induced by 10 or 60 mM KCl was significantly ($P < 0.05$) reduced by nicardipine, consistent with the previous results obtained and with the data obtained in the cell-contraction studies, shown in chapter 4. Figure 5.8 shows, however, that EPA significantly ($P < 0.05$) reduced influx in PSS, consistent with the previous results, but failed to reduce influx in 10 mM KCl and enhanced influx induced by 60 mM KCl. This latter observation contrasted with the previous results measured to EPA in which EPA simply failed to block influx at 60 mM KCl (shown in figure 5.4) and also with

the results in chapter 4, where EPA reduced the cell contraction induced by 60 mM KCl.

In view of this, the next objective was to determine whether the changes measured in influx were associated with corresponding changes in intracellular calcium concentrations ($[Ca^{2+}]_i$).

Table 5.1. Effect of EPA, ethanol or nicardipine on calcium influx. Cells were pre-incubated for 30 min with PSS (10 μ l) or nicardipine (0.1 μ M; NIC) added to the culture medium. Cells exposed to nicardipine were then incubated for 40 min in PSS containing nicardipine (0.1 μ M; NIC) at 37°C whilst cells not pre-incubated with nicardipine were incubated for 40 min in PSS or in PSS containing EPA (50 μ M) or ethanol (1%, EPA vehicle; ETH) at 37°C. ^{45}Ca and PSS or PSS with 10 or 60 mM KCl were present. Following removal of the solution, the cells were lysed and radioactivity measured.

KCl concentration (mM)	Calcium influx (nmol μ g ⁻¹ protein)			
	CON	NIC (0.1 μ M)	ETH (1%)	EPA (50 μ M)
PSS	14.61 \pm 0.73 (n=10)	4.07 \pm 0.38 ^a (n=4)	14.44 \pm 1.21 (n=12)	9.35 \pm 2.68 ^b (n=12)
10	18.69 \pm 1.48 (n=16)	10.30 \pm 0.86 ^a (n=4)	19.09 \pm 2.74 (n=18)	31.65 \pm 3.67 (n=18)
60	23.10 \pm 4.17 (n=16)	14.60 \pm 0.76 ^a (n=4)	24.72 \pm 6.10 (n=18)	51.23 \pm 6.12 (n=18)

^a indicates significant difference from CON, ^b indicates significant difference from ETH. Values are expressed as nmol μ g⁻¹ protein \pm s.e.mean for 4-18 experiments (n \geq 4).

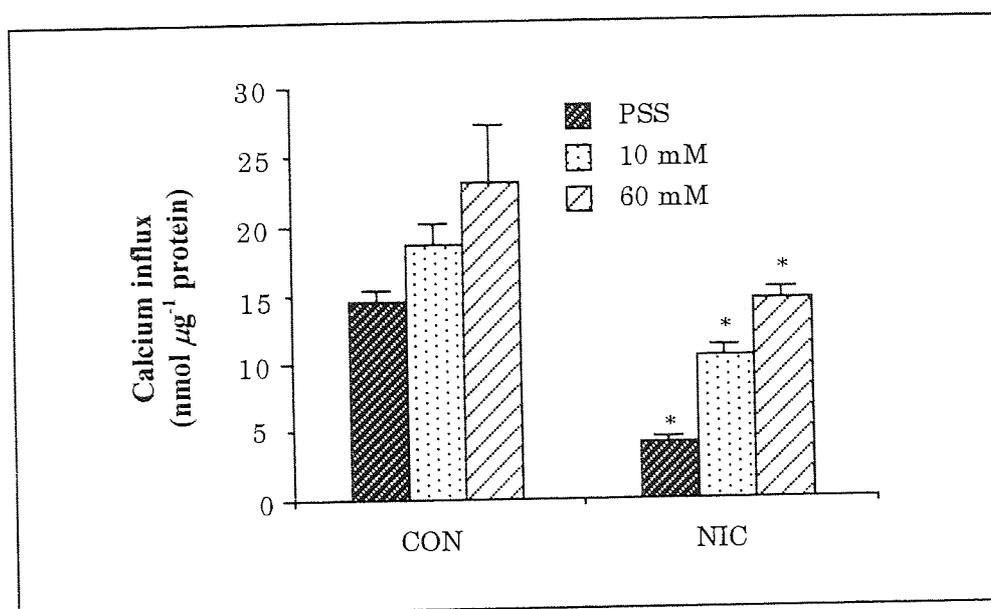


Figure 5.7. Effect of a 30 min pre-incubation with nicardipine on calcium influx. Cells were pre-incubated for 30 min with PSS (10 μ l) or nicardipine (0.1 μ M; NIC) added to the culture medium. Cells exposed to nicardipine were then incubated for 40 min in PSS containing nicardipine (0.1 μ M; NIC) at 37°C whilst cells not pre-incubated with nicardipine were incubated for 40 min in PSS at 37°C. ⁴⁵Ca and PSS (5 mM KCl) or PSS with 10 or 60 mM KCl were present. Following removal of the solution, the cells were lysed and radioactivity measured. Values are expressed as nmol μ g⁻¹ protein \pm s.e. mean for 4-12 experiments ($n \geq 4$).

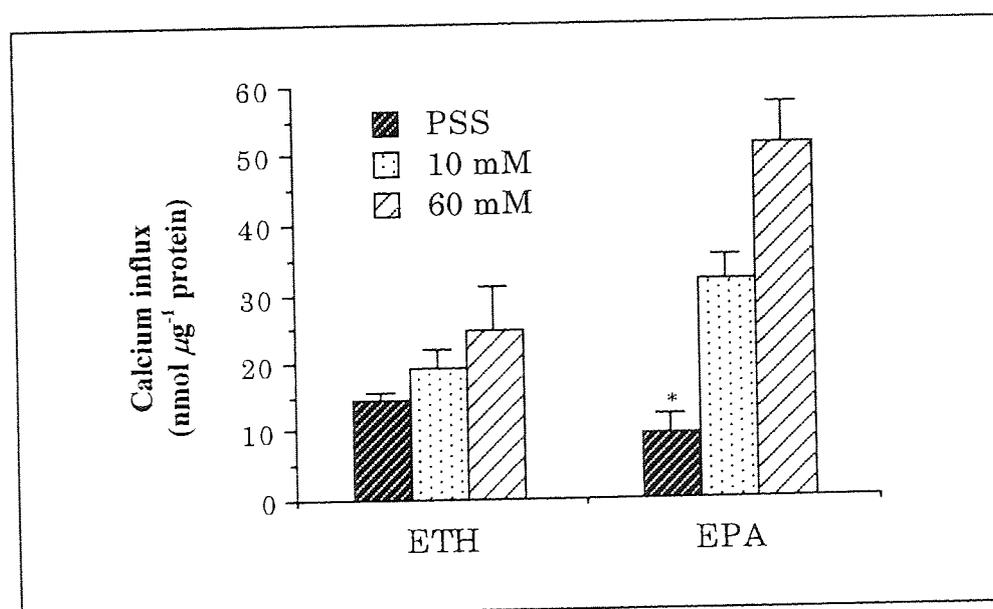


Figure 5.8. Effect of EPA on calcium influx. Cells were incubated for 40 min in PSS containing EPA (50 μ M) or ethanol (1%, EPA vehicle; ETH). ⁴⁵Ca and PSS (5 mM KCl) or PSS with 10 or 60 mM KCl were present. Following removal of the solution, the cells were lysed and radioactivity measured. Values are expressed as nmol μ g⁻¹ protein \pm s.e. mean for 12-18 experiments ($n > 12$).

5.2.7. Effect of KCl on $[Ca^{2+}]_i$.

A preliminary study in this investigation measured the response of the AOCs to KCl (60 mM). AOC's were isolated and cultured for 10-13 days and then prepared for $[Ca^{2+}]_i$ measurements. The cells were incubated for 60 min in PSS at 37°C containing 0.5 μ M fura-2/AM, washed in PSS at 37°C and then re suspended in either PSS at 37°C or calcium-free PSS at 37°C (at a density of 5×10^5 cells ml⁻¹) to allow complete hydrolysis of the indicator from the acetoxymethyl ester. After 30 min, 2 ml of the cell suspension was transferred to a cuvette and placed in the luminescent spectrometer (Perkin Elmer LS50). Excitation wavelengths at 340 and 380 nm and emission at 510 nm were set. The cells were stirred and equilibrated for 5 min at 37°C, then the effect of the addition of KCl (60 mM) to the PSS or calcium-free PSS was examined. The methodology is described fully in chapter 2, section 2.3.1 and 2.3.2.

The initial finding in these studies was that basal $[Ca^{2+}]_i$ showed variability between 61.5 and 307.6 nM, shown in table 5.3. This made it difficult to average any subsequent responses to stimulation with KCl, therefore, the data presented in figure 5.9 is a representation of the responses obtained.

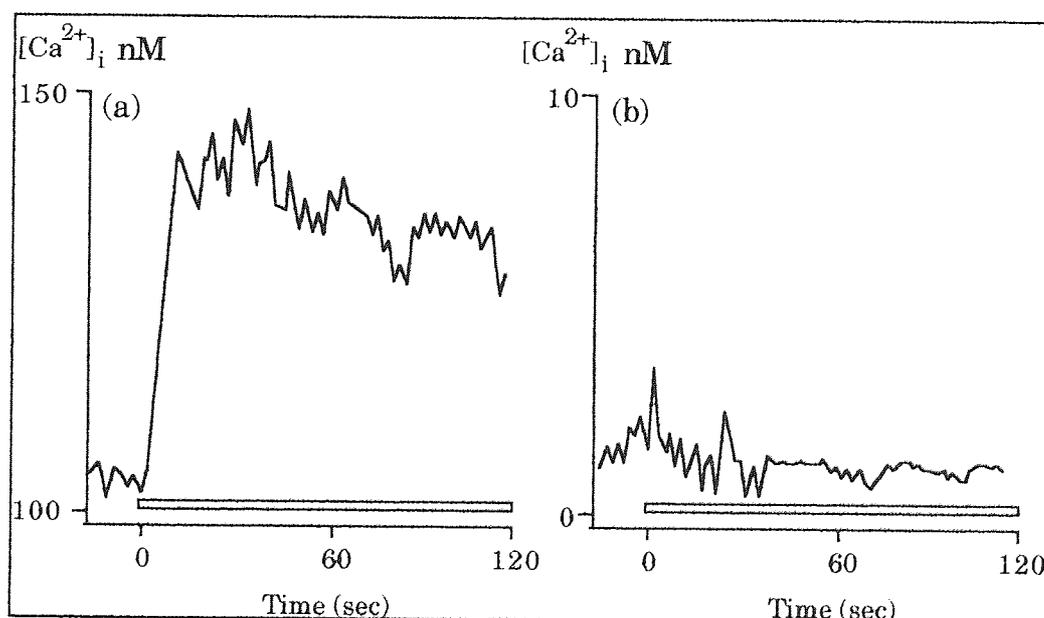


Figure 5.9. Representative traces of the KCl-induced $[Ca^{2+}]_i$, determined using fura-2. In the presence of PSS at 37°C (a), KCl (60mM; ---) induced a rapid rise of $[Ca^{2+}]_i$ followed by a sustained phase lasting over 2 min. When the cells were exposed to calcium-free PSS (b) at 37°C, $[Ca^{2+}]_i$ was not elevated in response to KCl (60 mM). Each trace is representative from 6 experiments

Despite the variation in $[Ca^{2+}]_i$ measured, the range of results was consistent with those reported elsewhere (Bukoski *et al*, 1994). It can be seen in Figure 5.9. that KCl induced a rapid rise of $[Ca^{2+}]_i$, which was followed by a sustained phase. During the sustained phase, $[Ca^{2+}]_i$ remained elevated above baseline for at least 2 min in the presence of PSS. Conversely in Ca^{2+} -free PSS, KCl did not induce a rise in $[Ca^{2+}]_i$. These results demonstrated that KCl elevated $[Ca^{2+}]_i$ by activating calcium entry, so the effect of EPA and nicardipine on this response were examined.

Table 5.2. Effect of EPA or nicardipine on $[Ca^{2+}]_i$. After equilibration for 5 min in PSS at 37°C, a single dose of KCl (60 mM) was added to the PSS. $[Ca^{2+}]_i$ was determined according to the method of Grynkiewicz (1985) before (basal) and after KCl. In some experiments EPA (50 μ M) or nicardipine (0.1 μ M; NIC) was present in the equilibration.

Basal	$[Ca^{2+}]_i$ (nM)		
	KCl (60 mM)	NA (1 μ M) after equilibration with EPA (50 μ M)	NA (1 μ M) after equilibration with NIC (0.1 μ M)
61.5	77.3	47.8	0.5
152.4	168.2	100.3	2.0
100.0	154.2	74.3	0.0
307.6	266.0	265.0	5.0
71.6	57.6	53.2	0.0
268.5	256.0	197.0	3.1

5.2.8. Effect of KCl-induced $[Ca^{2+}]_i$ in the presence of EPA or nicardipine.

The study was extended to determine whether EPA had any effect upon the increase in $[Ca^{2+}]_i$ produced by KCl. The experiments described for the KCl study were repeated but the cells were equilibrated for 5 min in PSS at 37°C in the presence of nicardipine (0.1 μ M) or EPA (50 μ M). A single dose of KCl (60 mM) was then added to the PSS.

However, the $[Ca^{2+}]_i$ levels measured exhibited variability between experimental runs. Therefore, in each experiment, the responses to KCl in the presence of EPA or nicardipine was expressed as a percentage of the $[Ca^{2+}]_i$ measured to KCl in their absence, shown in table 5.3.

Nicardipine (0.1 μ M), decreased the peak KCl-induced $[Ca^{2+}]_i$ to a mean value of 0.04 ± 0.03 % of the KCl response obtained in the absence of nicardipine ($n=6$), whereas EPA reduced the response to a mean value of 73.1 ± 8.20 % ($n=6$). The response of the cells to nicardipine and EPA was consistent with the effects measured in the cell shortening experiments obtained previously, in chapter 3.

Moreover, the difference observed between the effect of EPA and nicardipine further suggested that EPA might not be associated with a mechanism of action at the L-type calcium channel, consistent with the previous suggestion made in response to the previous results on calcium influx. However, the results did not explain the observed increased influx with EPA in 60 mM KCl.

Therefore, as $[Ca^{2+}]_i$ is also associated with an effect at the α -adrenoceptor and because EPA reduced the effect of noradrenaline, shown in the cell contraction studies in chapter 3, the effect of EPA was examined on the noradrenaline response to intracellular calcium levels ($[Ca^{2+}]_i$). Initially, the effect of noradrenaline was examined.

5.2.9. Effect of noradrenaline on $[Ca^{2+}]_i$.

The same methodology was carried out in this study as that performed in the investigation on the effect of KCl, except that a single dose of noradrenaline (1 μ M) replaced the use of KCl. To exclude the effect of calcium influx on $[Ca^{2+}]_i$, it was necessary to measure $[Ca^{2+}]_i$ in fura-2 loaded cells in a calcium-free PSS. In performing this experiment, it was anticipated that a short duration of exposure of the cells to a decreased concentration of extra cellular calcium avoided a significant loss of intracellularly stored calcium.

The initial finding in these studies was that basal $[Ca^{2+}]_i$ showed variability between 45.7 and 258.0 nM, shown in table 5.4. This made it difficult to average any subsequent responses to stimulation with noradrenaline (1 μ M). Therefore, the data presented in figure 5.10 is a representation of the responses obtained. However, despite the variation in $[Ca^{2+}]_i$ measured, the range of results was consistent with those reported previously (Kitajima *et al*, 1996; Koh *et al*, 1994; Xuan & Glass, 1996; Batlle *et al*, 1991; Marsh & Hill, 1993; Touyz *et al*, 1994; Bukoski *et al*, 1994).

It is shown that noradrenaline (1 μ M) induced a transient increase in $[Ca^{2+}]_i$, suggesting release of calcium from intracellular stores, including the sarcoplasmic reticulum (Van Breeman, 1989), which was followed by a sustained phase. During the sustained phase, $[Ca^{2+}]_i$ remained elevated above baseline for at least 2 min in the presence of PSS. Conversely, when the cells were exposed to Ca^{2+} -free PSS, noradrenaline induced a rise in $[Ca^{2+}]_i$ which returned to basal levels (at a similar rate of decline) within 2 min. This latter result suggested that noradrenaline- caused a release of calcium from intracellular stores and the difference between the two curves may represent entry of extra cellular calcium, assuming no calcium was bound to the cell membranes. The effect of EPA on this response was then studied and compared with phentolamine, the α -adrenoceptor antagonist.

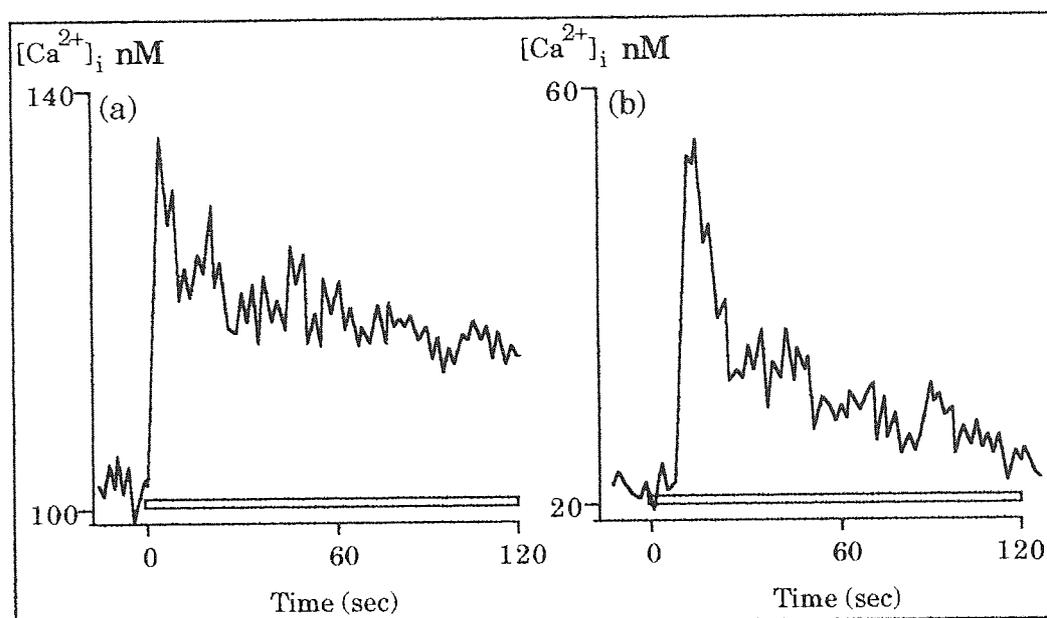


Figure 5.10. Representative traces of the noradrenaline-induced $[Ca^{2+}]_i$, determined using fura-2. In the presence of PSS at 37°C (a), noradrenaline (1 μ M; ---) induced a rapid rise of $[Ca^{2+}]_i$ followed by a sustained phase lasting over 2 min. When the cells were exposed to calcium-free PSS at 37°C (b), noradrenaline induced a rise in $[Ca^{2+}]_i$ which returned to basal levels within 2 min. Each trace is representative from 6 experiments.

Table 5.3. Effect of EPA or phentolamine on $[Ca^{2+}]_i$. After equilibration for 5 min in PSS at 37°C, a single dose of noradrenaline (1 μ M; NA) was added to the PSS. $[Ca^{2+}]_i$ was determined according to the method of Grynkiewicz et al., (1985) before (basal) and after NA. In some experiments EPA (50 μ M) or phentolamine (1 μ M; PHENT) was present in the equilibration.

Basal	$[Ca^{2+}]_i$ (nM)		
	NA (1 μ M)	NA (1 μ M) after equilibration with EPA (50 μ M)	NA (1 μ M) after equilibration with PHENT (1 μ M)
45.7	52.3	41.3	34.5
102.4	136.2	100.0	77.1
103.6	124.8	100.2	95.6
231.4	284.4	200.2	200.6
66.0	76.3	54.2	28.3
258.0	298.5	203.5	168.2

5.2.10. Effect of noradrenaline-induced $[Ca^{2+}]_i$ in the presence of EPA or phentolamine.

The cells were equilibrated for 5 min in PSS at 37°C in the presence of EPA (50 μ M) or phentolamine (1 μ M). A single dose of noradrenaline (1 μ M) was then added to the PSS.

The $[Ca^{2+}]_i$ levels measured exhibited variability between experimental runs. Therefore, in each experiment, the responses to noradrenaline in the presence of EPA or phentolamine was expressed as a percentage of the response measured to noradrenaline in their absence, shown in table 5.4.

EPA (50 μ M) decreased the maximal noradrenaline-stimulated $[Ca^{2+}]_i$ increase to a mean value of 80.9 ± 3.84 % of the noradrenaline-induced response ($n=6$), whereas in the presence of phentolamine, it was reduced to a mean value of 66.2 ± 5.58 % of the response ($n=6$).

The response of the cells to phentolamine and EPA was consistent with the effects measured in the cell shortening experiments obtained previously, in chapter 3. However, it was not clear whether EPA was affecting calcium influx through receptor-operated channels or the intracellular release of calcium. Therefore, a final study of calcium influx was performed to examine the effect of EPA on noradrenaline-induced calcium influx and the effect of EPA directly on $[Ca^{2+}]_i$ was investigated.

In the calcium influx study, the effect of noradrenaline was initially measured upon calcium influx in the AOCs.

5.2.11. Effect of noradrenaline on calcium influx.

Monolayers of cells were incubated with ^{45}Ca ($1\mu Ci ml^{-1}$) for 30 min in the presence of 10 nM, 0.1 μ M, 1 μ M, 10 μ M or 0.1 μ M noradrenaline, as described in 2.2.2.6. The results are presented in figure 5.11 and show that noradrenaline induced a concentration-dependent increase in calcium influx in the aortic cells.

5.2.12 Effect of EPA on noradrenaline-induced calcium influx.

The effect of EPA on noradrenaline-induced calcium influx in the AOCs was then examined. The experiment was repeated, but cells were pre-incubated in PSS at 37 °C for 30 min, with EPA (50 μ M) or 1% ethanol included in some wells. Also, the maximum concentration of noradrenaline used was 1 μ M as this was considered the upper physiological limit. The complete methodology is described in 2.2.2.7. The results are shown in figure 5.12

Calcium influx induced by 0.01 μ M, 0.1 μ M or 1 μ M NA was 5.80 ± 0.37 ($n=6$), 5.43 ± 0.33 ($n=6$) or 6.67 ± 0.56 nmol μg^{-1} protein ($n=6$) respectively in the presence of ETH (1%: EPA vehicle). EPA significantly decreased influx ($P<0.05$) in the presence of 0.01 μ M NA and 1 μ M NA to 3.86 ± 0.44 ($n=6$) and 5.34 ± 0.51 nmol μg^{-1} protein ($n=6$) respectively. The result at 0.1 μ M NA was not significantly decreased (4.52 ± 0.49 ; $n=6$), but this probably represented abnormally low values measured in the presence of ETH at this concentration. Therefore, the results

suggested that the mechanism of action of EPA may be associated with calcium influx through receptor-operated channels.

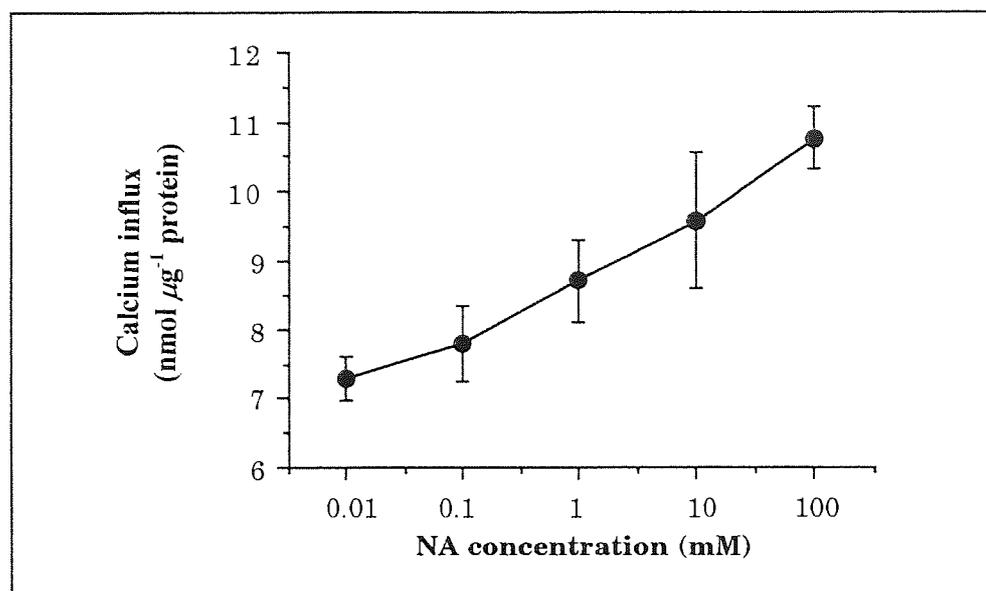


Figure 5.11. Effect of noradrenaline on calcium influx. Cells were incubated for 30 min in PSS containing ^{45}Ca and either 10 nM, 0.1 μM , 1 μM , 10 μM or 0.1 μM noradrenaline (NA). Following removal of the solution, the cells were lysed and radioactivity measured. Values are expressed as $\text{nmol } \mu\text{g}^{-1}$ protein \pm s.e.mean for 8 experiments ($n=8$).

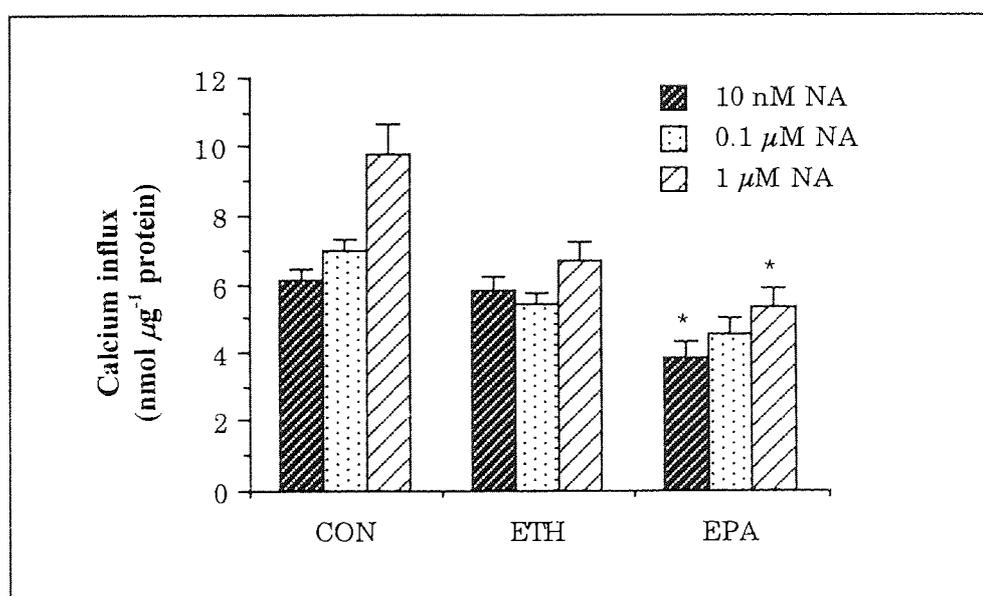


Figure 5.12. Effect of EPA on noradrenaline-induced calcium influx. Cells were incubated for 60 min in PSS containing ethanol (1%, EPA vehicle; ETH) or EPA (50 μM) or PSS (1 μl). ^{45}Ca (1 $\mu\text{Ci ml}^{-1}$) and 10 nM, 0.1 μM or 1 μM noradrenaline (NA) were present for the final 30 min. Following removal of the solution, the cells were lysed and radioactivity measured. Values are expressed as $\text{nmol } \mu\text{g}^{-1}$ protein \pm s.e.mean for 6 experiments ($n=6$). * indicates significant difference from corresponding ETH value.

5.2.13. Effect of EPA on $[Ca^{2+}]_i$.

The next objective in this investigation was to examine whether an effect of EPA on $[Ca^{2+}]_i$ could be observed in AOCs. To measure the background effect of EPA, a single dose of EPA ($50 \mu\text{M}$) replaced the use of noradrenaline or KCl, as described in the previous experiments. It can be seen in Figure 5.13 that when fura-2 loaded cells were exposed to EPA ($50 \mu\text{M}$) in PSS or calcium-free PSS, there was a transient rise in $[Ca^{2+}]_i$, which rapidly returned to basal levels within 1 min. These results suggested that EPA caused a release of intracellular calcium from storage sites.

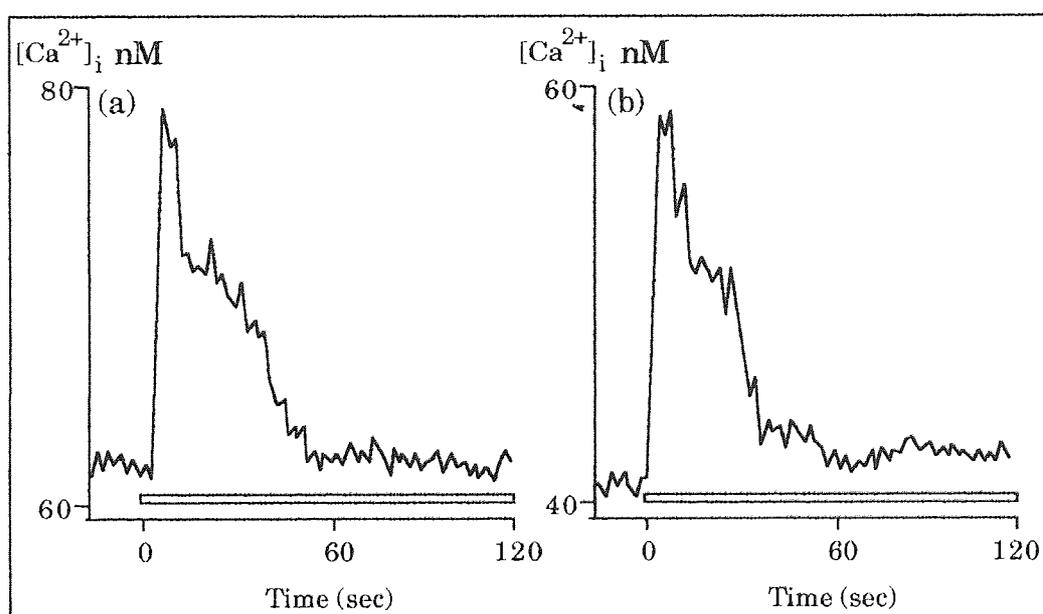


Figure 5.13. Representative traces of the EPA-induced $[Ca^{2+}]_i$, determined using fura-2. In the presence of PSS at 37°C (a) or calcium-free PSS (b) at 37°C, EPA ($50 \mu\text{M}$; —) induced a transient rise in $[Ca^{2+}]_i$ which rapidly returned to basal levels within 1 min. Each trace is representative from 6 experiments.

5.3. Discussion

In chapter 4, the partial inhibition by EPA, of the KCl- or noradrenaline-induced reduction of cell length measured in the AOCs indicates that EPA mediated an anti-vasoconstrictor effect which was endothelium-independent. Furthermore, the results suggested that the relaxatory action of EPA may be associated with the regulation of intracellular calcium *via* influx or mobilisation of stored calcium, however, the precise mechanism remained to be elucidated. The aim of this chapter, therefore, was to determine whether this anti-vasoconstrictor effect of EPA was associated with a block of calcium influx into the AOCs and whether the effect on influx altered the concentration of intracellular calcium.

Depolarisation of vascular smooth muscle induced by high concentrations of potassium is known to cause contraction (Droogmans *et al*, 1977) associated with increased calcium influx (van Breeman *et al*, 1972). Therefore, the preliminary study, presented in the results at the start of the present chapter demonstrated that over a 60 min period, calcium influx into the AOCs could be measured in the AOCs and was increased by depolarisation induced experimentally by the application of a solution to the cellular surface, containing potassium ions at a concentration of 60 mM. This was consistent with a previous study which measured a significant increase in calcium influx in monolayers of primary aortic cells derived from Wistar-Kyoto rats following exposure of the cells to 55 mM KCl (Yamada *et al*, 1992).

However, it was also noted that at the zero time point of the experiments reported at the start of this chapter and outlined above, calcium influx was approximately 30 % the total influx measured after 60 min. This was surprising, as it is widely recognised that intact rat aortic tissue maintained in PSS has a stable resting membrane potential of -50 - 55 mV, (Spedding & Paoletti, 1992) and the potential must depolarise to around -40 mV or -20 mV for T- or L-type calcium channels respectively to be activated. It has previously been shown that aortic smooth muscle cells have a resting membrane potential of -51 ± 1 mV (Bray *et al*, 1991), or -44.6 ± 7.4 mV (Asano *et al*, 1998) similar to that reported in the intact tissue. Therefore, the membrane potential of the AOCs was not considered to cause the observed effect on influx at the commencement of the experiments.

An alternative consideration, therefore, was that during the experimental procedure, some of the radiolabelled calcium (^{45}Ca) may be membrane-bound and thereby give rise to an inaccurately greater value for calcium influx. However, the repeated washing procedure following the termination of each experiment was performed in a lanthanum-containing solution. It is known that lanthanum displaces extracellularly bound calcium and inhibits calcium transport across

biological membranes, including transport through passive leak channels (Cauvin *et al*, 1983). Therefore, the incorporation of membrane-bound ^{45}Ca was disregarded as a likely contributory factor to the apparently high influx. A critical evaluation of the methodology was subsequently performed to try and account for the seemingly high influx; in performing the experiments, the AOCs were allowed to equilibrate for 30 min in PSS at 37°C prior to experimentation. As it has previously been reported that inactivation of excitable calcium channels can occur shortly after stimulation (Zschauer *et al*, 1987), equilibration occurred in the absence of ^{45}Ca , so that upon exposure to the tracer and stimulus, the influx of tracer would be clearly measured. Theoretically, this methodology did not allow for intra- and extracellular equilibration with ^{45}Ca and might favour the passive influx of ^{45}Ca down the concentration gradient. However, only tracer amounts of a high specific activity ^{45}Ca were added to the extracellular solution, so the balance of equilibrium should not be disturbed (Gurney, 1989). Consequently, upon exposure to depolarisation, a non-preferential influx of labelled and unlabelled calcium would ensue. A possibility may exist, however, that as the experimental incubation time increased, intra- and extracellular equilibration of ^{45}Ca might occur, thereby increasing the concentration of labelled calcium inside the cells. Consequently, following cell depolarisation, calcium may enter the cells as before, but with an inaccurate, greater measure of influx.

In considering whether this was likely, it was noted that a previous report in which the effect of depolarisation on calcium influx was measured in cells involved pre-equilibration of the cells with ^{45}Ca , however, the equilibration lasted for only 30 s (Zschauer *et al*, 1987). Furthermore, this report did not show measurements of ^{45}Ca influx at the zero time point. It was noted that after only 90 s exposure to either a depolarising or non-depolarising solution, influx was almost 30 % that measured after 60 min and furthermore was approximately 50 % that measured after 10 min, even in control PSS. The data presented in the results reported at the beginning of this chapter are consistent with these results. Moreover, this effect was consistently reported by Yamada *et al* (1992), in which no zero time point measurements were shown and in control PSS influx was approximately 30 % that at 60 min and greater than 50 % that measured at 10 min. Unlike Zschauer *et al* these researchers, along with others (Khoiyi *et al* (1993); Orlov *et al*, 1993; Yatani *et al*, 1987) did not pre-equilibrate the cells in ^{45}Ca prior to stimulation. Therefore, the seemingly high influx of calcium at the zero time point of the experiments is consistent with results presented prior to the investigation.

It was also noted that in the time course study reported at the beginning of this chapter, influx was only significantly greater ($P < 0.05$) in AOCs exposed to the

depolarising solution compared to those in PSS at the 20 and 40 min incubation time points, suggesting that the cells were responsive to depolarising solution, but not following immediate exposure to a depolarising solution. This was in contrast to the results shown in the cell-contraction studies in chapter 3 where the AOCs contracted under depolarising conditions within 1 min and remained contracted for a minimum 5 min period. The inconsistency between these results was considered to be a result of the variation in the data measured at the 5 and 10 min time points in the calcium influx study, rather than an effect of the cells, as the variability in data subsequently decreased with increasing repetition of the experiments. However, the calcium influx study was repeated after 5 min depolarisation to enable comparison with the cell contraction studies in chapter 3 and the effect of KCl concentration was also examined. The experiments were then repeated a further time, but influx was measured after 30 min, as a significant increase in calcium influx in cells exposed to the depolarising solution had been obtained at 20 min and 40 min in the previous results in this chapter.

After 5 min, the response to 60 mM KCl was significantly greater than the effect in PSS, consistent with the cell contraction studies performed in chapter 3. After 30 min, KCl caused a concentration-dependent increase in calcium influx, with maximal activation at 20, 40 or 60 mM KCl, consistent with previous studies on rabbit aortic strips (Iesaki *et al*, 1996) and primary cultured, aortic myocytes from the Wistar rat (Bodin *et al*, 1991).

The results therefore confirmed the data obtained in the cell contraction studies reported in chapter 3 which demonstrated that the cells were responsive to a depolarising solution. The influx experiment was repeated, therefore, but the effect of EPA on influx was examined in order to determine whether EPA might reduce influx by an action at the L-type calcium channel. The response to EPA was compared with the vehicle control (1 % ethanol) after 5 or 30 min.

The L-type calcium channel can exist in one of three distinct modes, although each channel switches randomly between these modes (Hess *et al*, 1984). The opening probability of the channel in response to depolarisation corresponds to the mode the channel is in; in mode 0 or *inactive*, the channel does not open; in mode 1 or *closed*, opening probability is low and the duration of opening is brief; in mode 2, or *open*, opening probability is high, and each opening is prolonged. As depolarisation increases, so the channel opens with increased frequency and duration, but the channel still switches between the modes. When it is in mode 0, dihydropyridine antagonists, if present, bind to the channel. This prevents the channel from switching modes, consequently rendering the channel inactive. As depolarisation increases, the inactivating effect of the dihydropyridine antagonists is more clearly observed.

This was not the observed response to EPA; after either 5 or 30 min (although measurements of calcium influx showed less variability after 30 min), EPA (50 μM) significantly decreased calcium influx measured in PSS or 10 mM KCl, but failed to reduce influx induced by the higher, depolarising concentrations of KCl at 20, 40 or 60 mM. These results suggested that EPA was not acting at the L-type channel. In a previous report by Hallaq *et al* (1992), EPA (5 μM), DHA (5 μM) and the dihydropyridine calcium blocker nitrendipine (0.03 nM) were all able to block ouabain toxicity (0.1 mM) in cardiac myocytes. Ouabain is a cardiac glycoside, used for the treatment of cardiac failure. Its primary action is on the K^+ -binding site of the $\text{Na}^{2+}/\text{K}^+$ -ATPase of the cell membrane, where it binds, thereby inhibiting the pump. As a consequence, there is increased intracellular Na^+ , which increases $\text{Na}^+/\text{Ca}^{2+}$ exchange. Thus, $[\text{Ca}^{2+}]_i$ increases, which leads to increased sequestration into storage sites, and increased Ca^{2+} influx. So they suggested an effect of EPA associated with block at L-type channels. However, it was also shown that EPA revealed a non-competitive decrease upon the binding of nitrendipine to the L-type channel (Hallaq *et al* ;1992). This demonstrated that EPA does not bind at the same subunit site as the dihydropyridines, so Hallaq *et al* (1992) suggested that the n-3 fatty acids or their metabolites may bind at a functionally associated site. However, non-competitive binding with dihydropyridines is shown by drugs including verapamil and diltiazem (Triggle, 1991) both of which selectively block the L-type calcium channel.

Therefore, the effect of an L-type calcium channel blocker on calcium influx in the AOCs was examined, using nicardipine. It was shown that after a 5 min exposure to nicardipine (0.1 μM) no block of calcium influx was measured. These results were in contrast to the results obtained in the cell contraction studies reported in chapter 4, which demonstrated that nicardipine (0.1 μM) significantly reduced KCl-induced cell contraction and that nicardipine (0.1 μM) blocked KCl-induced increases in $[\text{Ca}^{2+}]_i$ in the presence of extra cellular calcium. However, it was noted that in figure 5.2 the effect of depolarisation in the AOCs on calcium influx was clearer after 30 min incubation, so the effect of nicardipine on influx was examined after 30 min. It followed that nicardipine (0.1 μM) blocked calcium influx induced by all concentrations of KCl after 30 min. In smooth muscle with membrane potentials of -50 to -55 mV, L-type channels have a greater predominance of the inactivated state (Spedding & Paoletti, 1992). It is widely accepted that dihydropyridine blockers usually have high affinity for the inactivated state of the calcium channel, thereby keeping the channel inactive and consequently blocking calcium influx, although it should not be overlooked that it has been reported that they may have high affinity for the open state of the channel in smooth muscle cells (Cohen & McCarthy, 1987). Therefore, the effect

of nicardipine was anticipated. However, the effect of nicardipine to block calcium influx in PSS (figure 5.?) was not expected. This result suggested either that the AOCs were depolarised, even in PSS or that the methodology of the experiments resulted in a time dependent increase in calcium influx, since influx in PSS was greater after 30 min compared to 5 min. However, the results of nicardipine in PSS were consistent with a previous report by Sada *et al* (1990), in which nicardipine (0.1 μM) decreased resting tension and basal $[\text{Ca}^{2+}]_i$ in aortic strips from Wistar-Kyoto rats. Unfortunately, however, the results were not explained in the report by Sada *et al* (1990).

Earlier in this discussion, the membrane potential of aortic smooth muscle cells in PSS was assumed to be the same as the resting value. Without performing voltage-clamp experiments this could not be confirmed, but this technique was not available to the author. So, assuming no altered effect on the resting membrane potential of the cells, an alternative explanation was sought.

It has previously been reported that in radioligand binding studies, uptake of dihydropyridines and other calcium antagonists into biological membranes may be affected by the lipophilic nature of the drug. This results in very high uptake, with accumulation of 3,000-fold (nifedipine) to 19,000-fold (amlodipine), (Spedding & Paoletti, 1992). Therefore, this high concentration of dihydropyridines in the membrane may be in equilibrium with the receptor site, even under non-depolarising conditions. This might serve to explain the results obtained in the present study. So, assuming that the resting membrane potential of the aortic smooth muscle cells was not altered in PSS and that the block of influx by nicardipine in PSS was possibly due to equilibrium of the antagonist with the receptor site, a final experiment was performed to compare the effect of EPA and nicardipine.

The cells were bathed in PSS, a solution containing 10 mM KCl, or a solution containing 60 mM KCl. The latter represented the depolarising condition used on the cells earlier in the chapter and also in chapters 3 and 4, whilst EPA had previously been shown in the chapter to decrease influx in 10 mM KCl.

Calcium influx in PSS, or induced by 10 or 60 mM KCl was significantly ($P < 0.05$) reduced by nicardipine (0.1 μM). Conversely, EPA significantly ($P < 0.05$) reduced influx in PSS but not in 10 mM KCl and enhanced influx induced by 60 mM KCl, thereby suggesting that EPA is not associated simply with block of L-type channels. The enhanced influx induced by 60 mM demonstrated an additive effect on influx. It has previously been reported that that an additive effect on calcium influx can occur when a high potassium-induced calcium influx is combined with influx induced by receptor-operated channel agonists (Zschauer *et al*, 1987), leading to vasoconstriction. However, it is unreasonable to suggest that

the results observed in the present study were due to EPA acting as a receptor-operated channel agonist, since previous reports have demonstrated that EPA causes a vasorelaxatory effect. Furthermore, in chapter 4, EPA partially blocked the effect of 60 mM KCl in the cell contraction studies. Therefore, a discrepancy exists between the results obtained so far in this thesis. On the one hand, EPA blocked KCl-(60 μ M) induced cell contraction, yet on the other hand, it increased the KCl-(60 μ M) induced calcium influx. In considering the discrepancy, it was recognised that in the calcium influx studies, the methodology involved incubating EPA on the cells simultaneously with the depolarising solution. In the cell contraction studies, however, EPA was pre-incubated on the cells before KCl was added. Furthermore, the degree of block of some calcium ion channels increases with membrane depolarisation and frequency of stimulation. Some calcium channels are blocked (eg by verapamil) only after they are opened or inactivated. Diltiazem, in contrast to verapamil, inhibits cardiac calcium ion channels by binding mainly to the inactivated state, whereas nitrendipine exhibits some resting state block. If EPA was also to exhibit resting state block, then pre-incubating it on the AOCs, as performed in the cell contraction studies, might result in block of channels in the resting state. Furthermore, the cell contraction experiments in chapter 4 lasted a duration of only 2 minutes, which may have been long enough for EPA to still block the channel, even though the cells were exposed to 60 mM KCl for the last minute. In contrast to this methodology, the calcium influx studies did not involve a pre-incubation of the AOCs with EPA. Furthermore, the duration of the influx experiment was 40 min which may have ensured that the 60 mM KCl depolarised the cells, producing calcium influx and thereby overcame any block which may have been initially mediated by EPA.

Therefore, it is suggested that the discrepancy in the cell contraction and calcium influx results to 60 mM KCl be accounted for by the variation in the experimental methodologies, in particular, to pre-incubate, or not, with EPA.

Therefore, the results shown so far in this thesis suggested that EPA mediated a partial block of cell contraction induced by vasoactive agents and inhibited calcium influx, except when depolarised with 60 mM KCl for 40 min. The results also suggested that the effect of EPA was not associated with the block of L-type calcium channels but might be associated with block at the T-type channel. It was clear from the results, that the effect of EPA on calcium influx in 60 mM KCl was inconsistent with the other results obtained so far. In an attempt to elucidate whether the discrepancy was due to experimental variation in the methodologies used for all the studies, the next objective was to determine the effect of EPA on intracellular calcium concentrations ($[Ca^{2+}]_i$). In particular, the

study aimed to clarify which of the results, either the cell contraction studies, or the influx studies was representative of the true effect of EPA in 60 mM KCl.

A number of methods are available for measuring $[Ca^{2+}]_i$ directly including calcium-selective micro electrodes and nuclear magnetic resonance, but the most widely used methods employ optical probes which monitor calcium as a change in absorbance, luminescence or fluorescence (McCormack & Allen, 1991). Fluorescent calcium-sensing indicators are popular mainly because they are apparently incorporated easily into cells and they produce signals which can be monitored with fairly simple and widely available instrumentation. Such indicators absorb light at one wavelength and emit light at a longer wavelength (Gurney, 1989). The values at which this occurs with fura-2 are well separated, making it a useful fluorescent probe with which to study changes in $[Ca^{2+}]_i$. Fura-2 binds calcium, which shifts the excitation spectrum of fura-2 to shorter wavelengths (Grynkiewicz *et al*, 1985). The amount of shift produced depends on the amount of calcium binding.

In the present study, KCl (60 mM) induced a rapid rise of $[Ca^{2+}]_i$ which was followed by a sustained phase. During the sustained phase, $[Ca^{2+}]_i$ remained elevated above baseline for at least 2 min in the presence of PSS. Conversely, in Ca^{2+} -free PSS, KCl did not induce a rise in $[Ca^{2+}]_i$. These results were similar to other results (Abe *et al*, 1995; Ito *et al*, 1993) and indicated that KCl elevated $[Ca^{2+}]_i$ by activating calcium entry. The study was continued, to determine whether EPA had any effect upon the increase in $[Ca^{2+}]_i$ produced by KCl. The effect was also compared to the effect of nicardipine and it was shown that EPA significantly decreased the peak KCl-induced $[Ca^{2+}]_i$, but the effect was less than the effect of nicardipine. This was consistent with the effects of EPA and nicardipine measured in the cell shortening experiments obtained in chapter 4. Moreover, the difference observed between the effect of EPA and nicardipine confirmed that EPA was not associated simply with a mechanism of action at the L-type calcium channel, supporting the suggestion of Hallaq *et al* (1992). The results also provided further confirmation that the increased influx measured to EPA in 60 mM KCl at the beginning of this present chapter was likely due to the lack of pre-incubation with EPA.

$[Ca^{2+}]_i$ is also associated with an effect at the α -adrenoceptor and in the cell contraction studies shown in chapter 4, EPA reduced the effect of noradrenaline, an agonist at the α -adrenoceptor receptor. Therefore, the effect of EPA was examined on the noradrenaline response to $[Ca^{2+}]_i$. This would also serve to establish that EPA did not act as a receptor-operated channel agonist. Initially, the effect of noradrenaline was examined.

The initial finding in these studies was that basal $[Ca^{2+}]_i$ showed variability between 45.7 and 258.0 nM. This made it difficult to average any subsequent responses to stimulation with noradrenaline (1 μ M), which induced a transient increase in $[Ca^{2+}]_i$, suggesting release of calcium from intracellular stores, including the sarcoplasmic reticulum (Van Breeman, 1989). Therefore, the data presented in figure 5.10 is a representation of the responses obtained. However, despite the variation in $[Ca^{2+}]_i$ measured, the range of results was consistent with those reported elsewhere; in rat aortic strips, Kitajima *et al* (1996) measured 84 ± 3.7 nM ($n=4$); in rat aortic cells, Koh *et al* (1994) measured approximately 100 nM and in calcium-free solution, basal levels were approximately 50 nM; Xuan & Glass (1996) measured between 50 -100 nM in rat aortic cells; Batlle *et al* (1991) measured 124 nM in rat aortic cells; Marsh & Hill (1993) measured 69 ± 2 nM ($n=353$) and in calcium-free PSS, basal $[Ca^{2+}]_i$ was 33 ± 2 nM ($n=140$), in bovine tracheal smooth muscle cells; Touyz *et al* (1994) measured 96 ± 10 nM in rat mesenteric artery cells from Wistars; Bukoski *et al* (1994) measured 65 ± 9.3 nM in rat mesenteric arteries and Erdbrügger *et al* (1993) measured 40 to 120 nM for noradrenaline-induced $[Ca^{2+}]_i$ levels in aortic smooth muscle cells.

The responses to noradrenaline in the present study were sustained above baseline for at least 2 min which is also consistent with results reported elsewhere (Erdbrügger *et al*; 1993) and suggest the opening of receptor-operated calcium channels. In calcium-free PSS, noradrenaline (1 μ M) induced a transient rise in $[Ca^{2+}]_i$ which was not sustained. Similar results in the absence of calcium were demonstrated by Abe *et al* (1995) and Marsh & Hill (1993).

Upon examination of the effect of EPA, responses were expressed as a percentage of the response measured to noradrenaline (1 μ M). Both EPA (50 μ M) and phentolamine (1 μ M) significantly ($P<0.05$) decreased the noradrenaline-induced $[Ca^{2+}]_i$, although the effect of EPA was less than that of phentolamine, consistent with the effects measured in the cell contraction experiments obtained in chapter 4. A previous report using porcine aortic smooth muscle cells revealed that phentolamine (10 μ M) inhibited noradrenaline-induced increases in $[Ca^{2+}]_i$ by 96 ± 6 % ($n=3$) (Erdbrügger *et al*, 1993) which was greater than the effect of phentolamine (1 μ M) measured in the present investigation (66.2 ± 5.58 % of the response; $n=6$). The difference is likely attributed to variation in the experimental procedure, including animal species and drug concentrations used, but the results serve to confirm that the AOCs are a viable model in which to study α -adrenergic mediated responses.

It was not clear at this stage of the study, whether EPA reduced the noradrenaline-stimulated $[Ca^{2+}]_i$ *via* an action linked to inhibition of receptor-operated channels or linked to decreased intracellular release of calcium.

Therefore the next objective in the present investigation was to determine whether the effect of EPA was associated with reduced calcium influx into AOCs through receptor-operated channels. If EPA failed to reduce $[Ca^{2+}]_i$ in the absence of extracellular calcium, then the effect of EPA would not be associated with receptor-operated channels. Consequently, the effect of EPA on the noradrenaline response in calcium-free PSS was eagerly awaited. Unfortunately, failure of the equipment at this point did not allow for further measurements to be taken. Therefore, a final study of calcium influx was performed to examine the effect of EPA on noradrenaline-induced calcium influx. Obviously, it was not appropriate to remove extracellular calcium in an influx experiment so the line of thought for this experiment was reversed; if EPA reduced the noradrenaline-induced calcium influx in the presence of extracellular calcium, then the effect of EPA could be associated with receptor-operated channels. A preliminary study confirmed a measurable effect of noradrenaline upon calcium influx in the AOCs.

The results of these experiments revealed that the noradrenaline-induced, concentration-dependent increase in calcium influx was significantly ($P < 0.05$) reduced by EPA. This was consistent with a previous report by Asano *et al* (1997) who measured vasopressin- and endothelin-induced calcium currents (I_{CAT}) in rat A7r5 aortic smooth muscle cells using the whole-cell voltage clamp technique and found that EPA, DHA and DPA (docosapentaenoic acid) inhibited I_{CAT} . It has been reported that I_{CAT} are mediated partly by calcium-permeable receptor-operated channels (Benham & Tsien, 1987; Byrne & Large, 1988). Furthermore, in vascular smooth muscle, the hormones vasopressin and endothelin induce calcium release from internal stores and calcium influx through receptor-operated calcium channels. Therefore, it is suggested that the results of the experiments with noradrenaline in the present chapter are consistent with an action of EPA to reduce calcium influx through receptor-operated channels.

However an action solely associated with receptor-operated channels does not explain the effect of EPA to block the KCl-induced cell contraction in chapter 3, nor the effect of EPA to significantly decrease the peak KCl-induced $[Ca^{2+}]_i$ in the present chapter. It also does not explain the subsequent results obtained in the present study in which EPA ($50 \mu M$) induced a transient rise in $[Ca^{2+}]_i$, which rapidly returned to basal levels within 1 min, in the presence or absence of extracellular calcium.

An increase in $[Ca^{2+}]_i$ can be mediated by an influx of extracellular calcium or by the release of intracellular calcium from internal storage sites. Therefore, these latter results suggested that EPA also caused a release of intracellular calcium from storage sites, which is not immediately recognised as being associated with a vasorelaxatory response. However, it is noteworthy that a previous report

has shown that $[Ca^{2+}]_i$ is not always effectively utilised to phosphorylate the myosin light chain and so mediate cell contraction (Kitajima *et al*, 1996). In smooth muscle cells, it has also been shown that compartmentalised calcium may locally activate membrane ion channels, ion pumps, exchange carriers and enzymes, without activating the contractile elements located in the central cytoplasm of the cell (Abe *et al*, 1995).

Therefore, it is suggested that the transient EPA-induced release of calcium may be from an internal binding site, possibly that of an intracellular membrane which may, upon its release, then serve in the longer term to prevent calcium influx and subsequently block further cell contraction, to noradrenaline or KCl.

In conclusion therefore, EPA (50 μ M) significantly decreased calcium influx measured in PSS or 10 mM KCl, but failed to reduce influx induced by the higher, depolarising concentrations of KCl at 20, 40 or 60 mM. Moreover, the effect was not consistent with that of nicardipine, so although the mechanism of action of EPA appeared to be associated with blocking calcium entry, it was not simply attributable to the block of L-type voltage-dependent calcium channels. This was confirmed when EPA inhibited noradrenaline-induced calcium influx, suggesting that the mechanism of action of EPA, at least in part, is consistent with an action to block calcium influx through receptor-operated channels. However, this does not explain the EPA-induced transient increase of $[Ca^{2+}]_i$.

A transient increase in $[Ca^{2+}]_i$ can occur following inositol 1,4,5-trisphosphate-induced releases of calcium from intracellular storage sites (Berridge, 1989) and has been shown to activate membrane-associated calcium-activated potassium channels. This hyperpolarises the plasma membrane, moving the membrane potential farther from the activation range for calcium channels. This effect might serve to explain the effect of EPA to reduce calcium influx induced by low concentrations of KCl. Therefore, the mechanism by which the transient increase of $[Ca^{2+}]_i$ occurred, and the effect of EPA upon membrane-associated potassium channels remained for further clarification.

Chapter 6

The Effect Of EPA On Inositol Phosphate Formation

6.1. Introduction

Results described so far in this thesis have demonstrated that EPA partially blocks the contraction of cells to the vasoactive agents, KCl and noradrenaline (chapter 4). As previously discussed, EPA-induced relaxation of vascular smooth muscle preparations may reflect a reduction in intracellular calcium ion concentration as a result of a decreased influx of extracellular calcium or the blockade of intracellular calcium release. Subsequent studies (chapter 5) examined the effect of EPA on the influx of extracellular calcium where it was found to significantly decrease influx. However, the results also showed that EPA was not associated simply with the block of L-type calcium channels, confirming that it did not bind at the same subunit site as the dihydropyridine calcium channel blockers as recently suggested (Hallaq *et al*, 1992). However, the results did suggest that EPA could reduce calcium influx through receptor-operated channels, consistent with a previous report by Asano *et al* (1997) since EPA significantly reduced the noradrenaline-induced, concentration-dependent increase in calcium influx.

However, it was also shown in chapter 5, that when extracellular calcium was absent, EPA induced a transient increase in $[Ca^{2+}]_i$. The results in chapter 5 did not demonstrate that this increase was associated with calcium influx, therefore the aim of the studies reported in this chapter was to determine whether it was linked to a mechanism associated with increasing intracellular calcium release.

Calcium entry into the cytosol from the sarcoplasmic reticulum is under the control of second messengers (Van Breeman, 1989), including inositol 1,4,5-trisphosphate (IP₃; Berridge & Irvine, 1989) which is formed *via* hydrolysis of membrane phosphoinositides following α_1 -adrenoceptor stimulation (Han *et al*, 1987). The importance of the phosphatidylinositol (PtdIns) cycle on the free intracellular calcium ion concentration $[Ca^{2+}]_i$ and cellular function has been described in the general introduction. However, experimental studies have suggested that the n-3 fatty acids inhibit α -adrenoceptor stimulation (Engler, 1992b), although the extent of this effect has not been described, nor whether the effect was α -adrenoceptor specific. It has previously been shown that dietary intake of n-3 and n-6 polyunsaturated fatty acids for 8 weeks reduced total release

of inositol phosphates in isolated rat left atria (Woodcock *et al*, 1995), but there are no reports demonstrating an acute effect of EPA on IP₃-mediated intracellular calcium release in aortic smooth muscle cells. Therefore, the objectives of the present study were to demonstrate an effect of EPA, α -adrenoceptor agonists and α -adrenoceptor antagonists on inositol phosphate formation in AOCs.

Preliminary studies involved demonstrating that formation of total inositol phosphates occurred in the AOCs. The methodology involved in these studies is described in 2.4 and was based on the assay devised by Downes & Michell (1981) and developed by Berridge and co-workers (1983) to measure the incorporation of the radioactive label, tritium, into inositol phosphates. In summary, the AOCs were labelled with PSS containing radioactive *myo* [³H]-inositol for 24 hours, and then stimulated with an agonist. The PSS used throughout these studies contained BSA (3 mg ml⁻¹) to support cell survival, propranolol (1 μ M) to inhibit β -adrenoceptors, ascorbic acid (50 μ M) to prevent oxidation of catecholamines and EDTA (10 μ M) to chelate heavy metal impurities. The subsequent formation of *myo*-[³H] inositol phosphates (Berridge *et al*, 1983) were quantified after phase separation of the solution using 1,1,2-trichloro-1,2,2-trifluoroethane and tri-*n*-octylamine, by anion-exchange chromatography on columns containing 0.5 ml of Dowex-1 (X8; formate form) and elution of total [³H]inositol phosphates with formic acid (0.1 M) and ammonium formate (1 M). Radioactivity in the eluate was measured by liquid scintillation counting using a tritium program and inositol phosphate formation was expressed as counts per minute (cpm).

Studies of the effect of EPA on inositol phosphate formation followed the same methodology except that a single dose of EPA (50 μ M) or ethanol (1% final concentration) replaced the use of the α -adrenoceptor agonist and in some experiments a combination of noradrenaline (1 μ M) and a single dose of EPA (50 μ M) or ethanol (1% final concentration) was used. In some experiments, cellular protein was determined by the method of Lowry *et al* (1951), to normalise inositol phosphate formation as cpm mg⁻¹ protein.

Blanks, in which tracer was omitted from the extra cellular solution were performed routinely in each experiment and the measurements taken were subtracted from the corresponding measurements made in the presence of tracer. All experimental conditions were performed in duplicate or triplicate and the experiments were repeated using plates containing cells dispersed on different occasions to account for intra- and inter-experimental variation. Total inositol phosphates are presented as the means of *n* experiments \pm the standard error of the mean (s.e. mean). Statistical comparisons were made by Student's two-tailed *t* test for unpaired data, or in the case of multiple comparisons, analysis of variance

(ANOVA) followed by Scheffe's multiple range test. Significance was identified at $P < 0.05$

Second messengers are formed in very small amounts following receptor stimulation and are degraded rapidly after the signal has been transmitted (Oldham, 1991). Therefore, agonists used to stimulate formation of inositol phosphate were added in the presence of lithium ions which inhibit the enzymatic dephosphorylation of inositol monophosphates and so prevent the recycling of inositol (Berridge *et al*, 1982). To increase percentage incorporation of the label and to increase retrieval of labelled products from the cells, a high concentration of a high specific activity *myo*-[^3H] inositol was used on the cells.

It was shown in chapter 4 that EPA partially inhibited the noradrenaline-induced reduction in cell length, therefore, the objective of the next experiments was to examine whether any effect of noradrenaline on the inositol phosphate response was reduced by EPA. Similarly, it was shown in chapter 5 that EPA increased calcium influx in AOCs depolarised with 60 mM KCl, therefore, the objective of final experiments was to examine whether any effect of cell depolarisation on the inositol phosphate response was increased by EPA.

6.2. Results

6.2.1. The recovery of *myo*-[³H] inositol from the anion-exchange columns.

The anion-exchange columns were prepared according to the methodology described in 2.4.2 and their viability for use in the present investigation was ascertained. A solution of 5 μ Ci *myo*-[³H] inositol in 4 ml distilled H₂O was loaded into a column and the eluent collected, added to 10 ml scintillant (Hisafe 2) and counted for radioactivity. This was compared with controls, in which 5 μ Ci *myo*-[³H] inositol was added to 10 ml scintillant directly and then counted.

The mean count per minute of the radioactivity loaded into the columns was 1096783.1 ± 18379.16 ($n=20$), or 96% of the controls (1053750.0 ± 14900.15 ; $n=20$). Therefore, the use of the columns did not significantly reduce retrieval of radioactivity, consistent with the report by Downes & Michell (1981).

However, for ion-exchange chromatography to be used to quantify the amount of *myo*-[³H] inositol phosphate incorporated into AOCs following agonist-mediated hydrolysis of membrane phosphoinositides, the solution loaded into the columns was not simply distilled H₂O containing *myo*-[³H] inositol; the use of trichloroacetic acid (TCA) to precipitate cellular protein, followed by the separation of the TCA from the inositol-containing components of the cell using a mixture of freon and trioctylamine meant that any radioactivity contained in the AOCs would be added to a series of solutions prior to loading into the columns and prior to subsequent collection in the eluent.

Therefore, the next preliminary experiment, described in detail in 2.4.3.1, examined the effect of the steps used in the experiment on the retrieval of the radioactivity. 5 μ Ci *myo*-[³H] inositol was added to 0.5 ml trichloroacetic acid (TCA; 20 % w/v) which was then added to 5 ml of a 1:1 mixture of freon/trioctylamine. 0.5 ml of the upper phase was removed, added to 4 ml distilled H₂O and loaded into a column. Eluent was added to 10 ml scintillant and counted. This was compared with values obtained when 5 μ Ci *myo*-[³H] inositol was added directly to 5 ml freon/trioctylamine.

When added to the freon/trioctylamine, the mean counts per minute was 993584.5 ± 6501.38 ($n=20$), which was 91 % of the mean loaded value (1096783.1 ± 18379.16). When added to the TCA, the mean counts per minute was 941468.0 ± 5316.95 ($n=20$), which was 86 % of the mean loaded value. Therefore it was concluded that the method could be used with measurable retrieval of 5 μ Ci *myo*-[³H] inositol.

The next preliminary experiment therefore was to determine whether *myo*-[³H] inositol would be incorporated into the AOC membrane phosphoinositides.

6.2.2. Investigation of *myo*-[³H] inositol incorporation into the cells

In this study, AOCs were cultured for a maximum of 20 days in 24-well tissue culture plates, used for experimentation at 24 h intervals and exposed to the labelling solution for 24 h, according to the methodology described in 2.4.3.2 The experiment was repeated on cells isolated and dispersed on two other separate occasions.

Figure 6.1 shows that between 0 and 5 days in culture, there was a steady increase from 48.1 ± 3.84 to 77.53 ± 2.62 counts per minute (cpm) in the AOCs. Between 5 and 9 days in culture, incorporation of *myo*-[³H] inositol increased to 147.3 ± 8.26 cpm and steady state incorporation of around 160 cpm was achieved at 10 days.

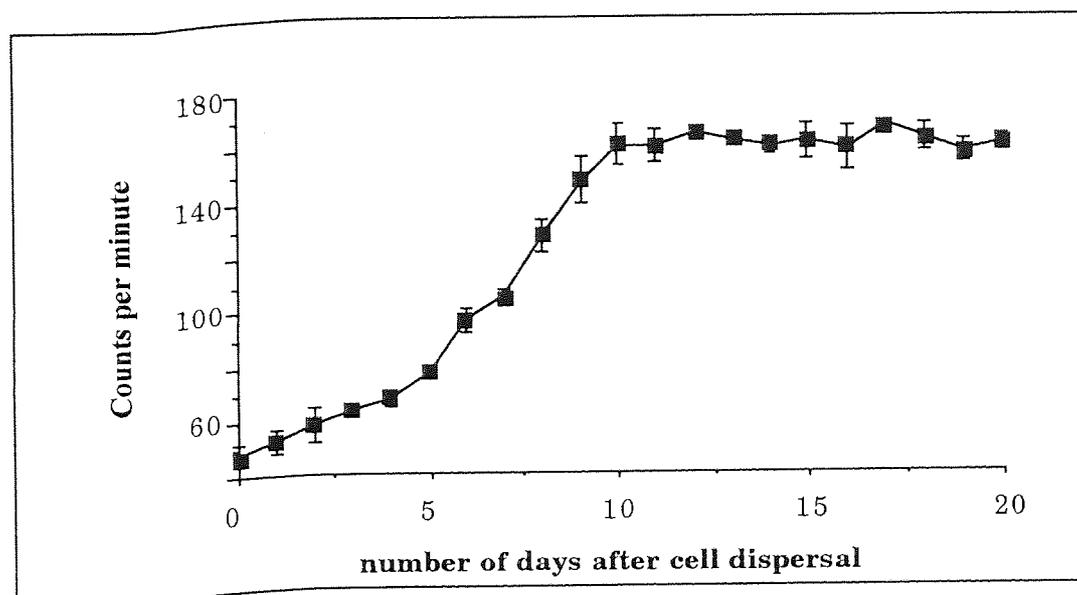


Figure 6.1. Determination of the incorporation of *myo*-[³H] inositol into AOCs. AOCs were incubated for 24 h with 5 μ Ci *myo*-[³H] inositol. Following washing and lysing the cells, *myo*-[³H]inositol phosphate incorporation was determined by anion-exchange chromatography. Radioactivity in the elutes was measured. All values are mean counts per minute \pm s.e.mean for 3 experiments ($n=3$).

6.2.3. Determination of labelling time with *myo*-[³H] inositol.

The next aim in the preliminary studies was to determine whether increasing the labelling time of the cells with *myo*-[³H] inositol significantly increased its incorporation into AOCs, as previous reports document different labelling times of 3 days (Locher *et al.*, 1989) or 48 h (de Jonge *et al.*, 1996). In the previous section, steady-state incorporation of the radio label was shown to occur in cells at day 10 in culture, therefore only AOCs cultured for 7, 10 or 14 days were used. The experiments were performed according to the methodology outlined in

the previous section, except that exposure time of the cells to the labelling solution was 24, 48 or 72 h.

In cells used at day 7, 10 or 14 of culture there was little variation in the inositol phosphate levels measured when the incubation period with *myo*-[³H] inositol was 24, 48 or 72 h, shown in figure 6.2.

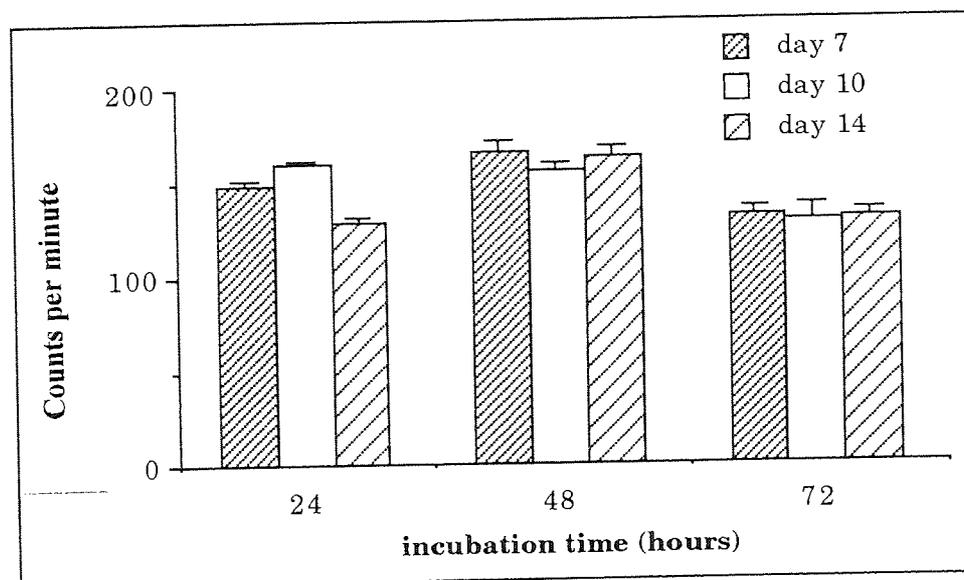


Figure 6.2. Effect of labelling time with *myo*-[³H] inositol on *myo*-[³H] inositol phosphate formation. AOCs at 7, 10 or 14 days in culture were incubated for 24, 48 or 72 h with 5 μ Ci *myo*-[³H] inositol. Following washing and lysing the cells, *myo*-[³H]inositol phosphate incorporation was determined by anion-exchange chromatography. Radioactivity in the elutes was measured. All values are mean counts per minute \pm s.e.mean for 3 experiments ($n=3$).

Therefore, there was no significant difference between incubation times of 24, 48 or 72 h in the absence of an agonist-induced response. However, a further preliminary study was performed which examined the effect of labelling time on the α -adrenoceptor agonist-mediated inositol phosphate response to determine the most appropriate labelling time for use in subsequent studies concerned with examining the effect of EPA upon inositol phosphate levels in the AOCs.

6.2.4. Effect of labelling time on α -adrenoceptor agonist-stimulated *myo*-[³H] inositol incorporation.

These studies followed the same procedure as that outlined in the previous section, except that following equilibration, the cells were exposed to a single dose of the agonist for the final 5 min of the LiCl-containing incubation. The complete methodology is described in 2.4.3.4.

Table 6.1. Effect of labelling time on α -adrenoceptor agonist-induced myo-[^3H] inositol on myo-[^3H] inositol phosphate formation. AOCs at 7, 10 or 14 days in culture were incubated for 24, 48 or 72 h with 5 μCi myo-[^3H] inositol. A single dose of noradrenaline (1 μM ; NA) or phenylephrine (1 μM ; PHE) was added to the LiCl-containing incubation. Following washing and lysing the cells, myo-[^3H]inositol phosphate incorporation was determined by anion-exchange chromatography. Radioactivity in the elutes was measured as counts per minute (cpm).

Control	Inositol phosphate levels (cpm)		
	24 h	48 h	72 h
7 days	148.70 \pm 2.17	164.85 \pm 6.46	131.21 \pm 4.40
10 days	159.51 \pm 2.24	156.21 \pm 3.25	128.83 \pm 8.30
14 days	128.44 \pm 2.99	161.95 \pm 5.82	129.84 \pm 4.22
<i>NA (1 μM)</i>			
7 days	214.53 \pm 1.78	228.96 \pm 7.99	230.35 \pm 2.62
10 days	264.55 \pm 4.20	281.6 \pm 5.17	342.71 \pm 19.39
14 days	278.70 \pm 10.72	225.95 \pm 9.63	175.53 \pm 6.00
<i>PHE (1 μM)</i>			
7 days	183.14 \pm 2.84	202.35 \pm 8.65	220.85 \pm 4.61
10 days	188.48 \pm 3.19	254.60 \pm 4.91	252.75 \pm 11.79
14 days	271.26 \pm 15.38	237.68 \pm 8.34	141.90 \pm 5.84

All values are mean counts per minute \pm s.e.mean for 3 experiments ($n=3$).

It is evident from table 6.1 and figure 6.3 that there was no significant difference in inositol phosphate formation following labelling the AOCs with myo-[^3H]inositol for 24, 48 or 72 followed by exposure to noradrenaline (1 μM) or phenylephrine (1 μM) for 5 min. Consequently, a 24 h labelling time, contrary to 3 days (Locher *et al.*, 1989) or 48 h (de Jonge *et al.*, 1996) was subsequently used.

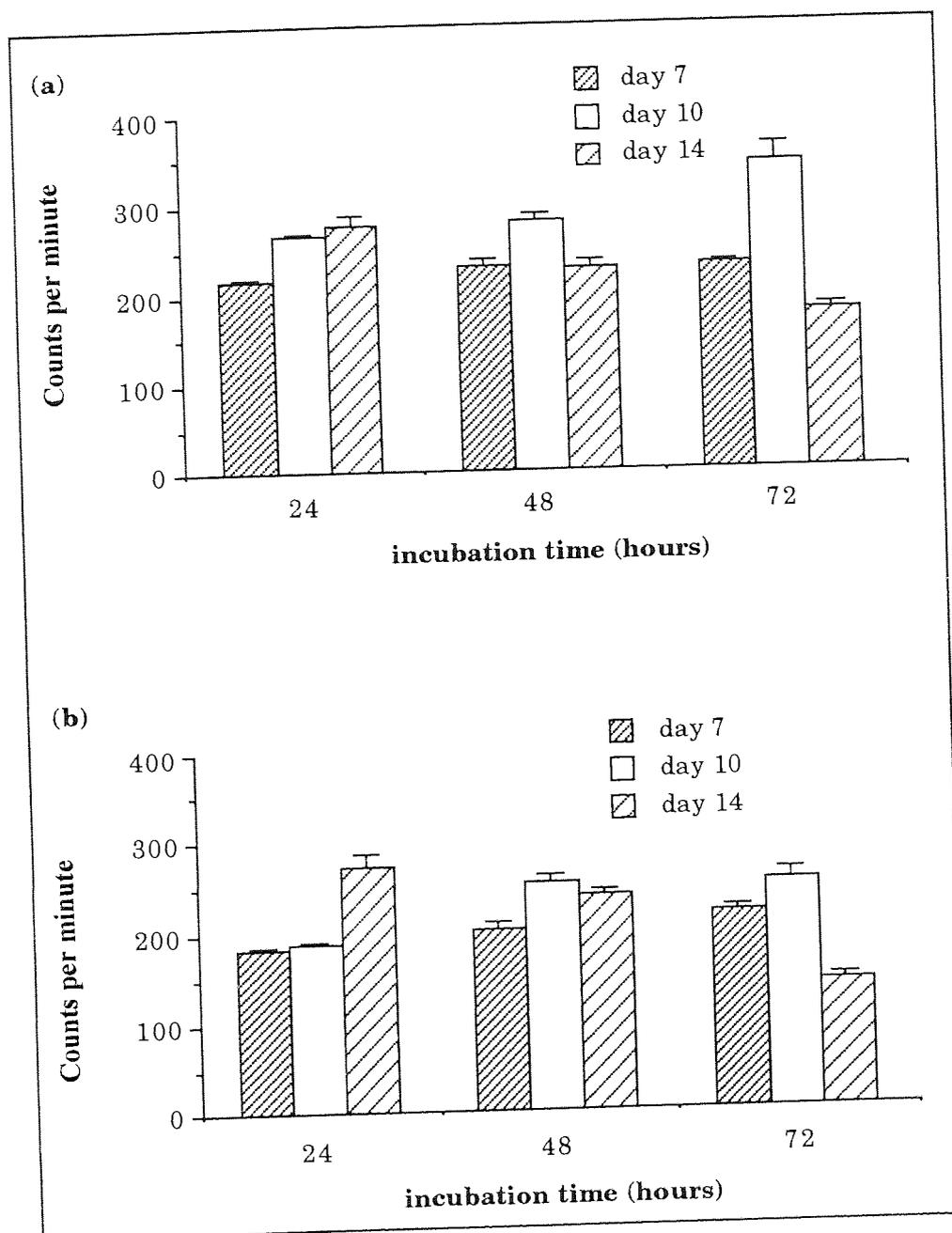


Figure 6.3. Effect of α -adrenoceptor agonists after 24, 48 or 72 h labelling with myo- ^3H inositol, on myo- ^3H inositol phosphate formation. AOCs at 7, 10 or 14 days in culture were incubated for 24, 48 or 72 h with 5 μCi myo- ^3H inositol. Following washing and equilibration, the cells were incubated in the presence of LiCl for 20 min. (a) Noradrenaline (1 μM) or (b) phenylephrine (1 μM) was added for the final 5 min after which time, cells were washed and lysed. Myo- ^3H inositol phosphate incorporation was determined by anion-exchange chromatography. Radioactivity in the elutes was measured. All values are mean counts per minute \pm s.e. mean for 3 experiments ($n=3$).

6.2.5. Effect of α -adrenoceptor agonists on *myo*-[^3H] inositol phosphate formation.

The results in the previous section indicated that exposure to noradrenaline (1 μM) or phenylephrine (1 μM) increased the hydrolysis of inositol phosphate in the AOCs, therefore, the next aim was to quantify these effects.

Throughout these experiments, cells were used between 10 and 13 days following dispersal from the aorta and then labelled with radioactive *myo* [^3H]-inositol for 24 hours (see previous section). The experiments were performed according to the methodology described in 2.4.3.5 and followed the same procedure as that outlined in the previous section, whereupon following equilibration, the cells were exposed to a single dose of the agonist for the final 5 min of the LiCl-containing incubation. Inositol phosphate formation was measured as counts per minute (cpm) and expressed as cpm mg^{-1} protein.

The basal formation of inositol phosphates (controls; CON) following a 24 hour incubation period and then addition of 10 μl distilled H_2O , was 269.8 ± 67.6 cpm mg^{-1} protein ($n=13$). It was found that the addition of NA (1 μM) or PHE (1 μM) produced significant increases in inositol phosphate formation compared with control levels (1033.1 ± 91.2 ; $n=14$ and 775.1 ± 131.3 ; $n=13$ cpm mg^{-1} protein respectively), shown in figure 6.4.

6.2.6. Effect of EPA on *myo*-[^3H] inositol phosphate formation.

To determine the control response of EPA on the formation of inositol phosphates in AOCs, a single dose of EPA (50 μM) or ethanol (ETH; 1% final concentration) replaced the use of the agonist, outlined in the previous section. All dosing with EPA or the agonists was performed in the same experiment.

It can be seen in figure 6.4. that after cells were exposed to ethanol (1%) for 5 min, inositol phosphate formation significantly increased to 706.3 ± 110.7 cpm mg^{-1} protein ($n=13$) when compared with basal levels (controls; CON). The effect of EPA was a significantly greater increase in inositol phosphate formation to 1168.9 ± 197.1 cpm mg^{-1} protein ($n=12$). The observed increase in inositol phosphate formation in the presence of EPA was significantly greater than ethanol alone and was surprising as there is no prior published data to support the view that EPA can act as an alpha-adrenoceptor agonist. However, it was noted previously in chapter 5, that when fura-2 loaded cells were exposed to EPA (50 μM) in PSS or calcium-free PSS, there was a transient rise in $[\text{Ca}^{2+}]_i$, which rapidly returned to basal levels within 1 min (figure 5.12), suggesting that EPA caused a transient release of intracellular calcium from storage sites. This transient effect of EPA on intracellular calcium might be explained by the apparent effect of EPA to increase the formation of inositol phosphate, however,

the mechanism by which this occurred remained unclear. Consideration was given to EPA not being simply associated with an agonist-like effect at the α_1 -adrenoceptor, so experiments were performed to investigate the effect of selective α -adrenoceptor antagonists on the responses of the cells to noradrenaline or EPA.

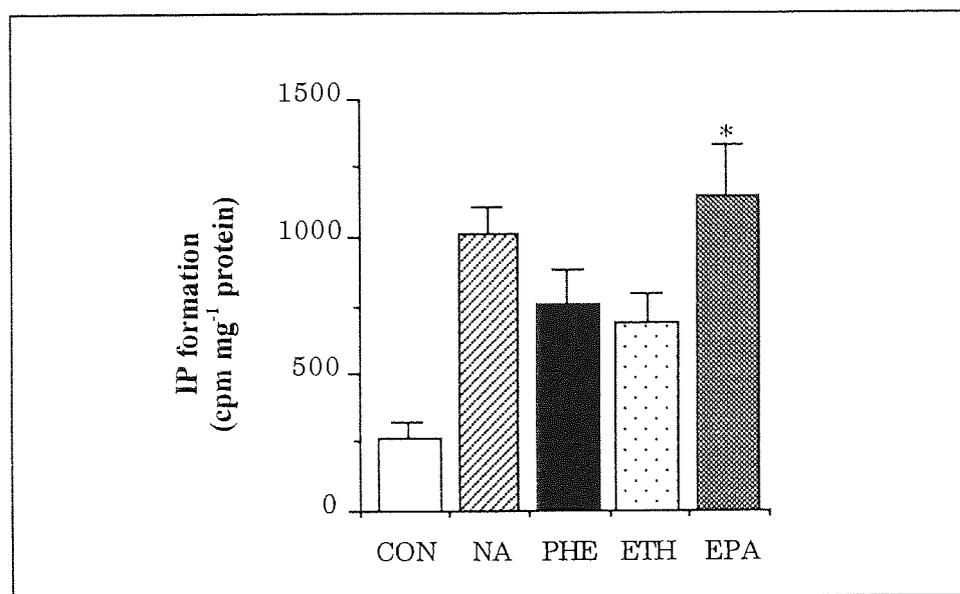


Figure 6.4. Effect of noradrenaline, phenylephrine, ethanol or EPA on myo-^[3H] inositol phosphate formation. AOCs at 10-13 days in culture were incubated for 24 h with 5 μ Ci myo-^[3H] inositol. Following washing and equilibration in PSS at 37°C, the cells were incubated in the presence of LiCl for 20 min. Noradrenaline (NA; 1 μ M), phenylephrine (PHE; 1 μ M), ethanol (ETH; 1%, the EPA vehicle) or EPA (50 μ M) was added for the final 5 min after which time, inositol phosphate formation was determined by anion-exchange chromatography. Radioactivity in the elutes was measured. All values are mean counts per minute mg⁻¹ protein \pm s.e.mean for $n \geq 12$. * indicates significant difference from ETH value using ANOVA followed by Scheffe's test, where $P < 0.05$.

6.2.7. Effect of α -adrenoceptor antagonists on noradrenaline-stimulated myo-^[3H] inositol phosphate formation.

It is well established that the noradrenaline-induced increase in PtdIns hydrolysis in the rat aorta is mediated through α_1 -adrenoceptors (Chiu *et al*, 1987; Hashimoto *et al*, 1986). Therefore, the next step in this investigation was to compare the effect of the selective α_1 -antagonist prazosin, the non-selective α_1 - and α_2 -antagonist phentolamine or the α_2 -antagonist idazoxan on responses of aortic cells to noradrenaline. The methodology for these experiments is described in 2.4.3.7, and followed the procedure outlined in the previous section except that a single dose of the antagonist was present for the duration of the 20 min LiCl-containing incubation and all antagonists were used for each experiment. A single dose of noradrenaline (1 μ M) was added for the final 5 min of this incubation.

It can be seen in figure 6.5 that in the presence of phentolamine ($1 \mu\text{M}$) or prazosin (5 nM) the noradrenaline-induced inositol phosphate formation was reduced to 463.6 ± 79.0 ($n=16$) and 461.4 ± 46.1 ($n=12$) cpm mg^{-1} protein respectively. Conversely, idazoxan ($0.5 \mu\text{M}$) had no effect on noradrenaline-induced inositol phosphate formation (1121.8 ± 112.6 ; $n=7$ cpm mg^{-1} protein). There was no significant difference between the results observed in the presence of phentolamine or prazosin. These results therefore suggest that in AOCs, the noradrenaline-induced formation of inositol phosphates is mediated through stimulation of α_1 -adrenoceptors. This agrees with the report by Manolopoulos *et al*, (1991) who showed that in rat aortic tissue α_1 -adrenoceptor-stimulated contraction with noradrenaline was associated with inositol phosphate formation.

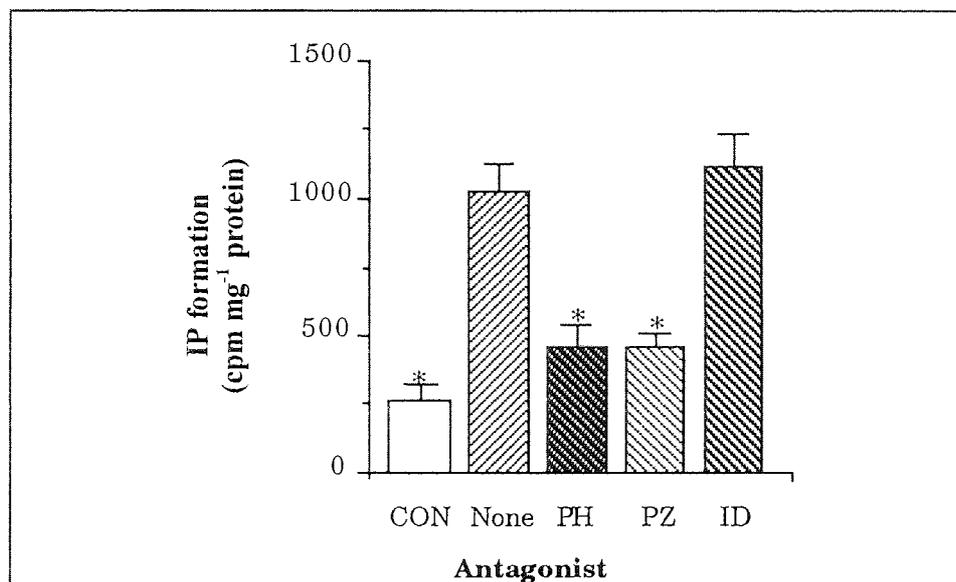


Figure 6.5. Effect of noradrenaline in the presence of phentolamine, prazosin or idazoxan on myo- $[\text{}^3\text{H}]$ inositol phosphate formation. AOCs at 10-13 days in culture were incubated for 24 h with $5 \mu\text{Ci}$ myo- $[\text{}^3\text{H}]$ inositol. Following washing and equilibration in PSS at 37°C , the cells were incubated in the presence of LiCl and phentolamine (PH, $1 \mu\text{M}$; $n=6$), prazosin (PZ, $0.1 \mu\text{M}$; $n=12$) or idazoxan (ID, $0.5 \mu\text{M}$; $n=7$) for 20 min. Noradrenaline (NA, $1 \mu\text{M}$) was added for the final 5 min after which time, inositol phosphate formation was determined by anion-exchange chromatography. Radioactivity in the elutes was measured. Control values (CON) were obtained in the absence of an antagonist or noradrenaline. All values are mean counts per minute mg^{-1} protein \pm s.e.mean for $n \geq 6$. * indicates significant difference from noradrenaline value (None) using ANOVA followed by Scheffe's test, where $P < 0.05$.

6.2.8. Effect of α -adrenoceptor antagonists on EPA-stimulated *myo*-[^3H] inositol phosphate formation.

Figure 6.6 shows the effect of pre-incubating the cells for 15 min in the presence of phentolamine (1 μM), prazosin (5 nM) or idazoxan (0.5 μM). It can be seen that the EPA-induced inositol phosphate formation was significantly reduced to 651.1 ± 78.1 ($n=15$) and 735.6 ± 107.5 ($n=10$) cpm mg^{-1} protein in the presence of phentolamine (1 μM) or prazosin (5 nM) respectively. Conversely, there was no significant effect of phentolamine (1 μM) or prazosin (5 nM) on the ethanol response (484.0 ± 98.7 ; $n=14$ and 557.3 ± 107.5 ; $n=9$ cpm mg^{-1} protein respectively). Idazoxan (0.5 μM) caused no reduction in either the EPA- or ethanol-stimulated formation of inositol phosphates, (1013.7 ± 53.5 ; $n=8$ and 847.7 ± 37.0 ; $n=8$ cpm mg^{-1} protein respectively), shown in figure 6.6.

Therefore, the results still appeared to suggest that EPA was able to stimulate the hydrolysis of PtdIns in the cultured AOCs of the rat and could be blocked by α_1 -adrenoceptor antagonists.

However, this interpretation of the results has not previously been published, since this suggests that EPA may act like a vasoactive rather than as a vasodilatory substance. Indeed, the results of studies that have investigated the effect of PUFAs on inositol phosphate levels are usually designed to examine a vasorelaxatory response of the PUFA and frequently compare agonist-induced inositol phosphate levels in the presence or absence of the PUFA, demonstrating that in the presence of the PUFA, the agonist response is reduced. Therefore, the effect of EPA on the noradrenaline response to inositol phosphate formation was examined in the present study.

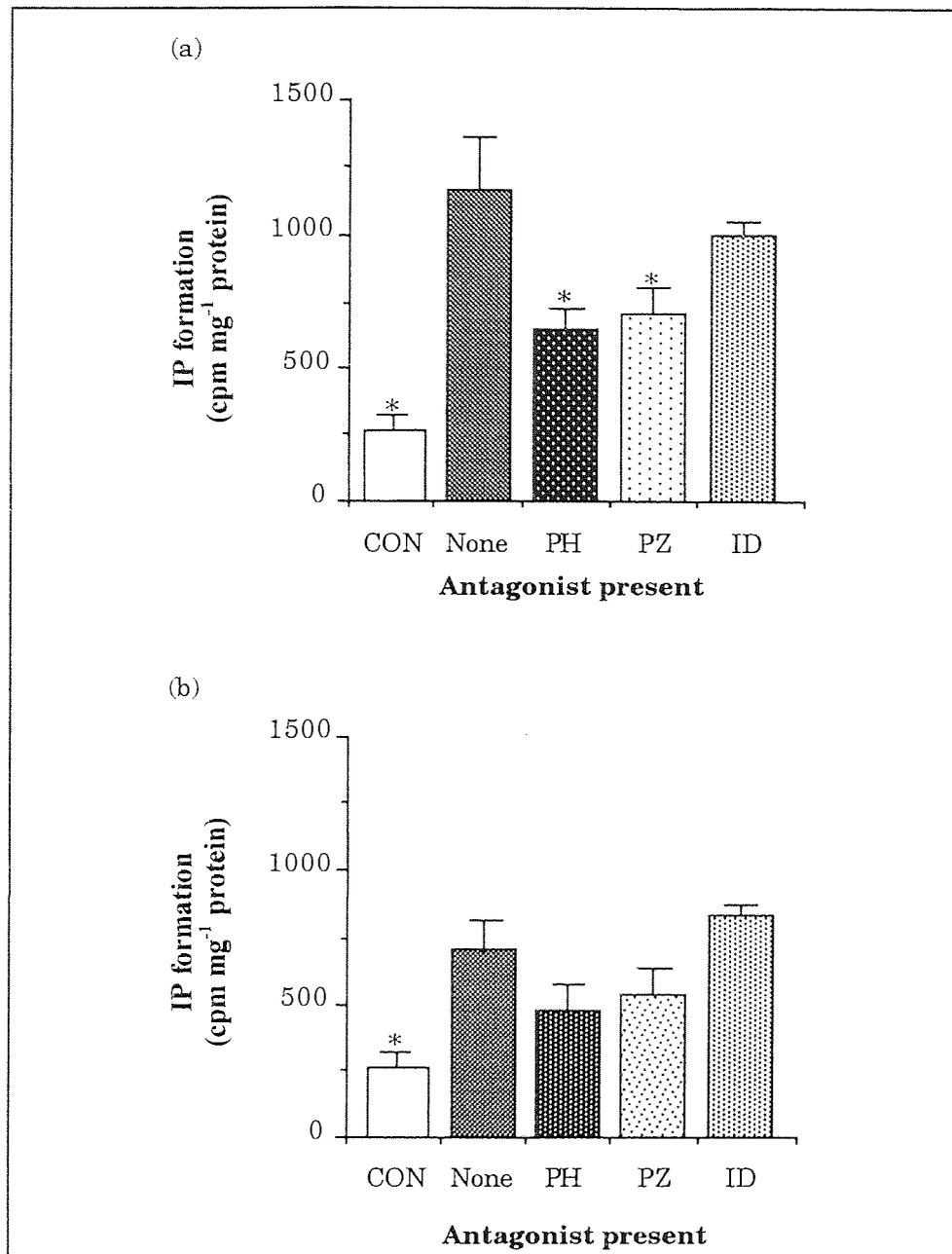


Figure 6.6. Effect of EPA in the presence of phentolamine, prazosin or idazoxan on myo-[³H] inositol phosphate formation in AOCs. AOCs at 10-13 days in culture were incubated for 24 h with 5 μCi myo-[³H] inositol. Following washing and equilibration in PSS at 37°C, the cells were incubated in the presence of LiCl and phentolamine (PH; 1 μM), prazosin (PZ; 0.1 μM) or idazoxan (ID; 0.5 μM) for 20 min. (a) EPA (50 μM) (b) ethanol (1%; EPA vehicle) was added for the final 5 min after which time, inositol phosphate formation was determined by anion-exchange chromatography. Radioactivity in the elutes was measured. Control values (CON) were obtained in the absence of an antagonist or EPA. All values are mean counts per minute mg⁻¹ protein ± s.e. mean for n ≥ 8. * indicates significant difference from value in absence of EPA or ethanol (None) using ANOVA followed by Scheffe's test, where P < 0.05.

6.2.9. Effect of the combination of EPA and noradrenaline on *myo*-[³H] inositol phosphate formation.

These experiments, which examined the effects of noradrenaline and EPA on inositol phosphate formation were performed according to the methodology described in 2.15.5. The experiments followed the procedure outlined in 6.2.6., except that a single dose of noradrenaline (1 μ M) and EPA (50 μ M) was simultaneously added to the cells for the final 5 min of the LiCl-containing incubation. The measured inositol phosphate formation was 1168.9 ± 197.1 cpm mg^{-1} protein ($n=6$), which was not significantly different from the inositol phosphate formation induced by either noradrenaline alone or EPA alone, determined by ANOVA, shown in figure 6.7.

It was considered likely that the lack of an additive effect determined from these results was due to a maximal effect being achieved with noradrenaline (1 μ M). The lack of a significant decrease in agonist-induced inositol phosphate formation by EPA was not seemingly consistent with previous reports.

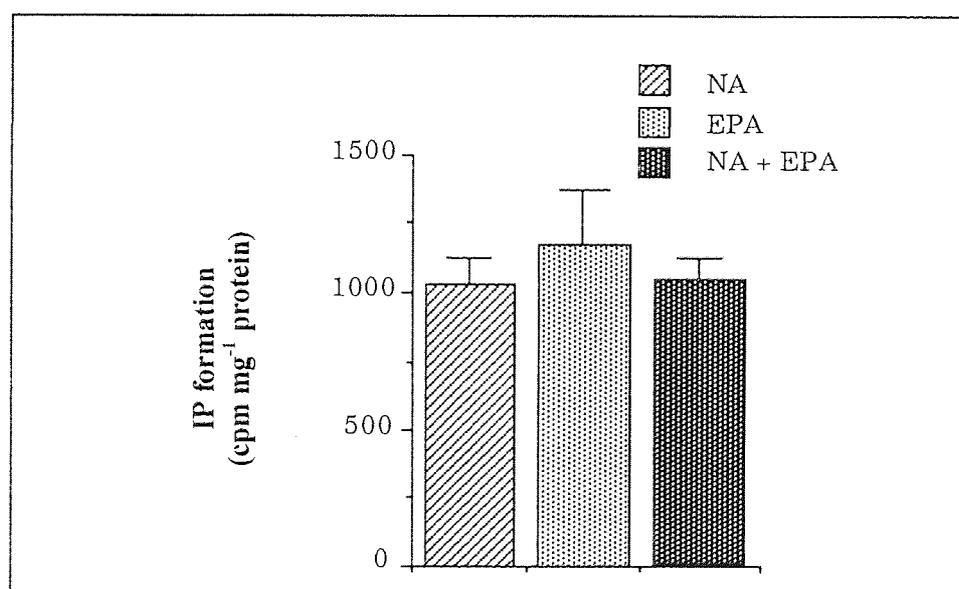


Figure 6.7. The effect of noradrenaline and EPA on *myo*-[³H] inositol phosphate formation. AOCs at 10-13 days in culture were incubated for 24 h with 5 μ Ci *myo*-[³H] inositol. Following washing and equilibration in PSS at 37°C, the cells were incubated in the presence of LiCl for 20 min. Noradrenaline (NA; 1 μ M) and EPA (50 μ M) were added for the final 5 min after which time, inositol phosphate formation was determined by anion-exchange chromatography. Radioactivity in the elutes was measured. All values are mean counts per minute \pm s.e.mean for $n \geq 6$.

6.3. Discussion

Initial studies in this thesis which examined the effect of EPA on AOCs demonstrated that EPA partially blocks cell contraction to the vasoactive agents, KCl and noradrenaline (chapter 4). As previously discussed, EPA-induced relaxation of vascular smooth muscle preparations may reflect a reduction in intracellular calcium ion concentration as a result of a decreased influx of extracellular calcium or the blockade of intracellular calcium release. Subsequent studies (chapter 5) examined the effect of EPA on the influx of extracellular calcium where it was found to significantly decrease influx. However, the results also showed that EPA was not associated simply with the block of L-type calcium channels, confirming that it did not bind at the same subunit site as the dihydropyridine calcium channel blockers as recently suggested (Hallaq *et al*, 1992). Furthermore, EPA significantly reduced the noradrenaline-induced, concentration-dependent increase in calcium influx, suggesting that EPA might reduce calcium influx through receptor-operated channels, consistent with a previous report by Asano *et al* (1997).

However, it was also shown in chapter 5, that when extracellular calcium was absent, EPA induced a transient increase in $[Ca^{2+}]_i$. The results in chapter 5 did not demonstrate that this increase was associated with calcium influx, therefore the aim of the studies reported in this chapter was to determine whether it was linked to a mechanism associated with increasing intracellular calcium release. Streb *et al* (1983) first proposed that hydrolysis of phosphatidylinositol (PtdIns) to the second messenger D-*myo* inositol 1,4,5-trisphosphate (IP₃) resulted in liberation of calcium from intracellular calcium stores. Since this time, the role of IP₃ has been well established (Berridge, 1987), outlined in the General Introduction.

Modification of the fatty acyl composition of the membrane phospholipids can influence many steps of the phosphatidylinositol signalling cascade. Hock *et al*, (1987) reported that feeding fish oils to rats resulted in alterations in the fatty acyl component of the myocardial membrane phospholipids. Soon afterwards, Reibel *et al* (1988) demonstrated that dietary n-3 and n-6 fatty acids were associated with reduced cardiac function and responsiveness to α -adrenoceptor stimulation. The latter report was confirmed subsequently by de Jonge *et al* (1996) who suggested that a causal relationship existed between the EPA-induced alterations of the physicochemical properties in the membrane bilayer and of the agonist-stimulated phosphatidylinositol cycle activity. However, the latter report did not explain the outstanding questions of Reibel *et al* (1988) who were unable to determine whether their observations were due to changes in the number or

affinity of myocardial α -adrenoceptors, modification of the receptor-coupling mechanism or alterations in the second messenger systems. The question was answered, in part, by Skúladóttir *et al.*, (1993) who demonstrated that incorporation of n-3 and n-6 PUFAs in the membrane phospholipids was associated with a decreased affinity of the α_1 -adrenoceptors for their antagonist ligand [^3H] prazosin in heart muscle. However, Reithmann *et al.* (1996) disagreed with this *receptor-coupling* effect of PUFAs, suggesting that the dissociation constant and maximal binding capacity of [^3H] prazosin was unchanged by PUFAs. Instead, Reithmann *et al.* (1996) suggested that the effect of PUFA exposure was related to an alteration of the second messenger system. Furthermore, the experiments described in chapter 5 concluded that the mechanism of action of EPA to block the vasoconstrictor effect of KCl and noradrenaline was not simply associated with an effect to decrease calcium entry through L-type voltage-operated calcium channels, whilst in chapter 4 it was shown that EPA transiently increased $[\text{Ca}^{2+}]_i$ in the absence of extracellular calcium.

Therefore, the experiments reported in the present chapter were designed to investigate whether the transient increase in $[\text{Ca}^{2+}]_i$ was caused by an increase in inositol phosphate and also to examine whether EPA mediates vasorelaxation through a reduction in IP_3 formation in vascular smooth muscle cells.

Initial experiments in this investigation established the viability of the experimental procedure. *Myo*- ^3H inositol was used as the radioactive tracer. Second messengers are produced in small amounts, so a high specific activity *myo*- ^3H inositol was added to the aortic smooth muscle cells to ensure detection of the low conversions of *myo*- ^3H inositol into *myo*- ^3H inositol phosphates following receptor stimulation. Also, *myo*- ^3H inositol with a polymer tablet, PT6-271, in contact with the aqueous solution was used in the present study. PT6-271 was developed by Amersham and specifically and continuously removes radiolytic decomposition products from *myo*- ^3H inositol as they are formed on storage. This has been shown to be effective in stabilising *myo*- ^3H inositols and reduce the co-elution of tritiated impurities. *Myo*- ^3H inositol produces negatively charged decomposition products that separate with inositol phosphates in the Berridge assay (Gurney, 1989) which provided the basis of the assay performed in the present study.

The anion-exchange columns prepared as described for use in the present study have previously been used in experiments associated with the measurement of inositol phosphates (Downes & Michell, 1981). These workers validated the use of the anion-exchange columns by confirmation of the identity and homogeneity of the inositol phosphates eluted using paper chromatography and high-voltage electrophoresis followed by auto radiography. Preliminary experiments in the

present study showed that retrieval of *myo*-[³H] inositol was 96% of that loaded into the anion-exchange columns, which was in agreement with the report by Downes & Michell (1981), who showed that recovery of radioactivity which was directly added to the columns, normally exceeded 90 % of that loaded.

The next preliminary study aimed to characterise loading of the AOCs with *myo*-[³H] inositol and to determine whether cell age following dispersal affected the incorporation of the tracer. After a lag phase for the first 9 days after dispersal, which probably reflected the number of cells exposed to the tracer, it was found that tracer incorporation into the cells reached a steady-state at 10 days. Therefore, cells at 10-13 days in culture were used in subsequent experiments.

The next preliminary study identified the duration of loading to be used subsequently, as previous reports document the use of different labelling times. Rat aortic smooth muscle cells were labelled with *myo*-[2-³H]inositol (4 μ Ci ml⁻¹) for 3 days (Locher *et al.*, 1989), cultured human aortic smooth muscle cells for 10-60 min (Okuda *et al.*, 1994) and ventricular myocytes were labelled for 48 h (de Jonge *et al.*, 1996). However, the results in the present investigation demonstrated labelling-saturation with *myo*-[³H] inositol occurred at 24 h in the AOCs. Therefore, the investigation continued, with the aim of characterising agonist-induced inositol phosphate formation in the AOCs.

The next study confirmed that α -adrenoceptor stimulation induced PtdIns hydrolysis in the AOCs. Noradrenaline acting on α_1 -adrenoceptors in rat aorta induces a biphasic response that consists of an initial phasic contraction, caused by IP₃-mediated mobilisation of intracellular calcium (Berridge, 1987), followed by the influx of extra cellular calcium through receptor-operated channels and/or voltage-dependent channels that participate in the tonic contraction (Cauvin & Malik, 1984; Chiu *et al.*, 1987; Scarborough & Carrier, 1984). Agonists used to stimulate formation of inositol phosphate were added in the presence of lithium ions that inhibit the enzymatic dephosphorylation of inositol monophosphates and so prevent the recycling of inositol (Berridge *et al.*, 1982). In the present investigation, noradrenaline or phenylephrine significantly increased total inositol phosphate formation, consistent with previous investigations (Chiu *et al.*, 1987; Legan *et al.*, 1985). Furthermore, Manolopoulos *et al.* (1991) demonstrated that the significant formation of IP₃ following 15 s of receptor stimulation correlated with the phasic response of the rat aorta to noradrenaline, confirming that the phasic component of noradrenaline contraction is mediated by IP₃ formation.

To confirm that the noradrenaline-induced inositol phosphate formation in the aortic smooth muscle cells used in the present investigation was mediated through an α_1 -adrenergic mechanism, the effect of α -adrenoceptor antagonists on the noradrenaline response was investigated. The results demonstrated that pre-

treatment of the cells with the selective α_2 -antagonist idazoxan did not reduce the subsequent noradrenaline-induced inositol phosphate formation. Conversely, inositol phosphate formation was significantly reduced in the presence of prazosin or phentolamine, in agreement with Manolopoulos (1991).

Having established an α_1 -adrenoceptor agonist-mediated effect on inositol phosphate levels in the AOCs, it was shown that EPA (50 μM) induced a significant increase in inositol phosphate formation (figure 6.4), similar to the response obtained with noradrenaline. The observed effect of EPA appeared to contrast with recent suggestions that PUFAs decrease inositol phosphate levels (Woodcock *et al*, 1995; Reithmann, *et al*, 1996), although it has also been reported that PUFAs do not decrease inositol phosphate levels (de Jonge *et al*, 1996). However, the result was consistent with the previous result in chapter 4, in which EPA caused a transient rise in $[\text{Ca}^{2+}]_i$, which rapidly returned to basal levels within 1 min (figure 5.12). The use of lithium in the methodology used in the inositol phosphate studies meant that even a transient increase in inositol phosphate formation would not result in the recycling to inositol and consequently would be measured as an increase in inositol phosphate. This, therefore, might serve to explain the transient increase in intracellular calcium observed in the fura-2 studies.

It is noteworthy, however, that the inositol phosphate formation which may have lead to a transient increase in $[\text{Ca}^{2+}]_i$, was not apparent in the cell length studies reported in chapter 4, as EPA did not cause the cells to contract (figure 4.3). This further supports the suggestion made in chapter 5, which was that the transient EPA-induced release of calcium may be from an internal binding site, possibly that of an inositol phosphate-dependent intracellular membrane that may, upon its release, then serve in the longer term to prevent calcium influx and subsequently block further cell contraction to noradrenaline or KCl.

As already outlined, no previous studies have demonstrated that EPA increases inositol phosphate, transiently or otherwise, however, it is possible that the inconsistency between previous reports and the present results arise from differences in the experimental procedures followed. Indeed, the result in the present study was measured following exposure of the AOCs to EPA for 5 min. Conversely, Reithmann *et al* (1996) exposed rat cardiac myocytes to DHA (60 $\mu\text{mol/l}$) for 3 days, before showing a decrease in α_1 -adrenoceptor agonist-induced inositol phosphate levels. Similarly, Woodcock *et al* (1995) fed rats with n-3 and n-6 PUFAs for eight weeks before showing a subsequent decrease. However, the effect of PUFAs on inositol phosphate formation cannot be explained simply on a time-dependent basis, since de Jonge *et al* (1996) showed that after only 4 days exposure to EPA (214 μM), IP_3 levels did not alter. But, it seems more likely when account is taken of the PUFA-exposure time in relation to the lithium exposure

time; when the PUFA is applied for hours (Reithmann *et al*, 1996), days (de Jonge *et al*, 1996) or weeks (Woodcock *et al*, 1995) before exposure to lithium, then the results tend to show a decrease, or at least, no increase, in inositol phosphate formation.

Furthermore, Reithmann *et al* (1996) showed that pretreatment of rat cardiomyocytes with DHA (60 μ mol/l) for 3 days decreased α_1 -adrenoceptor agonist-stimulated formation of IP₃ (and IP₂ and IP₁) by 55 %, whereas after only a 6 h pre-incubation with DHA (60 μ mol/l), the decrease was reduced to 17%. It might, therefore, be appropriate to suggest that if no pretreatment was performed, but was replaced by a single treatment of say, 5 min, consistent with the incubation in the present chapter, then the decrease might be reduced still further. It would, therefore, have been interesting to observe what results were obtained if Reithmann *et al* (1996) decreased the pre-incubation time still further.

It is also noteworthy that different methods of expressing results might produce different interpretations on the data. For instance, in the study by de Jonge *et al* (1996), the controls were all in the presence of the fatty acid vehicle, ethanol (0.3 %), fairly similar to the vehicle concentration used in the present investigation (1% ethanol). However, de Jonge *et al* (1996) expressed IP₃ levels as part of the total *myo*-[³H] inositol incorporated into cells. If EPA was able to increase *myo*-[³H] inositol uptake by smooth muscle cells as reported by Okuda (1994), it is possible that this increased uptake by the cells masked the effect of EPA on inositol phosphate formation. It is also worth considering that de Jonge *et al* (1996) used enzyme-dispersed cells that were primary cultured only four days before experimentation, which has been shown to result in a large reduction (85-95%) of the population of α_1 -adrenoceptor binding sites on vascular membranes. This also may have decreased the α_1 -adrenoceptor agonist-induced inositol phosphate formation, thereby allowing the effect of EPA to be overstated.

A further consideration in resolving the discrepancies between the report by Reithmann *et al* (1996) and the result in the present chapter is that the effect of the PUFA in the former study was examined when the concentration of noradrenaline was 100 μ M. In the presence of lower concentrations of noradrenaline, including 1 μ mol/l, the effect of DHA (20 μ mol/l) was not quantified, although it could clearly be seen in the report that the effect was considerably smaller than the 55 % value shown in the presence of 100 μ mol/l noradrenaline. This also raises the question of tissue sensitivity to agonists, discussed in chapter 3, in which reduced (Warshaw *et al*, 1986), greater (Bitar & Makhoulouf, 1982) or equal (Morgan *et al*, 1980) sensitivity occurs compared to that observed in intact tissue preparations.

However, although it appears likely that EPA may increase inositol phosphate, leading to a transient increase in $[Ca^{2+}]_i$, there is no previous evidence to suggest that EPA is associated with α_1 -adrenoceptor stimulation. Therefore, to establish this was the case, EPA-induced inositol phosphate formation was measured in the presence of the α -adrenoceptor antagonists used previously in this study and it was found that prazosin (1 nM) or phentolamine (1 μ M) significantly decreased the EPA response. This suggested that the increase in inositol phosphate was associated with an α_1 -adrenoceptor-stimulated response. In evaluating this result, consideration was taken of the results in the cell contraction studies reported in chapter 4 which suggested that in the unlikely event that the effect of EPA was to transiently increase inositol phosphate by an agonist-like effect at the α_1 -adrenoceptor, leading to a transient increase in intracellular calcium, the effect diminished within 1 min. Contrary to this effect, as outlined earlier in this discussion, a recent report demonstrated that the dissociation constant and maximal binding capacity of the α_1 -adrenoceptor antagonist [3 H] prazosin was unchanged by n-3 fatty acid exposure (Reithmann *et al.*, 1996), thereby suggesting an alternative mechanism of action for EPA exists, although, a receptor-mediated action of EPA has previously been proposed (Chin *et al.*, 1993), but not described in detail.

To extend the results obtained in chapter 4, in which the effect of EPA was examined upon the noradrenaline-induced cell contractions, the final objective in this investigation was to identify the effect of EPA on the noradrenaline-induced inositol phosphate response of the AOCs. This study would also limit the variations in the methodologies used between the experiments reported in this present study and those of previous investigations (Reithmann *et al.*, 1996; de Jonge *et al.*, 1996; Woodcock *et al.*, 1995), as the latter do not report any results for EPA in the absence of an α_1 -agonist. The ability of EPA to relax tissues pre-contracted to noradrenaline, or to prevent contractural responses by pre-incubating the tissue in EPA has previously been studied and shown to induce relaxation in aortic tissue (Juan & Sametz, 1986; Engler, 1989; Yin *et al.*, 1991; Lervang *et al.*, 1993). Therefore, in the present investigation, noradrenaline (1 μ M) and EPA (50 μ M) in combination were exposed to the cells. The result showed that EPA did not significantly decrease the effect to noradrenaline (figure 6.11) which was considered to be a maximal physiological effect in the experiment. However, it was noted that the EPA was not pre-incubated on the cells before exposure to noradrenaline, which might have affected the results. Previous reports that have demonstrated an effect of PUFAs on inositol phosphate formation have pre-exposed the cells to the PUFA before adding the agonist (Reibel *et al.*, 1988; Reithmann *et al.*, 1996; Woodcock *et al.*, 1995).

In conclusion, therefore, it has been shown in the present investigation that EPA (50 μM) induces an increase in inositol phosphate, likely to be transient, which might explain the transient increase in $[\text{Ca}^{2+}]_i$ measured in the absence of extra cellular calcium, in chapter 5. The transient nature of the effect of EPA might also serve to explain in part, the discrepancies between the results obtained in this chapter and those of previous studies in which the PUFA is exposed to cells for hours (Reithmann *et al* (1996), days (de Jonge *et al*, 1996) or weeks (Woodcock *et al*, 1995) before exposure to lithium. The action of EPA is associated with a vasorelaxatory response in the aorta (Juan & Sametz, 1986; Engler, 1989; Yin *et al.*, 1991; Lervang *et al*, 1993), and the inositol phosphate formation, leading to a transient increase in intracellular calcium ion concentration, did not lead to a contraction in the cell length studies, reported in chapter 4 (figure 4.3). This further supports the suggestion made in chapter 5, which was that the transient EPA-induced release of calcium may be from an internal binding site, possibly that of an intracellular membrane that may, upon its release, then serve in the longer term to prevent calcium influx and subsequently block further cell contraction to noradrenaline or KCl. The precise location from where this calcium is released remains unclear as the subsequent results obtained in this study with α -adrenoceptor antagonists suggest the release may be associated with an α_1 -adrenoceptor mechanism, yet, as outlined earlier in this discussion, a recent report demonstrated that the dissociation constant and maximal binding capacity of the α_1 -adrenoceptor antagonist [^3H] prazosin was unchanged by n-3 fatty acid exposure (Reithmann *et al*, 1996). This therefore appears to provide evidence against DHA binding to α -adrenoceptors although it is also worth considering that Reithmann *et al* (1996) utilised enzyme-dispersed cells which were primary cultured only one day before exposure to PUFA and four days before further experimentation, which has been shown to result in a large reduction (85-95%) of the population of α_1 -adrenoceptor binding sites on vascular membranes. No further experimental comparisons were made upon cells so it is possible that the similar binding extent of [^3H] prazosin after PUFA exposure in the studies by Reithmann *et al* (1996) may possibly be associated with a decreased population of α_1 -adrenoceptor binding sites on the cell membranes.

Nevertheless, a transient increase in $[\text{Ca}^{2+}]_i$ has been shown to activate calcium-activated potassium channels and cause hyperpolarisation of the plasma membrane, thereby moving the membrane potential farther from the activation range for calcium channels. This effect might serve to explain the effect of EPA to reduce calcium influx induced by low concentrations of KCl, shown in chapter 5. Therefore, the next aim was to determine an effect of EPA upon membrane potassium permeability.

Chapter 7

The Effect Of EPA On Membrane Potassium Permeability

7.1 Introduction

Results described so far in this thesis have demonstrated that EPA partially blocks the contraction of aortic smooth muscle cells (AOCs) to the vasoactive agent noradrenaline (chapter 4), possibly by decreasing calcium influx (chapter 5) through receptor-operated channels, consistent with a previous report by Asano *et al* (1997).

It was also shown in chapter 5, that EPA induces a transient increase in $[Ca^{2+}]_i$, which is independent of extracellular calcium and likely to be explained by the results obtained in chapter 6, which showed that EPA increased inositol phosphate levels. Therefore, it is suggested that the mechanism of action of EPA might involve stimulating a transient release of calcium from an inositol phosphate-dependent internal binding site, possibly that of an intracellular membrane and that following the release of this calcium transient, calcium influx may be prevented in the longer term, possibly by an action on receptor-operated calcium channels to subsequently block further cell contraction to noradrenaline, or by an action on voltage-dependent calcium channels, to subsequently block further cell contraction to KCl.

Calcium entry through the voltage-dependent calcium channels may be inhibited by potassium channel activators which induce the cellular efflux of potassium ions, thereby hyperpolarising the plasma membrane and moving the membrane potential farther from the activation range for calcium channels.

In 1990, Kim & Duff studied the ability of free fatty acids, including arachidonic acid, to modulate the K_{ATP} channel in rat cardiac myocytes using the patch-clamp technique and demonstrated inhibition of the channel by the fatty acids. Conversely, Muller *et al* (1992) demonstrated that arachidonic acid activated K_{ATP} channel activity and similarly, Asano *et al* (1997) confirmed that K^+ currents in the foetal rat aortic smooth muscle cells, A7r5, were activated by n-3 fatty acids, but they were unable to clarify which type of K^+ channel is activated. In an attempt to elucidate which K^+ channel may be affected, Gardener *et al* (1998), suggested an inhibitory effect of EPA (10 μ M) on a voltage-sensitive, delayed rectifier K^+ current, on a large conductance Ca^{2+} -activated K^+ channel and on K_{ATP} channels.

Alternative methods of study were adopted by Smith *et al* (1992) who measured potassium efflux from cells using the isotopic tracer, rubidium

chloride, ^{86}Rb . They examined the effect of a fish-oil supplemented diet on potassium efflux in the aorta of WKY and SHR rats, and compared the effects with those of diltiazem or sodium nitroprusside. They demonstrated that diltiazem and sodium nitroprusside, by reducing $[\text{Ca}^{2+}]_i$, also served to reduce potassium efflux, as determined using ^{86}Rb , and that this decrease was similarly observed in fish-oil fed rats. They concluded that fish oil modulates calcium metabolism in vascular smooth muscle to lower $[\text{Ca}^{2+}]_i$, decrease activation of calcium-activated potassium channels and thus decrease rubidium efflux through these channels.

Therefore, it was considered that an effect of EPA on K^+ channel activation might explain the effect of EPA to partially block the contraction of AOCs to KCl, shown in chapter 4. Therefore, the objective in the present chapter was to investigate the effect of EPA upon membrane potassium permeability.

The experiments were designed to examine the effect of EPA upon membrane potassium permeability in the AOCs using the isotope $^{86}\text{rubidium}$ chloride (^{86}Rb) as the marker. Leak and calcium-activated potassium channels ($\text{K}_{\text{Ca}^{2+}}$) in aortic smooth muscle are more selective for potassium than rubidium (Smith *et al*, 1986) however, the radioactive rubidium chloride isotope, $^{86}\text{RbCl}$ (^{86}Rb), was selected for use in this investigation as it has a longer half-life (18.7 days) than potassium (12.4 hours), making it easier to identify changes concerned with the mode of action of potassium channel-opening drugs (Greenwood & Weston, 1993).

Initial experiments characterised potassium efflux in the AOCs, adapting procedures performed on thoracic aortic segments by Weir & Weston (1986) and on human airway smooth muscle cells by de Lorenzi (1994).

The first objective was to determine the rate of potassium efflux in the AOCs, so preliminary experiments were performed to identify the incubation time required to load AOCs with the isotope. The methodology followed that described in 2.5.2.1 whereupon the cells were incubated in PSS containing ^{86}Rb ($1 \mu\text{Ci ml}^{-1}$) for different periods of time. After 0, 30, 60, 90, 120 or 150 min, the solution was aspirated and discarded. The cells were washed and lysed to retrieve any residual isotope. The solution was collected and radioactivity measured by γ counting on a Cerenkov program (Packard 1900TR) for 5 min. ^{86}Rb influx was expressed as counts per minute (cpm).

Blanks, in which tracer was omitted from the extra cellular solution were performed routinely in each experiment and the measurements taken were subtracted from the corresponding measurements made in the presence of tracer.

Following the preliminary experiments to determine maximal loading of the AOCs with ^{86}Rb ($1 \mu\text{Ci ml}^{-1}$) a further preliminary experiment was performed to determine that efflux occurred in the AOCs. Following the loading period, the

efflux of ^{86}Rb was monitored at 5 min intervals over a period of 60 min using a series of efflux solutions applied to the well. The efflux solutions were collected separately and when all were collected the remaining AOCs were lysed to determine the remaining ^{86}Rb content of the cells. *Blanks*, in which tracer was omitted from the extra cellular solution were subtracted from these measurements.

Radioactivity in all vials was measured as cpm and rubidium efflux from cells was calculated as the fractional loss of tracer from the cells for the duration of the efflux period and expressed in terms of a rate coefficient $\times 10^{-2} \text{ min}^{-1}$, according to equation 8, described in appendix 3. Statistical comparisons were made by Student's two-tailed *t* test for unpaired data, or in the case of multiple comparisons, analysis of variance (ANOVA) followed by Scheffe's multiple range test. Significance was identified at $P < 0.05$

Cromakalim is a smooth muscle relaxant which antagonises the action of ATP on ATP-sensitive potassium channels (K_{ATP}), thus opening them, causing potassium efflux and the tissue to relax (Greenwood & Weston, 1993). It has been shown previously to induce ^{86}Rb efflux in vascular tissue (Quast & Baumlin, 1988) and to produce relaxation of aortic segments (Greenwood & Weston, 1993). A similar action might explain the EPA-mediated block of cell contraction observed in the results in chapter 4, as it has been reported that DHA mediates vasodilation *via* K_{ATP} opening (Muller *et al*, 1992). However, in the event that EPA induces a transient increase in $[\text{Ca}^{2+}]_i$, which is independent of extracellular calcium (chapter 5) and likely to be explained by the increased inositol phosphate levels (chapter 6), then, the calcium transient might activate $K_{\text{Ca}^{2+}}$ to induce the cellular efflux of potassium ions. This would hyperpolarise the plasma membrane as outlined above and move the membrane potential farther from the activation range for voltage-dependent calcium channels, explaining the effect of EPA to partially block the contraction of AOCs to KCl, shown in chapter 4.

Therefore, the next objective compared the effect of EPA with that of cromakalim on ^{86}Rb efflux in the AOCs, in accordance with a modification of previously published methods (Weir & Weston, 1988; de Lorenzi, 1994) and determined whether the effect of EPA could be blocked by a K_{ATP} or a $K_{\text{Ca}^{2+}}$ blocker.

Glibenclamide, a drug in the sulphonylurea group, mimics the action of ATP on K_{ATP} channels, causing them to close and thereby reduce potassium efflux (Sturgess *et al*, 1985). Similarly, tetraethylammonium is effective at blocking $K_{\text{Ca}^{2+}}$ -channels (Inoue *et al*, 1985) and has been widely used to differentiate potassium-channel populations (Stanfield, 1983). In aortic cells, an alteration in membrane fluidity has been demonstrated to affect calcium-dependent potassium channel activity (Bolotina *et al*, 1989) and furthermore, it

has been reported that glibenclamide inhibits the effect of DHA in insulinoma cells (Muller *et al*, 1992). It has also been shown that EPA (30 μM) induces an outward potassium current in A7r5 cells, which was abolished by tetraethylammonium (Asano *et al*, 1997), a K_{Ca^+} -channel blocker (Halliday, 1995), thereby supporting the suggestion that the calcium transient from chapter 5 might activate $\text{K}_{\text{Ca}^{2+}}$ to induce cellular efflux of potassium ions to block the contraction of AOCs to KCl (chapter 4).

Therefore, final experiments investigated the effect of glibenclamide or tetraethylammonium on ^{86}Rb efflux in the AOCs to establish whether K_{ATP} or $\text{K}_{\text{Ca}^{2+}}$ channels were involved in the anti-vasoconstrictor responses measured to EPA in chapter 4 and also, the reduced calcium influx measured in chapter 5. In these experiments, a single dose of glibenclamide (1 μM) or tetraethylammonium (1 mM) was added to the ^{86}Rb -loading solution for the final 30 min. Then ^{86}Rb efflux was measured with cromakalim (1 μM) or EPA (50 μM) in the efflux solutions. *Blanks* were included in each experiment and after lysing the cells, radioactivity was measured and rubidium efflux determined as before.

7.2. Results

7.2.1. Determination of the ^{86}Rb loading period.

These experiments were designed to examine the effect of EPA upon membrane potassium permeability in the aortic smooth muscle cells (AOCs) using the isotope ^{86}Rb as the marker. The first objective was to determine the rate of potassium efflux in the AOCs, so preliminary experiments were performed to identify the incubation time required to load AOCs with the isotope.

The initial experiment followed the methodology described in 2.5.2.1 whereby the cells were incubated in PSS containing ^{86}Rb ($1 \mu\text{Ci ml}^{-1}$) for different periods of time. Figure 7.1 shows that in this preliminary experiment, there was no further increase in the ^{86}Rb content of the cells after 90 minutes (figure 5.14). Therefore, in all subsequent ^{86}Rb efflux experiments, AOCs were pre-loaded with the isotope for 90 min.

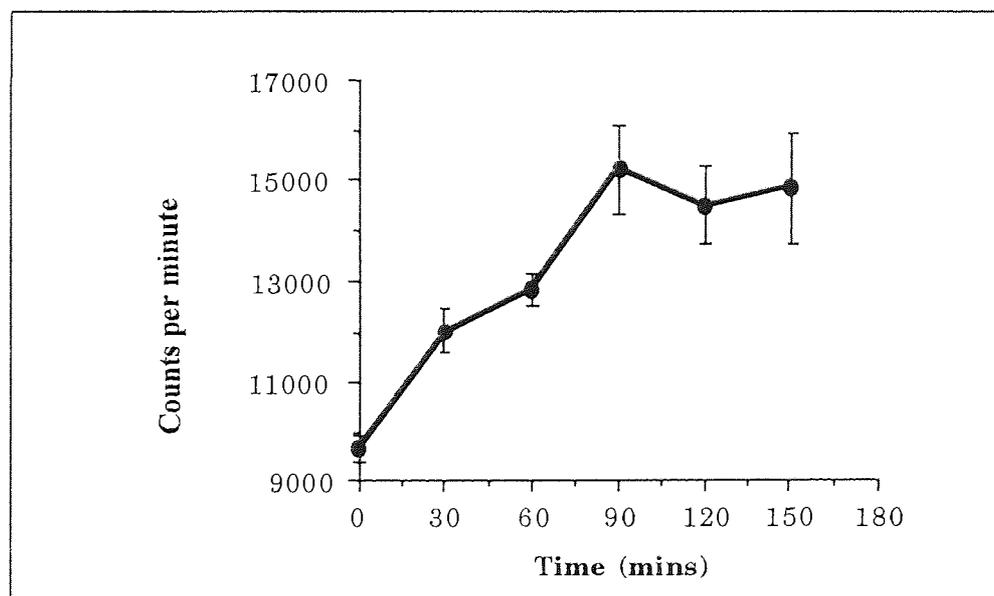


Figure 7.1 The determination of the ^{86}Rb loading period. PSS at 37°C containing ^{86}Rb ($1 \mu\text{Ci}$) was exposed to monolayers of aortic smooth muscle cells (AOCs) for 0, 30, 60, 90, 120 or 150 mins. The cells were washed and lysed. Radioactivity in the cells was measured by Cerenkov counting. Values are expressed as mean counts per minute (cpm) \pm s.e. mean from 4 experiments, each performed for 4 wells ($n=16$).

7.2.2. Investigation of basal ^{86}Rb efflux.

The study continued with the determination of ^{86}Rb efflux in the AOCs. Cells were loaded with ^{86}Rb ($1 \mu\text{Ci ml}^{-1}$) for 90 min, and then efflux of ^{86}Rb was monitored at 5 min intervals over a period of 60 min using a series of 1 ml aliquots of PSS applied to the well, according to the methodology described in 2.5.2.2.

From figure 7.2, the rate of efflux was 0.31 ± 0.053 , 0.03 ± 0.004 and $0.02 \pm 0.001 \times 10^{-2} \text{ min}^{-1}$ after 5, 10 and 15 min respectively. No change in efflux was observed between 15-60 min, in accordance with previous results (de Lorenzi, 1994).

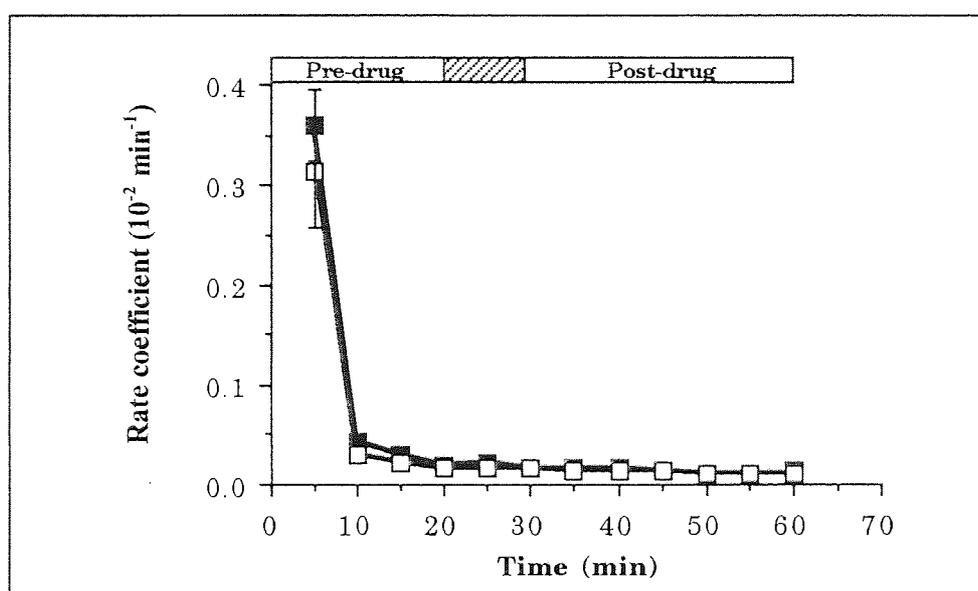


Figure 7.2. Effect of cromakalim on ^{86}Rb efflux. AOCs at 10-13 days in culture were loaded for 90 min with $1 \mu\text{Ci } ^{86}\text{Rb}$. The cells were washed, then a series of 1 ml PSS was applied to the cells, each for 5 or 10 min.  denotes period when $1 \mu\text{M}$ cromakalim (CRO; ) in the PSS. Radioactivity in each efflux solution was measured by Cerenkov counting. Values are expressed as the rate coefficient \pm s.e.mean from 6 experiments ($n=6$).

7.2.3. Effect of cromakalim on ^{86}Rb efflux.

To determine whether ATP-sensitive potassium channels (K_{ATP}) were present in the AOCs, cromakalim was exposed to the cells. The procedure followed that described in 2.5.2.3, outlined in 7.2.2, except that the solutions applied to the cells at 20 and 25 min contained a single dose of cromakalim ($1 \mu\text{M}$) and PSS added at 30, 40 or 50 min remained on the cells for 10 min. At the end of the experimental period, the cells were lysed to determine the remaining ^{86}Rb content and radioactivity was expressed in terms of a rate coefficient, calculated from equation 8 in appendix 3.

The results showed no effect of cromakalim following exposure to the cells at 20 or 25 min (figure 7.2). This suggested that either K_{ATP} -channels were not present on the AOCs or that there was so little rubidium in the cells after 10-15 min that no drug-induced influx could be demonstrated and that the experimental procedure required refinement in order to demonstrate the existence of such channels. It was therefore considered appropriate to modify the experimental methodology.

7.2.4. Effect of cromakalim or EPA immediately following loading.

This experiment followed the methodology described in the previous section, however, after the cells were washed, they were exposed to PSS containing cromakalim ($1 \mu\text{M}$; CRO), EPA ($50 \mu\text{M}$) or ethanol (1%, the EPA vehicle; ETH) for 5 or 10 min intervals throughout the efflux period measured.

The rate of efflux determined at 5 min was $0.40 \pm 0.041 \times 10^{-2} \text{ min}^{-1}$ ($n=6$) with CRO ($1 \mu\text{M}$). This was significantly greater ($P<0.05$) than the efflux measured in the controls ($0.26 \pm 0.013 \times 10^{-2} \text{ min}^{-1}$; $n=6$), suggesting that K_{ATP} channels were present in the AOCs. At 10 min, the rate of efflux had decreased to 0.03 ± 0.004 ($n=6$) and $0.03 \pm 0.005 \times 10^{-2} \text{ min}^{-1}$ ($n=6$) in CON and CRO respectively. There was no further difference measured in the rate of efflux between CON and CRO (see figure 7.3).

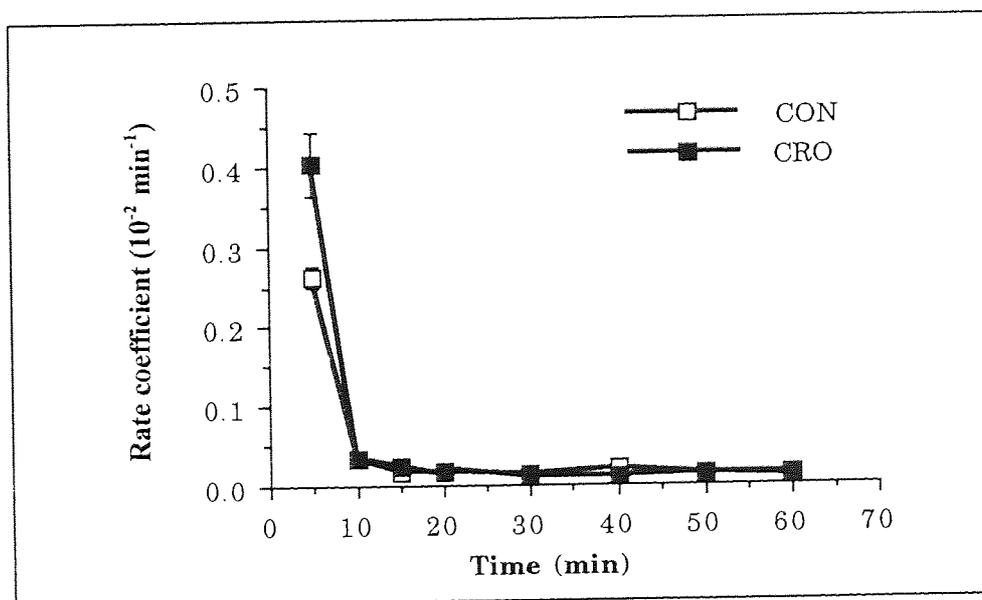


Figure 7.3. Effect of immediate exposure to cromakalim on ^{86}Rb efflux. AOCs at 10-13 days in culture were loaded for 90 min with $1 \mu\text{Ci } ^{86}\text{Rb}$. The cells were washed, then exposed to PSS (CON) or PSS containing cromakalim ($1 \mu\text{M}$; CRO) for 5 or 10 min intervals. Radioactivity in each efflux solution was measured by Cerenkov counting. Values are expressed as the rate coefficient \pm s.e.mean from 6 experiments ($n=6$).

Similarly, it can be seen in figure 7.4 that at the 5 min time interval, EPA significantly increased the rate of efflux to $1.35 \pm 0.115 \times 10^{-2} \text{ min}^{-1}$ ($n=6$) compared to that determined for ETH ($0.40 \pm 0.019 \times 10^{-2} \text{ min}^{-1}$; $n=6$). At 10 min, the rate of efflux plateaued at 0.09 ± 0.009 ($n=6$) and $0.03 \pm 0.002 \times 10^{-2} \text{ min}^{-1}$ ($n=6$) for EPA and ETH respectively, except at 30 and 40 min, when EPA induced a slight significant increase ($P<0.05$) in efflux compared to ETH.

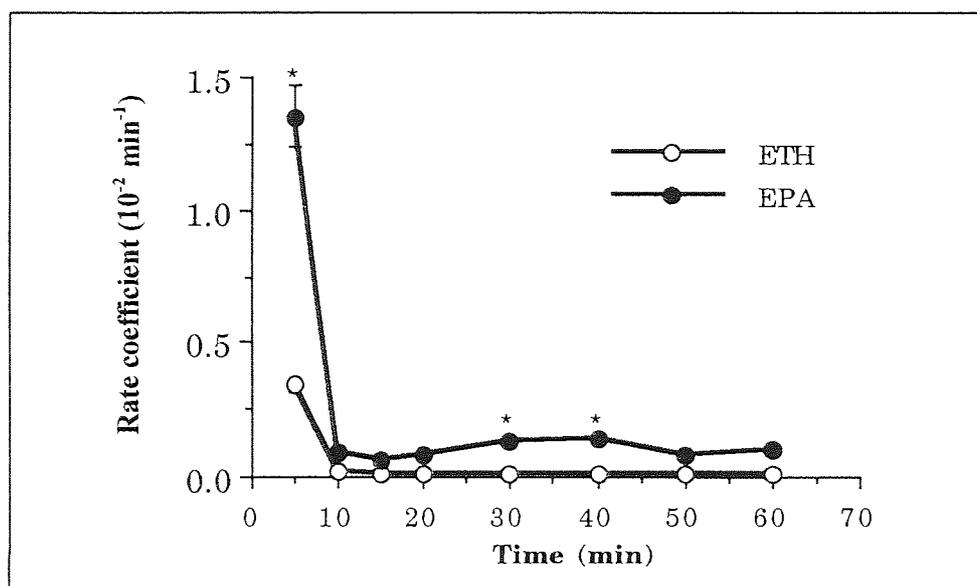


Figure 7.4. Effect of immediate exposure to EPA on ^{86}Rb efflux. AOCs at 10-13 days in culture were loaded for 90 min with $1\mu\text{Ci } ^{86}\text{Rb}$. The cells were washed, then exposed to PSS containing $50 \mu\text{M}$ EPA (EPA) or ethanol (1%, EPA vehicle; ETH) for 5 or 10 min intervals. Radioactivity in each efflux solution was measured by Cerenkov counting. Values are expressed as the rate coefficient \pm s.e.mean from 6 experiments ($n=6$). * indicates significant difference from corresponding ETH value ($P<0.05$).

The results obtained showed that efflux was not clearly being measured. therefore, the objective in the next set of experiments aimed to improve measurement of efflux. It has previously been reported that exposing the cells in a well to a single efflux solution allows for the determination of an effect of DHA on ^{86}Rb efflux (Muller *et al*, 1992), therefore the methodology in the present study was modified and involved exposing the cells in each well to a single efflux solution.

7.2.5. Effect of a single efflux solution on ^{86}Rb efflux.

These experiments followed the procedure described in 2.5.2.4 whereby one efflux solution alone was applied to a well for 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 60 min. Radioactivity in the solutions was measured as cpm and the rate of efflux for each time interval was estimated from the rate coefficient, described in equation 8, appendix 3.

The rate of efflux decreased steadily from $0.40 \pm 0.057 \times 10^{-2} \text{ min}^{-1}$ ($n=3$) at 5 min to $0.12 \pm 0.028 \times 10^{-2} \text{ min}^{-1}$ ($n=3$) at 20 min, after which time, efflux steadied for a further 20 min prior to fluctuating around $0.05 \times 10^{-2} \text{ min}^{-1}$ for the remaining 20 min of the experiment (figure 7.5). These results confirmed that the modification to the methodology decreased the rapid rate of efflux observed in the previous experiments (figure 7.4). The experiment was therefore extended to include efflux measurements taken at 1 min intervals following loading.

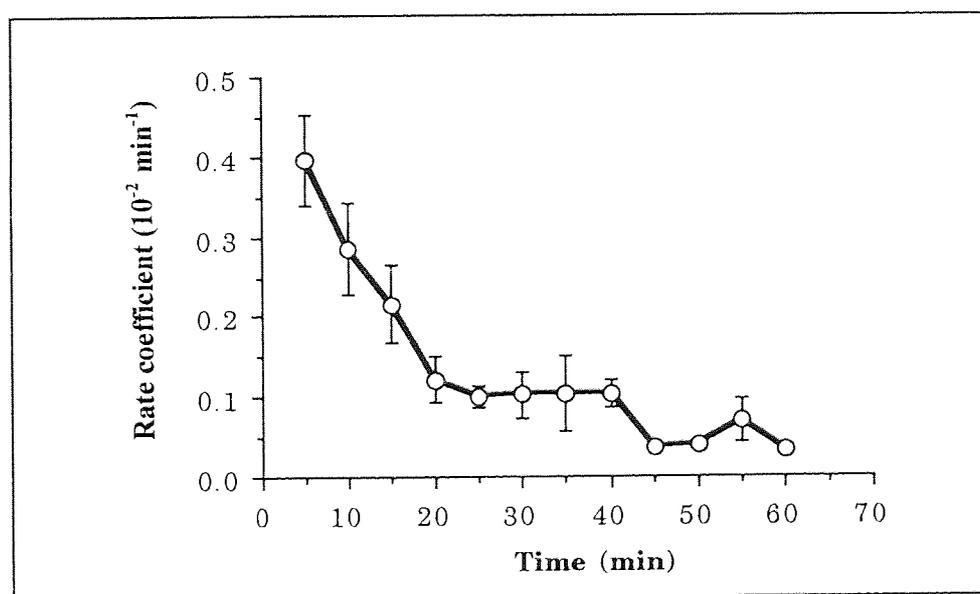


Figure 7.5 Effect of a single efflux solution on ^{86}Rb efflux between 0-60 min. AOCs at 10-13 days in culture were incubated for 90 min with $1\mu\text{Ci } ^{86}\text{Rb}$. Cells were washed and exposed to an efflux solution of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 60 min. Radioactivity in each wash solution was measured by Cerenkov counting. Rate of efflux was expressed as the rate coefficient \pm s.e.mean from 4 experiments, each performed for 4 wells ($n=16$).

Initially, a study on efflux between 0 and 12 min was made. It can be seen in figure 7.6 that the rate of efflux was $3.36 \pm 0.80 \times 10^{-2} \text{ min}^{-1}$ ($n=6$) after 1 min, decreasing to $1.06 \pm 0.120 \times 10^{-2} \text{ min}^{-1}$ ($n=6$) by 2 min. Consistent with the previous experiment, efflux at 5 min was $0.40 \pm 0.052 \times 10^{-2} \text{ min}^{-1}$ ($n=6$). Efflux rate then continued to decrease without reaching steady state. Therefore, the rate of efflux was determined over the initial 5 min period following exposure of the cells to cromakalim or EPA (EPA or cromakalim was contained in the PSS added to each well and present throughout the incubation). The values for the rates of efflux are detailed in table 7.1 and are summarised in figure 7.7. The results revealed no significant effect of CRO ($1 \mu\text{M}$) or EPA ($50 \mu\text{M}$) on the rate of efflux compared with the controls. This might suggest that the cells were anoxic after the loading period so that even without cromakalim, the resultant low levels of intracellular ATP might already induce K_{ATP} to be fully open. However, one final attempt was made to identify whether K_{ATP} or K_{Ca^+} blockers could be used to decrease rate of efflux and thereby expose an effect of EPA.

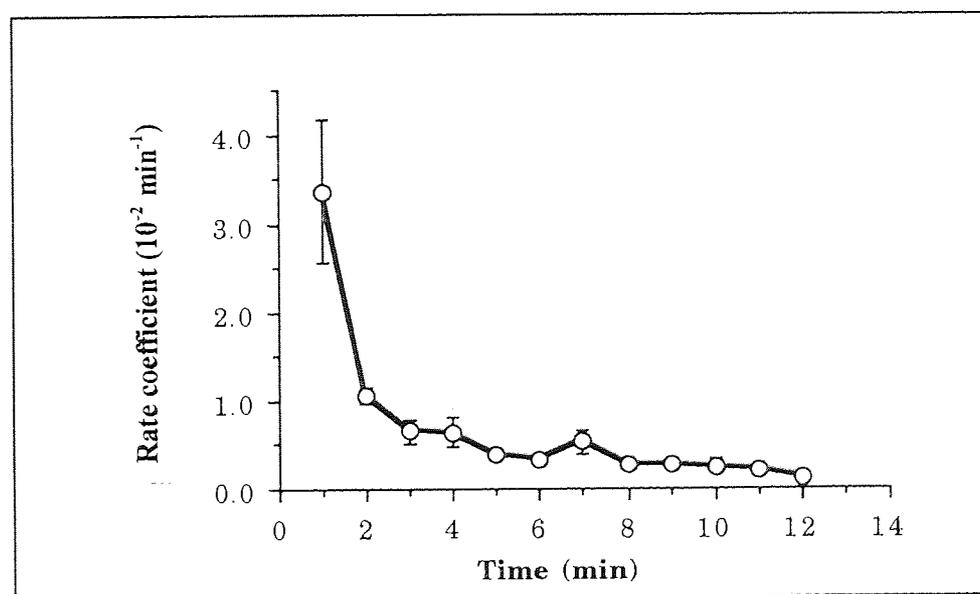


Figure 7.6 Effect of a single efflux solution on ^{86}Rb efflux between 0-12 min. AOCs at 10-13 days in culture were incubated for 90 min with $1\mu\text{Ci } ^{86}\text{Rb}$. Cells were washed and exposed to an efflux solution of 0,1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 min. Radioactivity in each wash solution was measured by Cerenkov counting. Rate of efflux was expressed as the rate coefficient \pm s.e.mean from 6 experiments ($n=6$).

Table 7.1. Effect of a single efflux solution on ^{86}Rb efflux between 0-5 min. AOCs at 10-13 days in culture were incubated for 90 min with $1\mu\text{Ci } ^{86}\text{Rb}$. Cells were washed and exposed to an efflux solution containing $10\ \mu\text{l}$ PSS (Control; CON), cromakalim (CRO), ethanol (ETH) or EPA ($50\ \mu\text{M}$) for a time interval of 0-300 sec. Radioactivity in each wash solution was measured by Cerenkov counting. Rate of efflux was expressed as the rate coefficient \pm s.e.mean from 4 (*6) experiments.

Time interval (sec)	Rate coefficient ($10^{-2}\ \text{min}^{-1}$)			
	CON*	CRO ($1\ \mu\text{M}$)	ETH (1%)	EPA ($50\ \mu\text{M}$)
15	4.46 ± 0.699	5.08 ± 0.590	4.45 ± 0.583	5.90 ± 1.130
30	3.45 ± 0.599	4.55 ± 1.197	3.12 ± 0.519	3.05 ± 0.355
45	3.51 ± 1.022	2.44 ± 0.426	3.03 ± 0.929	1.96 ± 0.209
60	3.22 ± 0.270	2.13 ± 0.392	2.15 ± 0.613	1.49 ± 0.185
90	1.88 ± 0.594	1.74 ± 0.697	1.19 ± 0.519	0.91 ± 0.087
120	1.00 ± 0.210	1.09 ± 0.264	1.33 ± 0.549	0.63 ± 0.086
180	0.59 ± 0.075	0.55 ± 0.151	0.56 ± 0.113	0.75 ± 0.142
240	0.42 ± 0.054	0.30 ± 0.052	0.41 ± 0.072	0.42 ± 0.067
300	0.36 ± 0.033	0.24 ± 0.042	0.50 ± 0.107	0.64 ± 0.032

Rate of efflux was expressed as the rate coefficient \pm s.e.mean from 4 (*6) experiments.

7.2.6. Effect of glibenclamide or tetraethylammonium on ^{86}Rb efflux.

These experiments followed the methodology described in 2.5.2.5 in which, a single dose of glibenclamide ($1\ \mu\text{M}$) or TEA ($1\ \text{mM}$) was added to the loading solution for the final 30 min. The cells were washed twice with PSS at 37°C and then one efflux solution alone was applied to a well for 0, 15, 30, 60, 90, 120, 180, 240 or 300 sec.

It is shown in figure 7.8 that when a glibenclamide ($1\ \mu\text{M}$) pretreatment was used on the AOCs, an effect of cromakalim was observed. Glibenclamide significantly decreased the response from 5.03 ± 0.590 ($n=4$) to $2.38 \pm 0.531 \times 10^{-2}\ \text{min}^{-1}$ ($n=4$) at 15 sec, from 4.55 ± 1.197 ($n=4$) to $1.78 \pm 0.464 \times 10^{-2}\ \text{min}^{-1}$ ($n=4$) at 30 sec, from 2.44 ± 0.426 ($n=4$) to $1.17 \pm 0.315 \times 10^{-2}\ \text{min}^{-1}$ ($n=4$) at 45 sec, from 2.13 ± 0.392 ($n=4$) to $0.92 \pm 0.280 \times 10^{-2}\ \text{min}^{-1}$ ($n=4$) at 60 sec and from 1.74 ± 0.697 ($n=4$) to $0.516 \pm 0.159 \times 10^{-2}\ \text{min}^{-1}$ ($n=4$) at 90 sec. Conversely, however, TEA did not decrease the response to cromakalim. These results showed that exposing the cells to glibenclamide in the loading solution revealed an effect of cromakalim. This effect was reduced by glibenclamide but not TEA confirming that cromakalim activates K_{ATP} channels.

Glibenclamide, but not TEA reduced ($P < 0.05$) the EPA-induced efflux significantly from 5.90 ± 1.130 ($n=4$) to $3.14 \pm 0.561 \times 10^{-2} \text{ min}^{-1}$ ($n=4$) at 15 secs and from 3.05 ± 0.355 ($n=4$) to $1.89 \pm 0.200 \times 10^{-2} \text{ min}^{-1}$ ($n=4$) at 30 secs, shown in figure 7.9. This suggested that EPA induced efflux by activation of K_{ATP} channels. There was no effect of glibenclamide or TEA on the ethanol response, (figure 7.10).

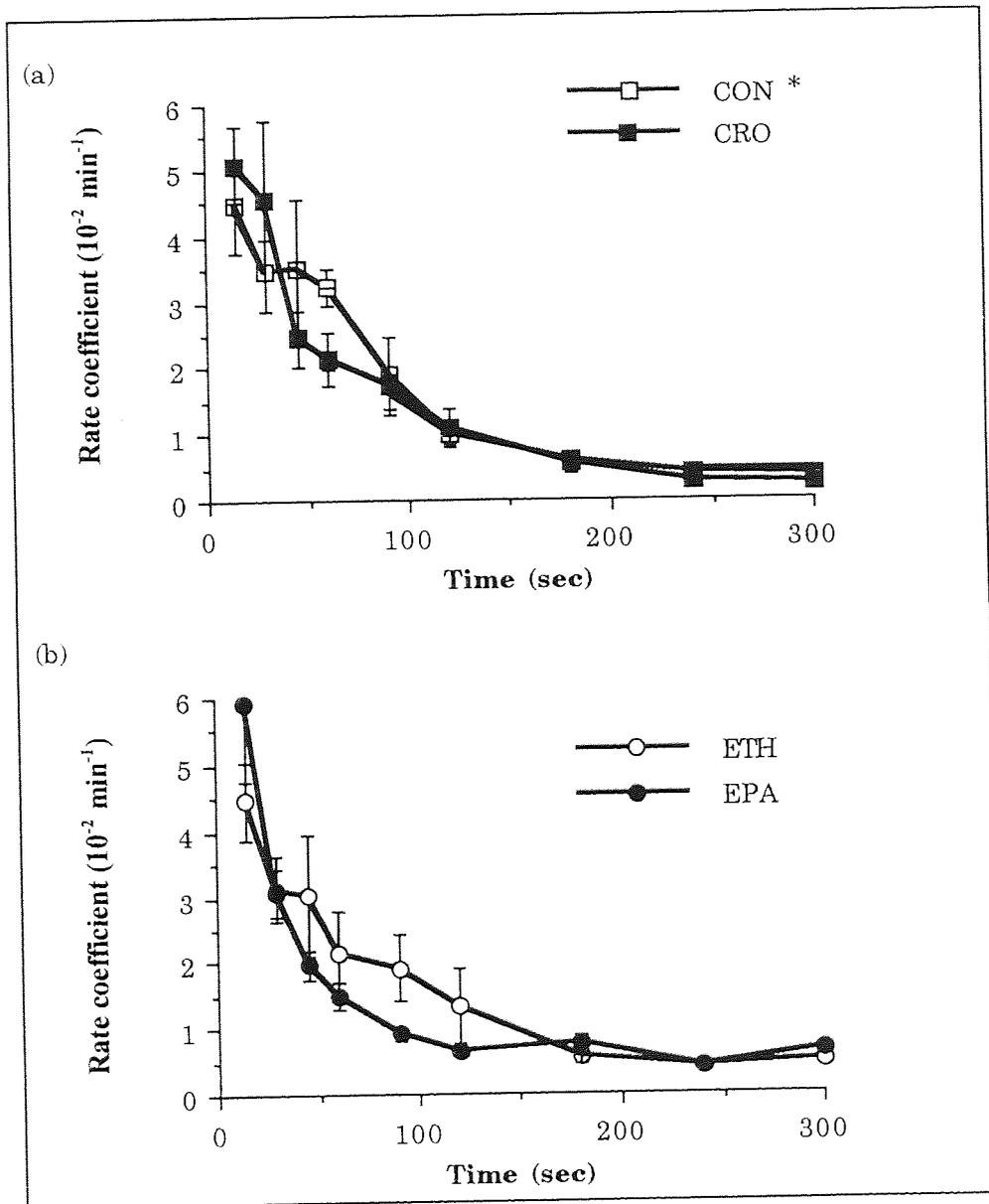


Figure 7.7. Effect of a single efflux solution on ^{86}Rb efflux between 0-5 min. AOCs at 10-13 days in culture were incubated for 90 min with $1\mu\text{Ci } ^{86}\text{Rb}$. Cells were washed and exposed to an efflux solution of 0, 15, 30, 45, 60, 90, 120, 180, 240 or 300 sec at 37°C containing (a) cromakalim ($1\mu\text{M}$; CRO), or $10\mu\text{l}$ PSS (CON), (b) EPA ($50\mu\text{M}$) or ethanol (1%, the EPA vehicle; ETH). Radioactivity in each wash solution was measured by Cerenkov counting. Rate of efflux was expressed as the rate coefficient \pm s.e.mean from 4 (*6) experiments.

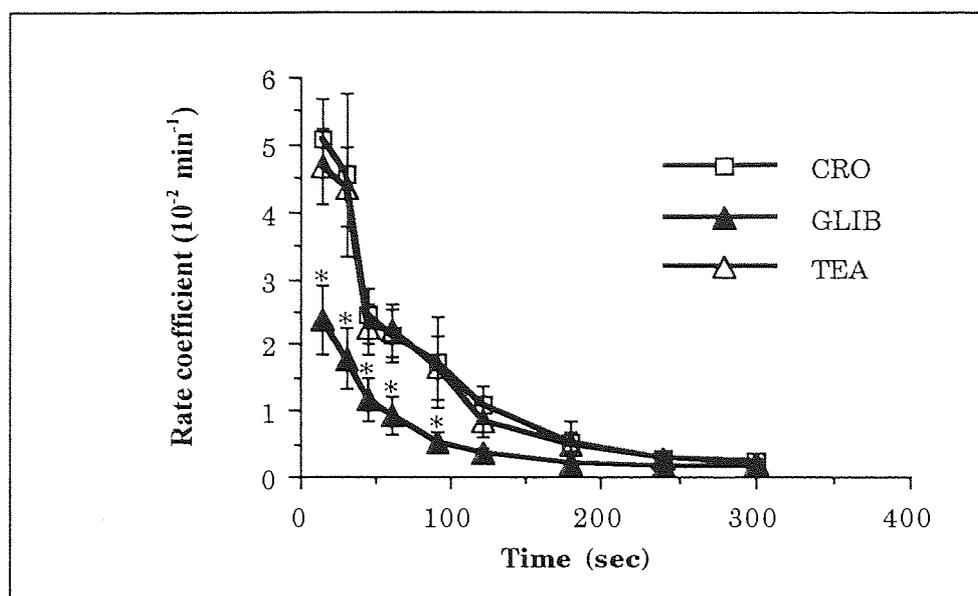


Figure 7.8 Effect of glibenclamide or tetraethylammonium on the cromakalim response. AOCs at 10-13 days in culture were incubated for 120 min with $1\mu\text{Ci } ^{86}\text{Rb}$ and glibenclamide ($1\mu\text{M}$; GLIB) or tetraethylammonium (1 mM ; TEA) for the final 30 min. Following removal of the incubation solution, cells were bathed in a single efflux solution containing cromakalim ($1\mu\text{M}$) for 15, 30, 45, 60, 90, 120, 180, 240 or 300 sec and GLIB or TEA. Radioactivity in each efflux solution was measured by Cerenkov counting. Rate of efflux was expressed as the rate coefficient \pm s.e.mean from 4 experiments ($n=4$). * indicates significant difference from corresponding CRO value.

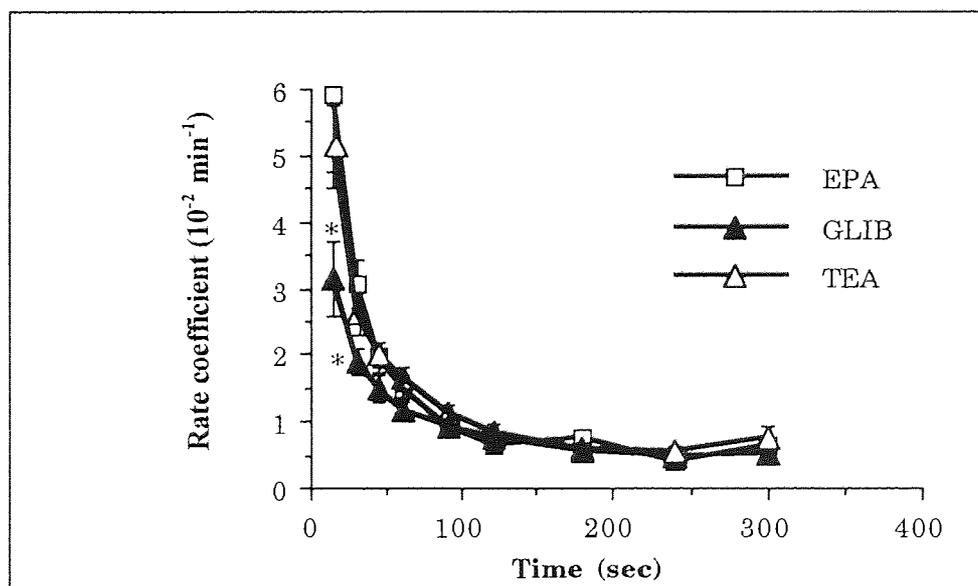


Figure 7.9. Effect of glibenclamide or tetraethylammonium on the EPA response. AOCs at 10-13 days in culture were incubated for 120 min with $1\mu\text{Ci } ^{86}\text{Rb}$ and glibenclamide ($1\mu\text{M}$; GLIB) or tetraethylammonium (1 mM ; TEA) for the final 30 min. Following removal of the incubation solution, cells were bathed in a single efflux solution containing EPA ($50\mu\text{M}$) for 15, 30, 45, 60, 90, 120, 180, 240 or 300 sec and GLIB or TEA. Radioactivity in each efflux solution was measured by Cerenkov counting. Rate of efflux was expressed as the rate coefficient \pm s.e.mean from 4 experiments ($n=4$). * indicates significant difference from corresponding CON value.

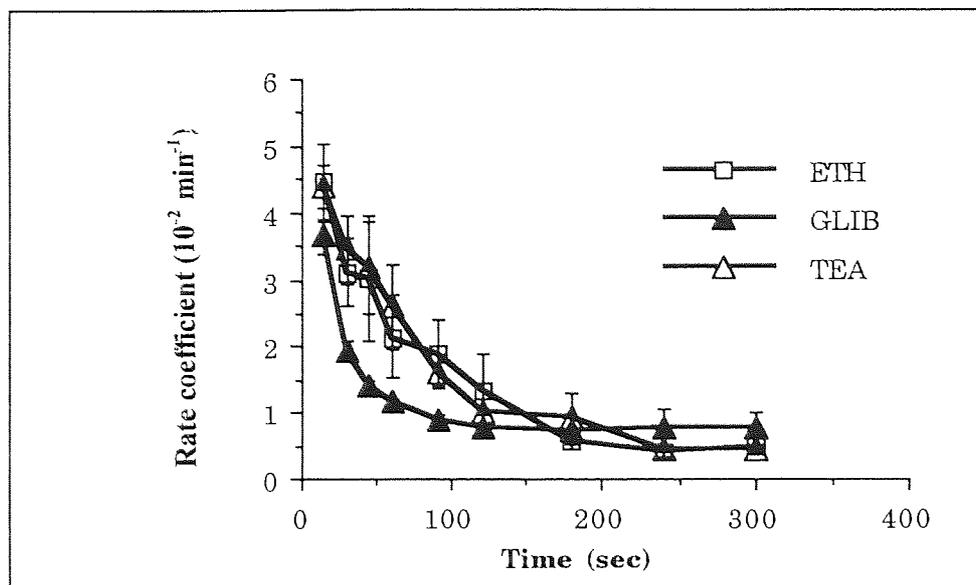


Figure 7.10. Effect of glibenclamide or tetraethylammonium on the ethanol response. AOCs at 10-13 days in culture were incubated for 120 min with $1\mu\text{Ci}$ ^{86}Rb and glibenclamide ($1\mu\text{M}$; GLIB) or tetraethylammonium (1mM ; TEA) for the final 30 min. Following removal of the incubation solution, cells were bathed in a single efflux solution containing ethanol (1%, the EPA vehicle) for 15, 30, 45, 60, 90, 120, 180, 240 or 300 sec and GLIB or TEA. Radioactivity in each efflux solution was measured by Cerenkov counting. Rate of efflux was expressed as the rate coefficient \pm s.e.mean from 4 experiments ($n=4$).

7.3 Discussion

The initial investigation in this thesis examined the effect of EPA on cell contraction and revealed that EPA partially blocks the contraction of aortic smooth muscle cells (AOCs) to the vasoactive agent noradrenaline (chapter 4). This was shown to be due to EPA decreasing calcium influx (chapter 5) through receptor-operated channels, consistent with a previous report by Asano *et al* (1997). However, it was also shown in chapter 5, that EPA induces a calcium-independent transient increase in $[Ca^{2+}]_i$, likely explained by the results in chapter 6 which demonstrated an EPA-induced increase in inositol phosphate levels. Therefore, it is suggested that the mechanism of action of EPA might involve stimulation of a transient release of calcium from an inositol phosphate-dependent internal binding site, possibly that of an intracellular membrane and that following the calcium transient, subsequent calcium influx may be prevented by an action on receptor-operated calcium channels to block further cell contraction to noradrenaline, or by an action on voltage-dependent calcium channels, to subsequently block further cell contraction to KCl.

Calcium entry through the voltage-dependent calcium channels may be inhibited by potassium channel activators which induce the cellular efflux of potassium ions, thereby hyperpolarising the plasma membrane and moving the membrane potential farther from the activation range for calcium channels. This might explain the effect of EPA to partially block the contraction of AOCs to KCl, shown in chapter 4.

In a variety of tissues including vascular smooth muscle, dietary supplementation with fish oil has been shown to affect membrane fluidity as a result of the subsequent substitution of n-3 fatty acids for n-6 fatty acids as the fatty acyl component of the membrane phospholipid (Hornstra *et al*, 1981; Lockette *et al*, 1982). In AOCs, an alteration in membrane fluidity has been demonstrated to affect Ca^{2+} -dependent potassium channel ($K_{Ca^{2+}}$) activity (Bolotina *et al*, 1989) although it has also been suggested that potassium conductance in myocardial cells is not influenced by the n-3 fatty acid composition of membrane phospholipids (Grynberg *et al*, 1995). However, it is known that the n-3 fatty acid, arachidonic acid and its metabolites are direct modulators of potassium channels in smooth muscle cells (Ordway *et al*, 1989) and it has also been demonstrated that EPA modulates potassium channels in vascular smooth muscle cells (Bolotina *et al.*, 1989).

In view of these previously published results and the results reported in this thesis, the next aim, therefore, was to investigate the effect of EPA upon membrane potassium permeability.

The rubidium isotope ^{86}Rb has frequently been used as a marker to indicate changes in membrane potassium permeability in studies concerned with the mode of action of the potassium-channel-opening drugs. The use of ^{86}Rb as a tracer is frequently preferred to ^{42}K primarily because rubidium and potassium have very similar physical and chemical properties (Ussing, 1960) but the half-life of ^{86}Rb is more convenient (18.7 days) than that of ^{42}K (12.4 hours). Potassium channels in vascular smooth muscle are more selective for potassium than rubidium (Smith *et al* 1986) but the kinetics of the two ions appear to be similar. When present in trace amounts, the two isotopes yield very similar values for the sodium-potassium pump-mediated fluxes of potassium in smooth muscle cell preparations (Widdicombe, 1977; Smith, *et al*, 1986) and skeletal muscle (Clausen *et al*, 1987).

The aim of initial experiments was to characterise potassium efflux in the AOCs, so preliminary experiments were performed which demonstrated maximal loading of the AOCs with ^{86}Rb ($1 \mu\text{Ci ml}^{-1}$) occurred by 90 minutes (figure 5.14), consistent with the loading period adopted previously (Greenwood & Weston, 1993). It was noted that the rate coefficient of efflux was lower than that of previous reports in dog and human airway smooth muscle cells (de Lorenzi, 1994) or rat isolated aorta strips (Greenwood & Weston, 1993), but was attributed to species and tissue variation.

To examine for the presence of K_{ATP} channels shown to occur in the aorta (Bray *et al*, 1991, Kovacs & Nelson, 1991), cromakalim was exposed to the AOCs as it has previously been shown to induce ^{86}Rb efflux in vascular tissue (Quast & Baumlin, 1988) and to produce relaxation of aortic segments (Greenwood & Weston, 1993). However, in the present investigation, the exposure of AOCs to cromakalim ($1 \mu\text{M}$) in accordance with the previously published methods produced no increase in the rate coefficient of K^+ efflux (figure 7.2). The considered explanation for this was that either the cells were anoxic after the loading period so that even without cromakalim, the residual low levels of intracellular ATP activated K_{ATP} or that the species and tissue variation outlined above required modification of the methodology used to enable the demonstration of the existence of K_{ATP} -channels in the AOCs. Therefore the experimental methodology was modified.

At this stage of the study, it was noted that over the experimental time course of 60 min, the greatest proportion of efflux occurred immediately following termination of the loading period and that steady state efflux was never achieved. Therefore, it was considered appropriate to examine immediate efflux from the AOCs and whether an effect of cromakalim or EPA could then be identified. So the methodology was modified to include EPA or cromakalim in the PSS throughout efflux. After 5 min the rate of efflux was significantly greater ($P < 0.05$)

with CRO (1 μM) than the efflux measured in the controls, however, inter-experimental variation was measured for the controls (figure 7.3). After a further 5 min there was no significant difference measured in the rate of efflux between control cells or those treated with CRO (1 μM).

Similarly, EPA significantly increased the rate of efflux compared to ETH (1%) over the initial 5 min efflux period, but then, except at 30 and 40 min, when EPA induced a temporary significant increase ($P < 0.05$) in efflux, there was no difference in the efflux with EPA or ETH.

It was apparent at this stage of the investigation, that the rate of efflux remained highest immediately following the termination of the loading period but then rapidly declined. Muller *et al* (1992) measured ^{86}Rb efflux using only a single efflux solution on the cells in each well and was able to demonstrate that over 50 % of the loaded isotope remained in the cells after 20 min. As a consequence, the methodology adopted in the present study was modified again. The cell preparation and 90 min loading procedure remained unchanged, however, only one aliquot of PSS was applied per well and consequently, the results demonstrated a more gradual rate of efflux. Therefore, the methodology was extended to include efflux over the initial 5 min period of efflux and was utilised in the examination of the effect of EPA.

The results revealed that neither cromakalim nor EPA (50 μM) significantly ($P < 0.05$) increased the rate of efflux yet Muller *et al.*, (1992) showed that 20 μM DHA induced an increase in efflux, indicating that DHA activated K_{ATP} -channel opening. K_{ATP} may be activated by a variety of endogenous mediators including prostanoids (Bouchard *et al*, 1994), β -adrenoceptor agonists (Randall & McCulloch, 1995) and adenosine (Dart & Standen, 1993), however, it has previously been reported that during exposure to K^+ channel openers, measurements of potassium efflux differ when ^{86}Rb and ^{42}K are used; studies indicate that a greater measurable efflux occurs when ^{42}K is used as opposed to ^{86}Rb (Greenwood & Weston, 1993). A comparison of the transport of ^{42}K and ^{86}Rb in rat and mouse soleus muscle and in rat erythrocytes revealed that for studies of potassium efflux, ^{86}Rb was not an acceptable tracer for the detection of qualitative changes (Dørup & Clausen, 1994). Therefore, the possibility may exist that in the present study, a change in efflux may have occurred but was not detected. This, however, seemed unlikely as it has been reported previously that measurable increases in K_{ATP} -channel opening using ^{86}Rb efflux kinetics are produced with arachidonic acid and DHA and furthermore that glibenclamide inhibited the effect of DHA (Muller *et al*, 1992). The thought that a similar action might explain the EPA-mediated block of cell contraction observed in the results in chapter 4 and that the EPA-induced transient increase in $[\text{Ca}^{2+}]_i$, (chapter 5) likely explained by the increased inositol phosphate levels (chapter 6), might

activate $K_{Ca^{2+}}$ to induce the cellular efflux of potassium ions prompted a final study to be undertaken. As no EPA or cromakalim-induced increase in efflux had, so far, been demonstrated, the effect of pre-treating the cells with a K_{ATP} or a $K_{Ca^{2+}}$ blocker was examined, to determine whether this would decrease efflux enough to reveal an effect of cromakalim or EPA.

Glibenclamide, a drug in the sulphonylurea group, mimics the action of ATP on K_{ATP} channels, causing them to close and thereby reduce potassium efflux (Sturgess *et al.*, 1985). Similarly, tetraethylammonium is effective at blocking $K_{Ca^{2+}}$ -channels (Inoue *et al.*, 1985) and has been widely used to differentiate potassium-channel populations (Stanfield, 1983). In aortic cells, an alteration in membrane fluidity has been demonstrated to affect calcium-dependent potassium channel activity (Bolotina *et al.*, 1989) and furthermore, it has been reported that glibenclamide inhibits the effect of DHA in insulinoma cells (Muller *et al.*, 1992). It has also been shown that EPA (30 μ M) induces an outward potassium current in A7r5 cells, which was abolished by tetraethylammonium (Asano *et al.*, 1997), a $K_{Ca^{2+}}$ -channel blocker (Halliday *et al.*, 1995), thereby supporting the suggestion that the calcium transient from chapter 5 might activate $K_{Ca^{2+}}$ to induce cellular efflux of potassium ions to block the contraction of AOCs to KCl (chapter 4).

Therefore, final experiments investigated the effect of glibenclamide or tetraethylammonium on ^{86}Rb efflux in the AOCs to establish whether K_{ATP} or $K_{Ca^{2+}}$ channels were involved in the anti-vasoconstrictor responses measured to EPA in chapter 4 and also, the reduced calcium influx measured in chapter 5.

Glibenclamide significantly decreased the efflux measured to cromakalim, consistent with the report by Beech *et al.* (1993) who demonstrated that potassium channels were not sensitive to glibenclamide in the absence of cromakalim. This suggested that the presence of glibenclamide in the final stage of loading was sufficient to reduce the activation of K_{ATP} channels during loading and then during efflux. Glibenclamide (1 μ M) also reduced the effect of EPA (figure 7.9), suggesting that EPA increased membrane potassium permeability through K_{ATP} channels. This suggestion supports a report by Muller *et al.* (1992) who measured increases in K_{ATP} -channel opening using ^{86}Rb efflux kinetics with arachidonic acid and DHA and furthermore that glibenclamide inhibited the effect of DHA (Muller *et al.*, 1992). Further support can be seen from reports using n-6 fatty acids (Ordway *et al.*, 1989; Kim & Clapham, 1989) and n-3 fatty acids (Bregestovski *et al.*, 1989; Smith *et al.*, 1992, Asano *et al.*, 1997; Gardener *et al.*, 1998) which were shown to have a direct effect on potassium ion channels situated in vascular membranes, activation of which resulted in membrane stabilisation towards the non-contractile state (Halliday *et al.*, 1995). This contrasts with a report by Kim & Duff, (1990)

however, who stated that arachidonic acid produced inhibitory effects on currents elicited by K_{ATP} -channels.

Halliday *et al.* (1995) referred to studies in which it was suggested that there are many types of voltage-sensitive potassium channels which exist in smooth muscle. Three of these groups have been extensively characterised, distinguished by their sensitivities to membrane potential and pharmacological agents and include the voltage-gated potassium current and the delayed rectifier channels. The third channel is the calcium-activated potassium-channel ($K_{Ca^{2+}}$) the opening probability of which increases with rises in $[Ca^{2+}]_i$ and depolarisation above the resting potential. These channels serve to terminate periods of Ca^{2+} entry by repolarising or hyper the cell (Beech & Bolton, 1989). It was suggested earlier that from the results presented in this thesis, in the event that EPA induces a transient increase in $[Ca^{2+}]_i$, which is independent of extracellular calcium (chapter 5) and likely to be explained by the increased inositol phosphate levels (chapter 6), then, the calcium transient might activate $K_{Ca^{2+}}$ to induce the cellular efflux of potassium ions. This would hyperpolarise the plasma membrane as outlined above and move the membrane potential farther from the activation range for voltage-dependent calcium channels, explaining the effect of EPA to partially block the contraction of AOCs to KCl, shown in chapter 4.

Therefore, the effect of tetraethylammonium, a $K_{Ca^{2+}}$ -channel blocker (Halliday *et al.*, 1995), was examined in the present investigation. Tetraethylammonium has been used commonly to differentiate potassium-channel populations (Stanfield, 1983) and it has been shown previously that low concentrations (< 3 mM) are effective at blocking $K_{Ca^{2+}}$ -channels (Inoue *et al.*, 1985). However, in the present study, there was no significant effect ($P < 0.05$) of tetraethylammonium (1 mM) on ^{86}Rb efflux in control cells. Consistently, the results by Beech & Bolton (1989) indicated that in intact strips of rabbit aortic tissues, tetraethylammonium clearly produced an increase in tension, but when using the whole cell patch clamp technique, found that it only blocked potassium currents activated above 0mV.

In the present investigation, tetraethylammonium did not decrease the effect of cromakalim nor EPA, indicating that the role of $K_{Ca^{2+}}$ was not associated with the efflux observed with cromakalim or EPA. This contrasted with previous reports which showed that fish oil modulates activity at $K_{Ca^{2+}}$ (Smith *et al.*, 1992). An elevation in $[Ca^{2+}]_i$ has been proposed to precipitate the vasoconstriction and increased potassium efflux observed through calcium-activated channels in vascular smooth muscle from both genetic (Smith & Myers, 1990) and mineralocorticoid hypertensive rats (Smith & Jones, 1990). However, when Smith *et al.* (1992), studied the effect of menhaden oil on calcium-dependent ^{86}Rb efflux from the aorta of stroke-prone, spontaneously hypertensive rats, they showed that

menhaden oil decreased the diltiazem or sodium nitroprusside-induced inhibition of ^{86}Rb efflux in the animals. Their results demonstrated that calcium-sensitive ^{86}Rb efflux was decreased with fish oil. However, it was discussed in chapter 6, that EPA may exert a time-dependent effect upon inositol phosphate formation, whereby the effect increased following chronic exposure to the fatty acid. This effect might also occur in studies of membrane potassium permeability and might explain the difference in the results obtained in the studies of Smith *et al* (1992) and those in the present investigation.

In conclusion therefore, EPA (50 μM) induced ^{86}Rb efflux from the AOCs which was blocked by glibenclamide (1 μM) but not tetraethylammonium (1 mM), confirming that EPA activated K_{ATP} channels in the AOCs. Therefore, it is suggested that the mechanism of action of EPA involves stimulation of a transient release of calcium from an inositol phosphate-dependent internal binding site, possibly that of an intracellular membrane and following the calcium transient, subsequent calcium influx is prevented by an action on both receptor-operated calcium channels, consistent with a previous report by Asano *et al* (1997), to block further cell contraction to noradrenaline (chapter 4) and on K_{ATP} channels (chapter 7) to induce the cellular efflux of potassium ions, thereby hyperpolarising the plasma membrane and moving the membrane potential farther from the activation range for calcium channels. This might explain the effect of EPA to subsequently block further cell contraction to KCl, shown in chapter 4.

However, having identified possible mechanisms by which EPA mediates a vasorelaxatory response in AOCs, it was considered appropriate to examine whether the resultant effect was also apparent in the intact aortic tissue since EPA and DHA have previously been shown to inhibit the contraction induced by α -adrenoceptor agonists in rat aortic rings (Juan & Sametz, 1987; Engler *et al*, 1990; Engler, 1992a).

Chapter 8

The Effect Of EPA On Aortic Tissue Contraction.

8.1 Introduction

Results described so far in this thesis have demonstrated that EPA partially blocks the contraction of cells to the vasoactive agents, KCl and noradrenaline (chapter 4). As it was reported that relaxation of vascular smooth muscle may be a result of blockade of intracellular calcium release or a decrease of influx of extracellular calcium, or be due to desensitisation of the contractile apparatus to calcium (Somlyo & Himpens, 1989), subsequent studies examined the effect of EPA on these parameters. It was found in chapter 5, that EPA significantly decreased calcium influx but that EPA is not associated simply with block of L-type channels and does not bind at the same subunit site as the dihydropyridine calcium channel blockers, in support of a recent suggestion (Hallaq *et al*, 1992). However the noradrenaline-induced, concentration-dependent increase in calcium influx was significantly reduced by EPA, suggesting that EPA could be associated with calcium influx through receptor-operated channels, consistent with a previous report by Asano *et al* (1997).

However, it was also shown in chapter 5, that EPA induces a transient increase in $[Ca^{2+}]_i$, which is independent of extracellular calcium. This might be explained by the results obtained in chapter 6, which showed that EPA increased inositol phosphate levels. In the event that the transient EPA-induced release of calcium may be from an inositol phosphate-dependent internal binding site, possibly that of an intracellular membrane, it is suggested that upon the release of this calcium transient, stimulation of membrane-associated K^+ channels may hyperpolarise the cell membrane, moving the membrane potential away from that required to allow calcium influx and subsequently blocking further cell contraction to noradrenaline or KCl.

The role of EPA appeared to multifactorial, therefore, the aim of the experiments reported in this chapter, was to confirm whether the effects of EPA observed on the cells were apparent in intact tissue preparations and whether the mechanism(s) of action of EPA was also linked to causing desensitisation of the contractile filaments in the cells.

EPA and DHA have previously been shown to inhibit the contraction induced by α -adrenoceptor agonists in rat aortic rings (Juan & Sametz, 1987; Engler *et al*, 1990; Engler, 1992a), so the objectives of the initial experiments described in this chapter was to confirm that EPA caused a reduction of the contractural response to noradrenaline in isolated, endothelium-denuded aorta.

The procedures used for the experiments are described fully in 2.7, but in summary the technique involved sectioning rat thoracic aorta into rings (5 mm), removing the endothelium and suspending the rings horizontally between two wire triangles in an organ bath. One triangle was connected *via* a wire hook to a transducer (Pioden Isometric UF1) whilst the other was clamped in place in the organ bath (see figure 2.1) containing Krebs solution at 37°C.

Following equilibration of all tissues used in these experiments, a sensitising response to NA (3 μ M) was elicited to determine tissue viability. The determination of the absence of endothelium using acetylcholine was followed by a washout period.

The effect of EPA on noradrenaline-induced contractures was determined using non-cumulative concentration-response curves. The sensitivity of the aorta preparations to noradrenaline was assessed as the negative logarithm of the concentration required to cause 50 % of the maximum response (pD_2). All data are presented as the means of n aortic rings \pm the standard error of the mean (s.e. mean). Statistical comparisons were made by Student's two-tailed t test for paired or unpaired data as appropriate, or in the case of multiple comparisons, analysis of variance (ANOVA) followed by Scheffe's multiple range test. Significance was identified at $P < 0.05$.

To confirm that the effect of EPA was not to inhibit the α -adrenoceptor in rat aortic rings the objectives of the next experiments was to investigate whether the affect of EPA on the tissue could be blocked by α -adrenoceptor antagonists. In the experiments which examined the effect of α -adrenoceptor antagonists, the antagonist was added to the bath at least 15 min prior to EPA or noradrenaline.

In view of the effect of EPA to transiently increase calcium and to increase inositol phosphate, a further study was performed to investigate whether EPA affected the contracture resulting from release of intracellular calcium. It is well recognised that a biphasic form of the contractile response of the rat aorta is observed following selective agonist stimulation. This response consists of an initial phasic contraction as the consequence of a rapid rise in calcium due to calcium release from intracellular stores (Godfraind & Kaba, 1972), followed by a slow, sustained tonic contraction which is dependent upon calcium entry from the extra cellular solution (Cauvin & Malik, 1984; Scarborough & Carrier, 1984).

In these experiments the effect of EPA on noradrenaline-induced contractures was measured following calcium removal from the solution bathing the tissue. The tissues were pre-treated with EPA (50 μ M) for 30 min before replacement of Krebs with calcium-free Krebs containing EPA (50 μ M) and EGTA (2 mM) to chelate extracellular calcium, thereby ensuring the tissue is bathed in a

calcium-free solution. Following stimulation, any resulting contraction must be due to the release of intracellular calcium.

It was outlined above, that a vasorelaxatory response may be due to desensitisation of the contractile filaments to calcium (Somlyo & Himpens, 1989), so a final study was performed to determine whether the transient increase in calcium, which appeared not to increase the contractile status of the cells (chapter 4), was due to a decreased sensitivity of the contractile filaments to calcium. To study this, the tissue was depolarised whilst in a calcium-free solution; this ensured that voltage-operated calcium channels were open. Calcium was then added to the solution bathing the cells, which should cause the tissue to contract if the sensitivity of the contractile machinery to calcium was normal. The effect of EPA upon this response was examined. In performing these experiments, extracellular calcium was added to a nominally calcium-free high potassium solution (KPSS) and the sensitising response was elicited to 60 mM KCl instead of 3 μ M noradrenaline. After washout, PSS was replaced with KPSS and EPA (50 μ M) or ethanol (ETH, 1 %; the EPA vehicle) was added. Cumulative concentration-response curves to calcium were then constructed.

8.2 Results

8.2.1. Effect of EPA on agonist-induced contractures

Initial experiments aimed to determine whether eicosapentaenoic acid (EPA) caused a reduction of the contractural responses in the aorta of the rat. Aortic tissue was prepared and mounted for isometric recording according to the methodology described in 2.7.1. Briefly, following the sensitising response to the non-selective α -adrenoceptor agonist, noradrenaline (NA; 3 μM), a single dose of EPA (50 μM) or ethanol (1%, ETH; the EPA vehicle) was added to the bathing PSS during the final 30 min of the washout and then following every subsequent washout. Non-cumulative concentration-response curves were constructed to NA. Each dose of NA was left in contact with the tissue for a minimum period of 5 min and successive doses of agonist were added after a minimum period of 15 min had elapsed, during which time the tissue was washed every 5 min. The contact times and washout times varied with the concentration of agonist used to induce contractures, as the higher concentrations often required a longer period of time to induce a maximum response and to wash out.

Isometric responses were measured as grams of tension. NA (3 μM) induced a biphasic response, consisting of a brief fast response followed by a slowly developing component. Maximal contracture was reached within 2 min after drug addition (Figure 8.1) and could be maintained for more than 30 min in the presence of the agonist. It was found that EPA and ETH affected the contracture induced after stimulation with NA. In the presence of EPA the addition of a low concentration of agonist (1-3 nM) induced a contracture which was maintained, but peak tension was 3-fold lower than the peak attained in the absence of EPA. Figure 8.2 shows concentration-response curves for EPA and ETH. It can be seen that in the presence of ETH, there was a rightward shift in the concentration-response curve to NA and a reduction in the maximum response. There was a further significant reduction of the contractures in the presence of EPA.

The sensitivity of the aorta preparations to NA was assessed as the negative logarithm of the concentration required to cause 50 % of the maximum response (PD_2). The PD_2 and peak contraction (E_{max}) values of the NA-induced concentration-response curves in the absence (control) or presence of EPA or ETH, are shown in table 8.1.

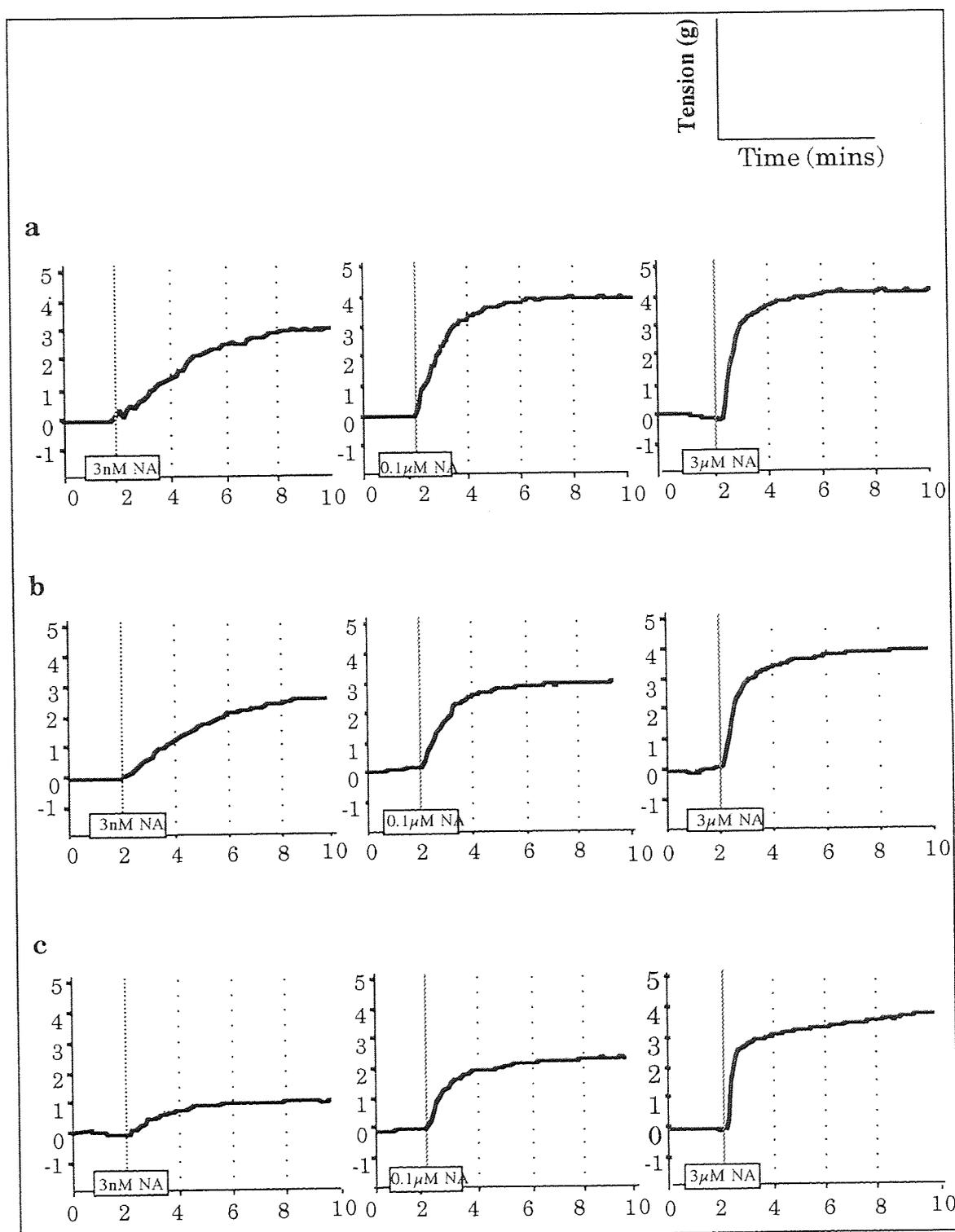


Figure 8.1. Representative traces showing noradrenaline-induced contractures in rat aorta. Responses elicited are (a) controls, (b) in the presence of ethanol (ETH, 1%; the EPA vehicle), (c) in the presence of EPA (50 μ M) and are expressed as grams tension. Addition of noradrenaline (NA) to the bathing PSS is indicated by the vertical line. Traces are representative of a minimum of 14 responses.

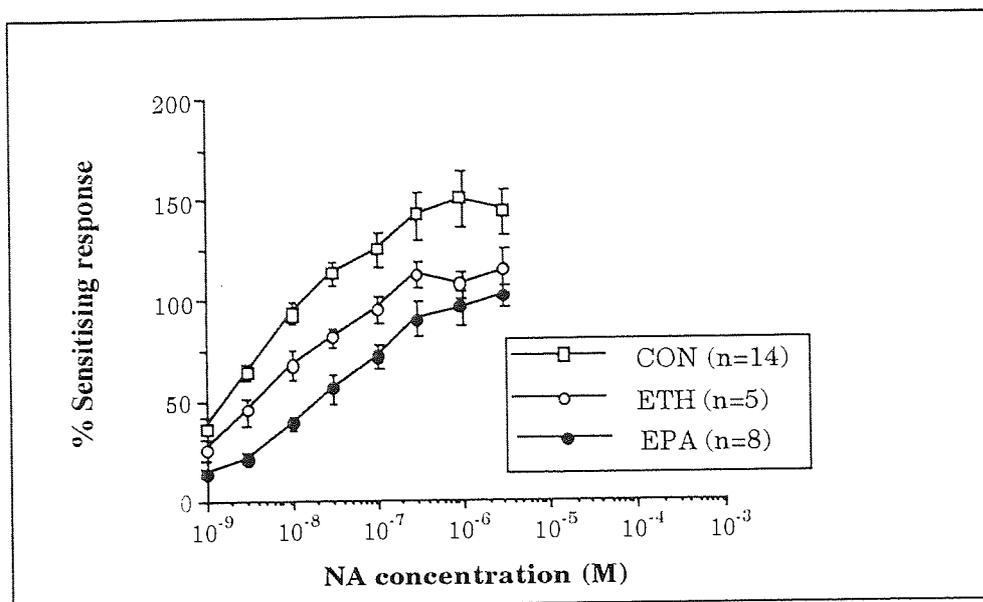


Figure 8.2. Effect of EPA or ETH on the concentration-response curve to noradrenaline. Following the sensitising response to noradrenaline (NA; 3 μ M) a single dose of EPA (50 μ M) or ethanol (1% final concentration, ETH; the EPA vehicle) was added to the bathing PSS during the final 30 min of the washout. Non-cumulative concentration-response curves were then constructed to NA. Responses are expressed as a percentage of the sensitising response to NA. All values are mean \pm s.e.mean for n tissues ($n \geq 5$).

Table 8.1. The effect of EPA or ethanol on the potency (PD_2) and peak response (E_{max}) of noradrenaline. A single dose of EPA (50 μ M) or ethanol (1% final concentration, ETH; the EPA vehicle) was added to the bathing PSS during the final 30 min of the washout, prior to the construction of non-cumulative concentration response curves to noradrenaline (NA).

	PD_2 values	E_{max} (% sensitising response)
Control ($n=14$)	8.54 ± 0.18	151.99 ± 13.44
ETH ($n=5$)	8.14 ± 0.21	120.51 ± 9.76
EPA ($n=8$)	$7.51 \pm 0.14^*$	107.91 ± 5.03

* indicates significant difference from ETH using ANOVA and Scheffe's multiple range test where $P < 0.05$.

8.2.2. Effects of α -adrenoceptor antagonists on agonist-induced contractures.

In these experiments, the effect of selective α -adrenoceptor antagonists on the response of the tissue to noradrenaline was investigated.

The experiments followed the methodology described in 2.7.2.2. and were similar to those outlined in the previous section. However, throughout the final 45 min of the washout following the sensitising response to NA (3 μ M), a single dose of antagonist was added to the bathing PSS. During the time the antagonist was incubated on the tissues, the bathing PSS was changed at 10 min intervals. Non-

cumulative concentration-response curves were then constructed to NA in the presence of the antagonist.

Contractures to NA were reduced in the presence of prazosin (PZ; 5 nM) or phentolamine (PH; 1 μ M), shown in figure 8.3 and indicated by the pD_2 values for the curves (see table 8.2). In the presence of PH or PZ, E_{max} was significantly reduced ($P < 0.05$), shown in table 8.2. The results are consistent with the existence of a population of postjunctional α_1 -adrenoceptors on the tissue.

Table 8.2. Effect of α -adrenoceptor antagonists on the potency (pD_2) and peak response (E_{max}) of noradrenaline. A single dose of prazosin (PZ; 5 nM) or phentolamine (PH; 1 μ M) was added to the bathing PSS throughout the final 45 min of washout. Non-cumulative concentration-response curves were then constructed to noradrenaline (NA) in the presence of the antagonist.

	PD_2 values	E_{max} (% sensitising response)
No antagonist	8.54 ± 0.18	151.99 ± 13.44
PZ	$6.36 \pm 0.12^*$	$98.36 \pm 8.49^*$
PH	$7.01 \pm 0.72^*$	$97.10 \pm 19.26^*$

* indicates significant difference from value obtained in the absence of an antagonist, using ANOVA and Scheffe's multiple range test where $P < 0.05$.

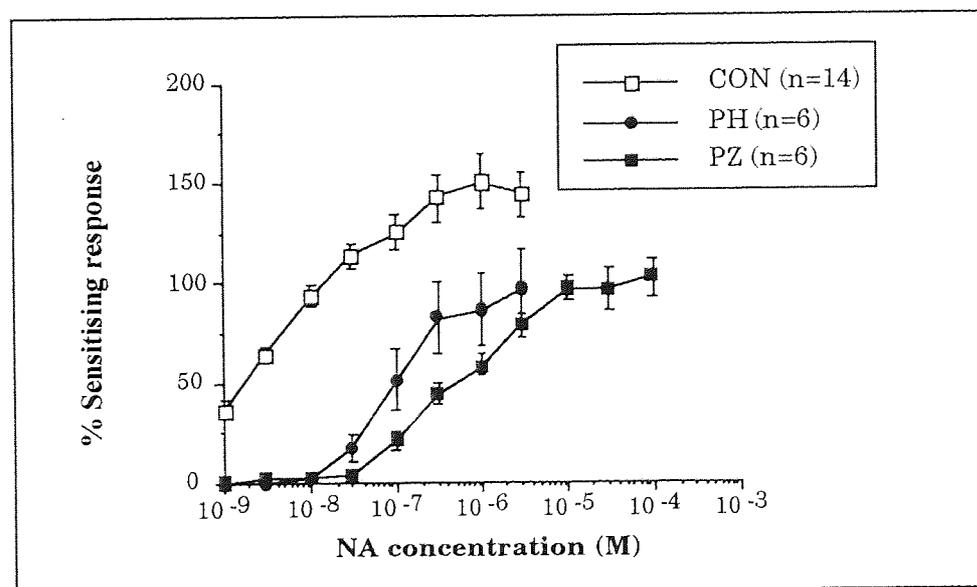


Figure 8.3. Effect of prazosin or phentolamine on the concentration-response curve to noradrenaline Throughout the final 45 min of the washout following the sensitising response to NA (3 μ M), a single dose of prazosin (PZ; 5 nM) or phentolamine (PH; 1 μ M) was added to the bathing PSS. Non-cumulative concentration-response curves were then constructed to NA in the presence of the antagonist. Responses are expressed as a percentage of the sensitising response to NA (3 μ M). All values are mean \pm s.e.mean for n tissues ($n \geq 6$).

8.2.3. Effects of EPA and α_1 -or α_2 -adrenoceptor antagonists on agonist-induced contractures.

Having established that EPA reduced the effect of noradrenaline on contractures of the aorta, the next objective was to determine whether the vasorelaxatory mechanism of action of EPA could be blocked by an α -adrenoceptor antagonist.

These experiments were similar to those of the preceding section, except that the tissue was pre-treated with an α -adrenoceptor antagonist. The aorta preparation was exposed to an antagonist in the PSS for 15 min prior to the addition of EPA (50 μ M) or ETH (1 %; the EPA vehicle) for a further 30 min. Non-cumulative concentration-response curves constructed to noradrenaline were performed as outlined in 8.2.2 and described completely in 2.7.2.3.

Following treatment with prazosin (PZ; 5 nM) or phentolamine (PH; 1 μ M), the noradrenaline-induced contractures were reduced in the presence or absence of ethanol (refer to pD_2 values shown in tables 8.2 and 8.3). Therefore, there was no effect by the ethanol on the blocking effect of the antagonist.

However, with EPA, the blocking effect of prazosin or phentolamine was enhanced, shown by a further rightward shift in the noradrenaline concentration-response curves (figures 8.5 and 8.6 respectively) and a decrease in the peak response being observed to prazosin. This was also indicated by the pD_2 values shown in table 8.3 and suggested that the relaxatory effect of EPA was not prevented by the antagonists. Therefore, it appeared that the effect of EPA occurred through a different mechanism than the α -adrenoceptor.

Table 8.3. *Effect of EPA or ethanol, in the presence of prazosin or phentolamine on the potency (pD_2) and peak response (E_{max}) of noradrenaline. The aorta preparation was exposed to a single dose of prazosin (PZ; 5 nM) or phentolamine (PH; 1 μ M) in the washout PSS for the final 30 min of washout. Non-cumulative concentration-response curves were then constructed to noradrenaline (NA) in the presence of the antagonists and EPA (50 μ M) or ethanol (ETH, 1%; the EPA vehicle).*

	pD_2 values	E_{max} (% sensitising response)
ETH	8.14 \pm 0.21 (n=5)	120.5 \pm 9.76
EPA	7.51 \pm 0.14 (n=8)	107.91 \pm 5.03
ETH + PZ	6.17 \pm 0.08 (n=8)	104.4 \pm 8.79
EPA + PZ	5.84 \pm 0.11 (n=7) *	65.1 \pm 7.72
ETH + PH	7.08 \pm 0.03 (n=7)	85.3 \pm 9.45
EPA + PH	6.62 \pm 0.10 (n=7) *	78.9 \pm 9.94

* indicates significant difference from corresponding antagonist value in presence of ETH, using ANOVA and Scheffe's multiple range test where $P < 0.05$.

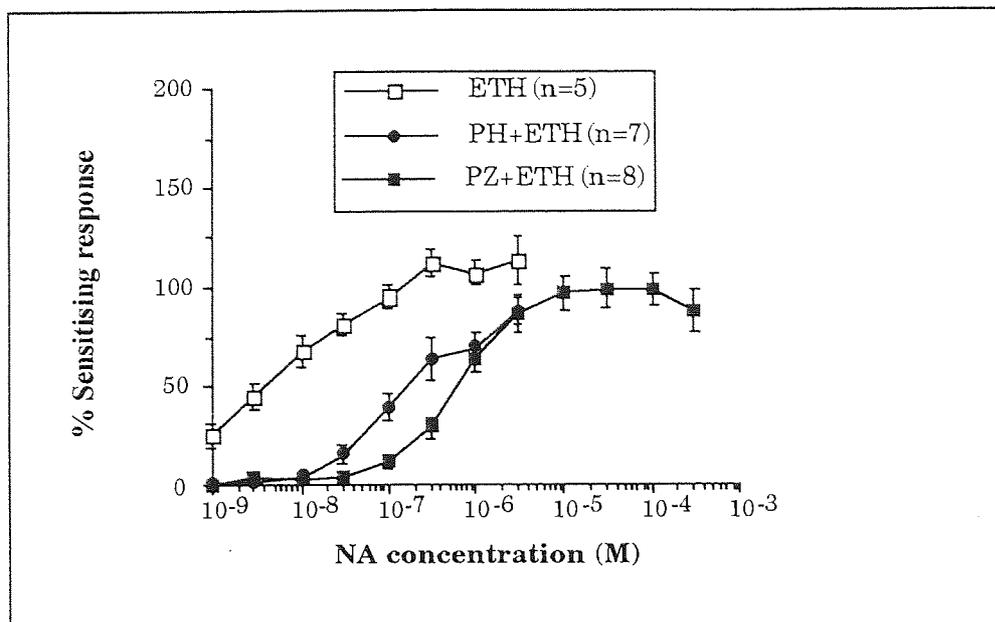


Figure 8.4. Effect of ethanol on the concentration-response curve to noradrenaline in the presence of prazosin or phentolamine. The aorta preparation was exposed to prazosin (PZ; 5 nM) or phentolamine (PH; 1 μ M) in the washout PSS for 15 min prior to the addition of ETH (1%; the EPA vehicle). 30 min later, non-cumulative concentration-response curves were then constructed to noradrenaline (NA) in the presence of the antagonist and ethanol (ETH, 1%; the EPA vehicle). Responses are expressed as a percentage of the sensitising response to 3 μ M NA.

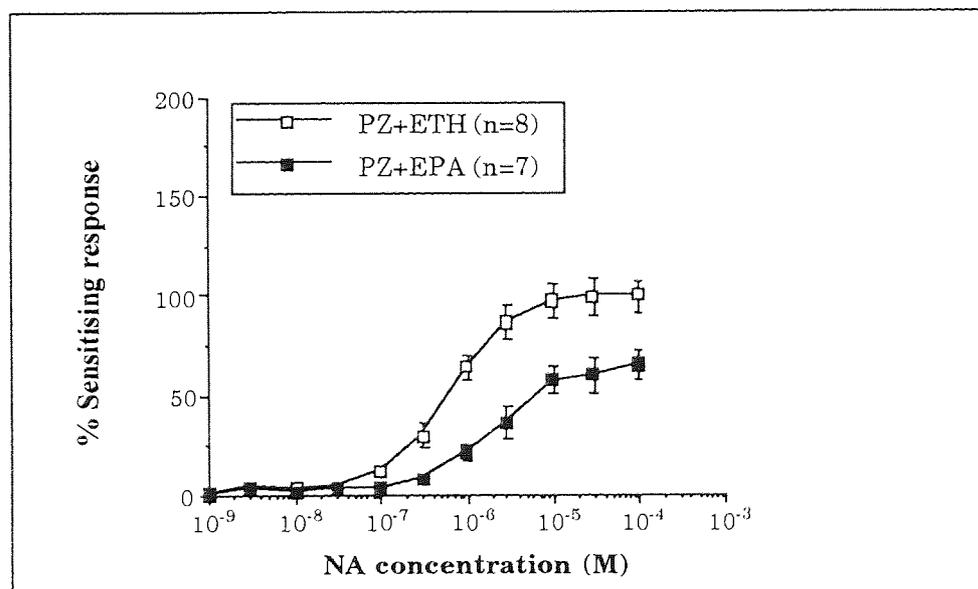


Figure 8.5. Effect of EPA on the concentration-response curve to noradrenaline in the presence of prazosin. The aorta preparation was exposed to prazosin (PZ; 5 nM) in the washout PSS for 15 min prior to the addition of EPA (50 μ M) or ethanol (1%; ETH, the EPA vehicle). 30 min later, non-cumulative concentration-response curves were then constructed to noradrenaline (NA) in the presence of the antagonist and EPA (50 μ M) or ethanol (1%; ETH, the EPA vehicle). Responses are expressed as a percentage of the sensitising response to 3 μ M NA.

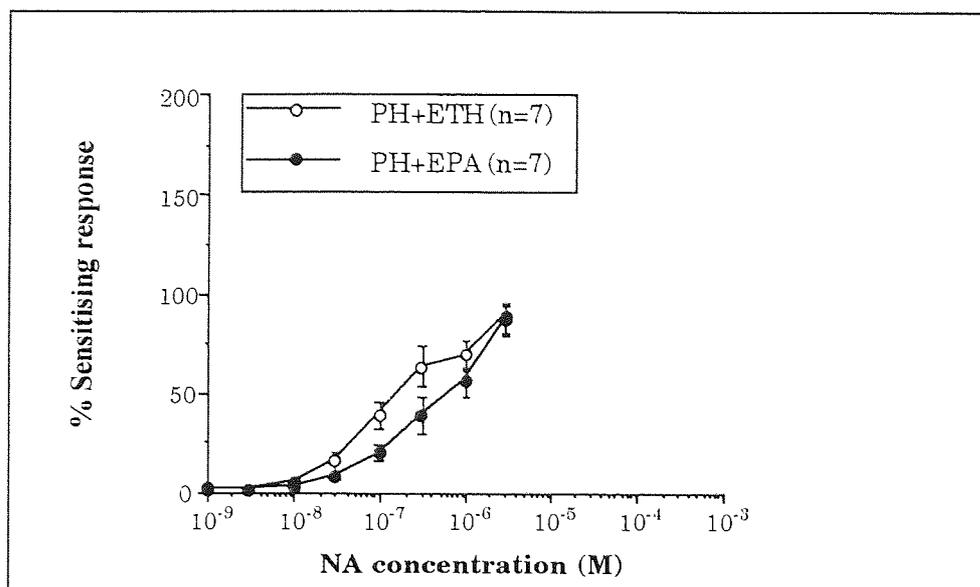


Figure 8.6. *Effect of EPA on the concentration-response curve to noradrenaline in the presence of phentolamine.* The aorta preparation was exposed to phentolamine (PH; 1 μ M) in the washout PSS for 15 min prior to the addition of EPA (50 μ M) or ethanol (1%; ETH, the EPA vehicle). 30 min later, non-cumulative concentration-response curves were then constructed to noradrenaline (NA) in the presence of the antagonist and EPA (50 μ M) or ethanol (1%; ETH, the EPA vehicle). Responses are expressed as a percentage of the sensitising response to 3 μ M NA.

8.2.4. Effects of calcium removal on agonist-induced contractures.

To show the effect of EPA on the contraction resulting from intracellular calcium release, control contractions to the agonist were compared with the EGTA-resistant response (ERR) obtained following incubation of the aortic preparation in calcium-free PSS containing the calcium ion chelator EGTA (2 mM) for 2 min. Any contraction remaining under these conditions was taken to be dependent on calcium released from intracellular stores (Cauvin and Malik, 1984).

Representative traces showing the effect of Ca²⁺-free, EGTA PSS on contractures to noradrenaline (NA) are shown in figure 8.7. It can be seen that the ERR to NA was transient with peak tension being achieved rapidly (within 1 min) followed by a fall to baseline tension during the subsequent 5 min period of agonist contact.

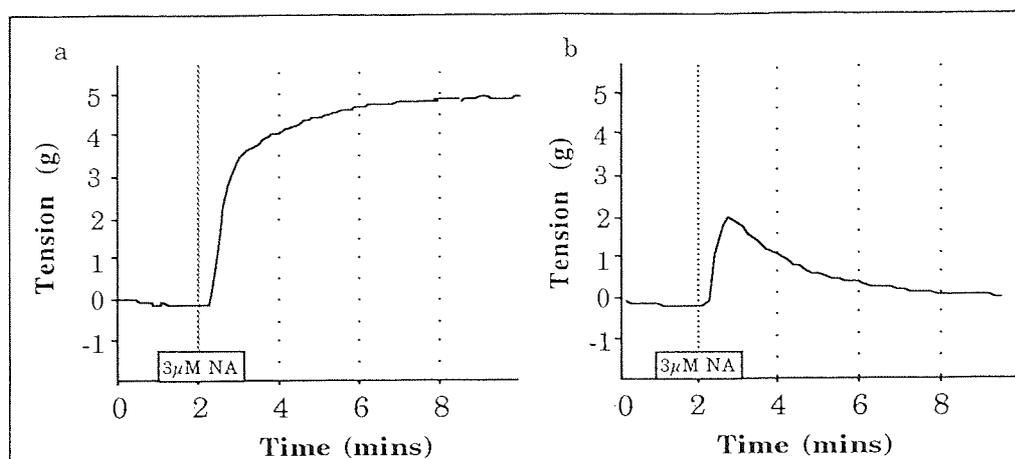


Figure 8.7. Representative traces showing the effect of $3 \mu\text{M}$ noradrenaline on the contractural response (a) in normal PSS and (b) in calcium-free PSS containing 2 mM EGTA. Responses are expressed as grams tension. Addition of NA to the bathing PSS is indicated by the vertical line. Traces are representative of a minimum of 9 responses.

Figure 8.8. shows that $3 \mu\text{M}$ noradrenaline produced an ERR which was $46.65 \pm 4.83 \%$ of the sensitising response. This was $34.66 \pm 3.12 \%$ of the control contracture observed to $3 \mu\text{M}$ noradrenaline in normal PSS.

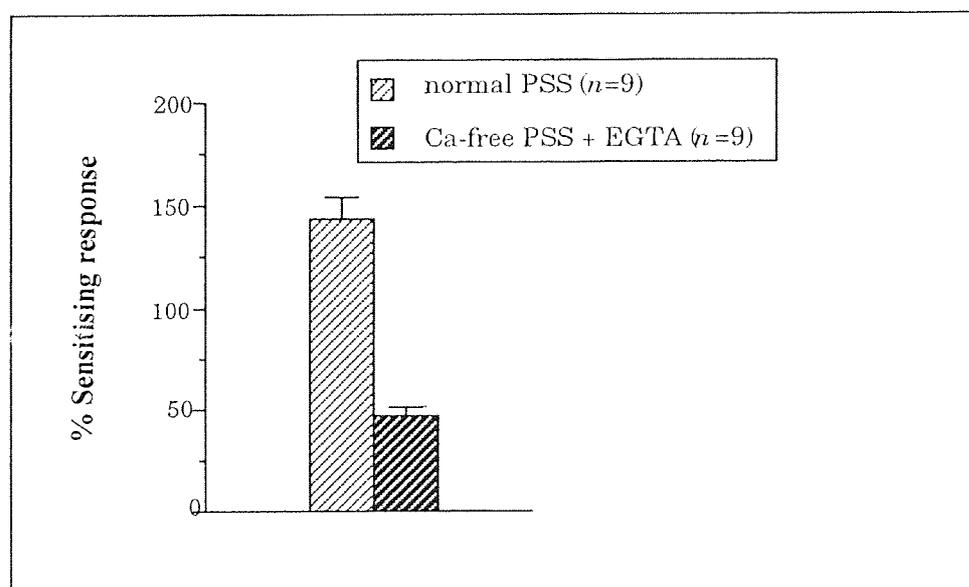


Figure 8.8. Effect of on the EGTA-resistant response to noradrenaline. Following the 90 min washout period, the bathing PSS was replaced with calcium-free PSS containing 2 mM EGTA. After 2 min, a single dose of noradrenaline (NA; $3 \mu\text{M}$) was added for a minimum period of 10 min. The maximal contraction (E_{max}) was measured, and responses are expressed as percentage of the sensitising response. Values are the mean \pm s.e. mean for n tissues ($n=9$).

8.2.5. Effect of EPA on the EGTA-resistant response to noradrenaline.

The objective of this part of the study was to investigate whether the reduction of NA-induced contractures by EPA was related to inhibition of intracellular calcium mobilisation, as suggested by Engler (1992b). Therefore the effect of EPA on NA-induced contractures in calcium-free PSS was determined. The experiments were similar to those performed in the previous section, however, during the final 30 min of the washout period, a single dose of EPA (50 μ M) was added to the bathing solution. The PSS containing EPA was replaced at 10 min intervals, as before. Following this, the bathing PSS was replaced with calcium-free PSS containing EGTA (2 mM) and EPA (50 μ M) or ethanol (ETH, 1%) for 2 min prior to the addition of a single dose of NA (3 μ M).

Following treatment with EPA (50 μ M) or ethanol (ETH, 1 %; the EPA vehicle), NA produced an ERR which was 42.51 ± 3.79 % or 44.75 ± 6.75 % of the sensitising response respectively (see figure 8.9). This was 40.81 ± 4.83 % or 36.58 ± 2.32 % of the NA-induced contraction in the presence of EPA or ETH observed in normal PSS, respectively.

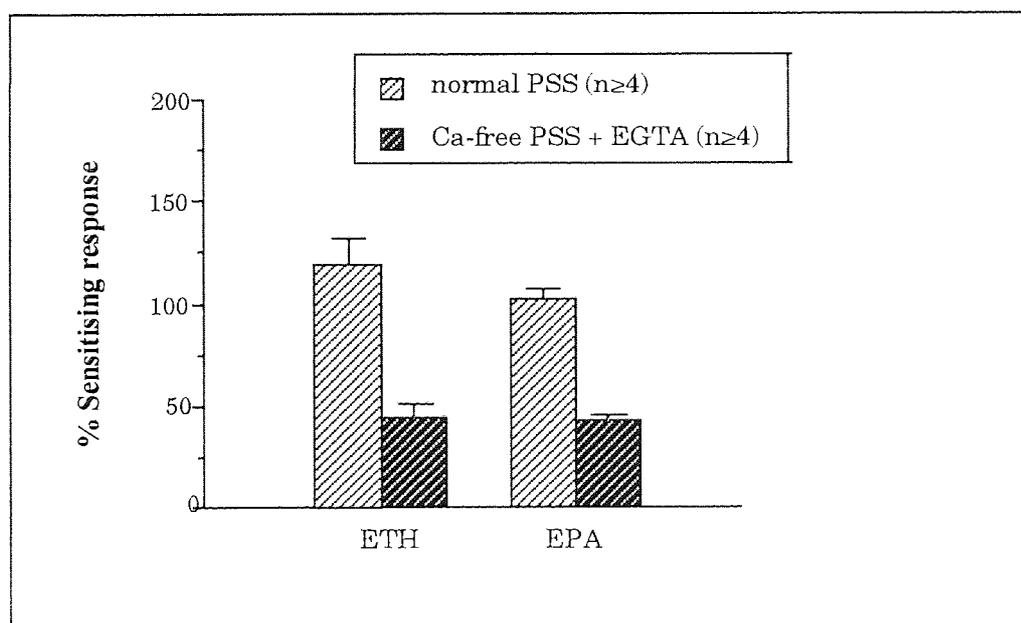


Figure 8.9. Effect of the EGTA-resistant response to noradrenaline in the presence of EPA. Following the sensitising response to noradrenaline (NA; 3 μ M), a single dose of EPA (50 μ M) or ethanol (1 %; ETH, the EPA vehicle) was added to the washout for the final 30 min. The bathing PSS was then replaced with calcium-free PSS containing 2 mM EGTA and EPA (50 μ M). After 2 min, a single dose of noradrenaline (3 μ M) was added for a minimum period of 10 min. The maximal contraction (E_{max}) was measured, and responses are expressed as percentage of the sensitising response. Values are mean \pm s.e. mean for n tissues ($n \geq 4$).

These results show that noradrenaline was still able to elicit an ERR in the presence of EPA, as the ERR, expressed as a percentage of the sensitising response to noradrenaline, was not significantly different, from that of ETH or the control response in normal PSS. This demonstrated that EPA did not affect the phasic component of the noradrenaline-induced contraction arising from the release of intracellular calcium. Noradrenaline-induced calcium release is thought to be an α_1 -mediated effect (Han *et al*, 1987), indicating that the transient contraction induced by α_1 -adrenoceptors was unaffected by EPA. To confirm this, the study was extended to examine what effect α_1 - and α_2 -adrenoceptor antagonists would have on the ERR.

8.2.6. Effects of EPA and α_1 - or α_2 -adrenoceptor antagonists on the EGTA-resistant response to noradrenaline.

The methods for these experiments are described fully in 2.7.2.6. and followed a similar protocol to that described in the previous section, however, throughout the final 45 min of the washout period, a single dose of the antagonist was added to the bathing PSS. When the bathing PSS was replaced with calcium-free PSS containing 2 mM EGTA and EPA (50 μ M) or ethanol (ETH, 1%: the EPA vehicle), the antagonist was also present. The single dose of noradrenaline (NA, 3 μ M) was added 2 min later.

It can be seen in figure 8.10 that in the presence of prazosin (PZ, 5 nM) the ERR for noradrenaline was small (6.88 ± 1.40 % of the sensitising response) and was significantly less than the ERR in the absence of the antagonist, demonstrating that the ERR could be blocked by an α_1 -adrenoceptor antagonist. The response to prazosin was also significantly less than the ERR obtained in the presence of phentolamine (PH, 1 μ M; 45.68 ± 9.04 % of the sensitising response) which was not significantly different from the ERR obtained in the absence of the antagonist, suggesting that phentolamine was less effective at the α_1 -adrenoceptor in this experiment.

In the presence of EPA, prazosin did not block the noradrenaline-induced ERR as much as it did when EPA was not added. However, this effect was also observed when ETH was added with the prazosin. There was no effect of EPA on the response to phentolamine. Therefore, these results demonstrated that the transient contraction to noradrenaline which is induced by α_1 -adrenoceptors and mediated by the release of intracellularly stored calcium, was unaffected by EPA.

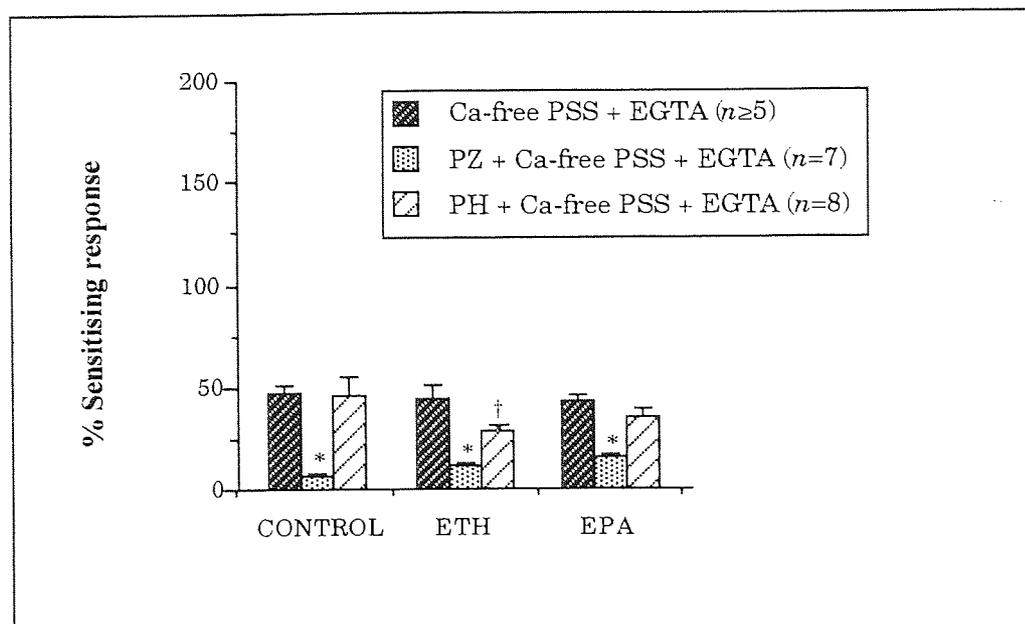


Figure 8.10. Effect of prazosin or phentolamine on the EGTA-resistant response to noradrenaline in the presence of EPA. The aorta preparation was exposed to prazosin (PZ; 5 nM) or phentolamine (PH; 1 μ M) in the washout PSS for 15 min prior to the addition EPA (50 μ M) or ethanol (ETH; 1%; the EPA vehicle). 30 min later, the PSS was replaced with calcium-free PSS containing 2 mM EGTA and EPA (50 μ M) or ETH (1%). After 2 min, noradrenaline (3 μ M) was added for a minimum period of 10 min. The maximal contraction (E_{max}) was measured, and responses are expressed as percentage of the sensitising response. Significant difference from corresponding control value (\dagger) or value in absence of antagonist (*). Values are mean \pm s.e. mean for n tissues ($n \geq 5$).

8.2.7. Effect of EPA on the response to $[Ca^{2+}]_e$ under depolarised conditions.

Vasorelaxation may be due to desensitisation of the contractile proteins to calcium (Somlyo & Himpens, 1989). To determine whether this effect might serve to explain the vasorelaxatory response observed to EPA in this tissue preparation, the final study in this investigation examined whether the sensitivity of the contractile filaments to $[Ca^{2+}]_i$ was altered by EPA. This can be studied by exposing the tissue to a nominally calcium-free high potassium solution (KPSS) and then increasing the calcium concentration of the KPSS. In the event that the contractile filaments are desensitised to calcium, then the response observed will be less than the usual response.

In these studies, KPSS was prepared as calcium-free, EGTA-containing PSS as for the experiments reported in the previous section, except that there was equimolar substitution of 60 mM KCl for NaCl. A sensitising response was elicited to 60 mM KCl instead of 3 μ M noradrenaline and after 30 min of washout, PSS was replaced with KPSS which remained exposed to the cells for a further 60 min. EPA (50 μ M) or ethanol (ETH, 1%; the EPA vehicle) was added for the final 30

min. Cumulative concentration-response curves to calcium were then constructed.

The results are shown in figure 8.11. The pD_2 value for the calcium-induced contraction in the presence of ETH was 7.0 ± 0.27 ($n=3$), which was not significantly different from the pD_2 value in the presence of EPA (6.7 ± 0.24 , $n=3$). The E_{max} values were not different. These results therefore demonstrated that the contractile filaments were not desensitised to calcium by EPA.

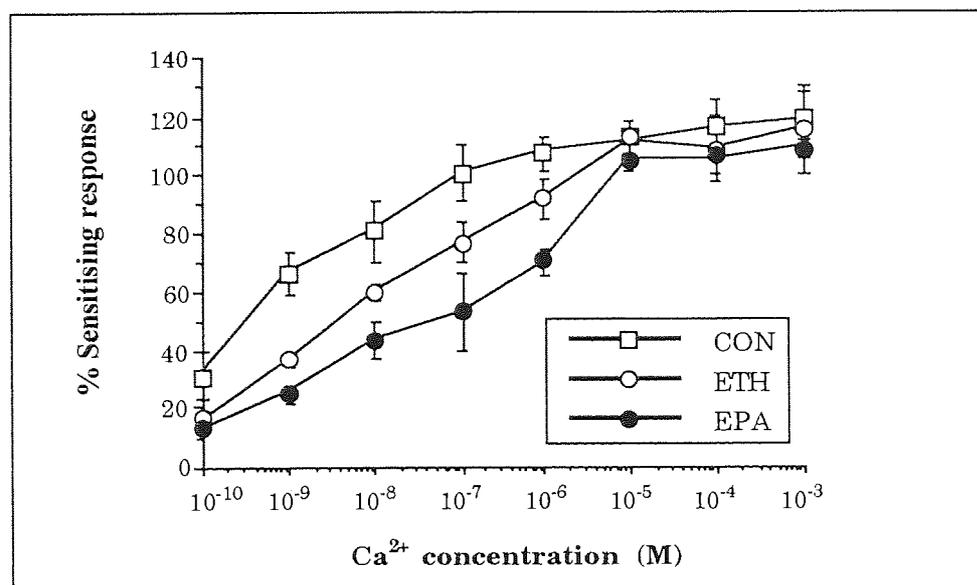


Figure 8.11. Effect of EPA on the response to extracellular calcium under depolarised conditions After 30 min of washout following the sensitising response to KCl (60 mM), PSS was replaced with calcium-free high potassium solution (KPSS) which remained exposed to the cells for a further 60 min. EPA (50 μ M) or ethanol (ETH, 1 %; the EPA vehicle) was added for the final 30 min. Cumulative concentration-response curves to calcium were then constructed. Responses are expressed as a percentage of the sensitising response to 60 mM KCl. All values are mean \pm s.e.mean for 3 experiments ($n=3$).

8.3. Discussion

It has previously been shown in this thesis, that EPA partially blocks the contraction of cells to the vasoactive agents, KCl and noradrenaline (chapter 4). As it was reported that relaxation of vascular smooth muscle may be a result of blockade of intracellular calcium release or a decrease of influx of extracellular calcium, or be due to desensitisation of the contractile apparatus within the cell to calcium (Somlyo & Himpens, 1989), subsequent studies examined the effect of EPA on these parameters. Influx of extracellular calcium can occur through voltage-dependent or receptor-operated calcium channels. It was found in chapter 5, that EPA significantly decreased calcium influx but that EPA was not associated simply with block of L-type channels, consistent with a previous suggestion by Hallaq *et al* (1992) that EPA did not bind at the same subunit site as the L-type dihydropyridine calcium channel blockers. Upon further investigation of the effect of EPA on calcium influx, it was discovered that EPA might reduce calcium influx through receptor-operated channels. This result supported a recent suggestion by Asano *et al* (1997), however, it failed to explain the result obtained in the fura-2 studies, in which EPA caused a transient increase in $[Ca^{2+}]_i$, in the absence of extracellular calcium. In an attempt to identify the reasons for this, and also to examine whether EPA reduced the release of intracellular calcium, the results obtained in chapter 6 showed that EPA increased inositol phosphate levels. It was suggested that this increase in inositol phosphate might cause the release of calcium from an inositol phosphate-dependent internal binding site, possibly that of an intracellular membrane, producing the transient increase in calcium observed in chapter 5. However, it was not clear at that stage, just how, if at all, the transient increase in calcium was associated with the decreased calcium influx observed in chapter 5. It was suggested that following the release of this calcium transient, a localised concentration of calcium would be generated that could activate many processes, including that to prevent calcium influx in the longer term, and so to block further cell contraction to noradrenaline or KCl, which was observed in the cell contraction studies in chapter 3.

The mechanism by which a localised concentration of calcium could prevent calcium influx in the longer term, and so to block further cell contraction to noradrenaline or KCl could be associated with activation of membrane-associated K^+ channels. Efflux of K^+ through these ion channels would hyperpolarise the cell membrane, which in turn would close voltage-operated calcium channels, thereby leading to cell relaxation.

The aim of the experiments reported in this chapter, was to confirm whether the effects of EPA observed on the cells were apparent in intact tissue

preparations and to determine whether relaxation of vascular smooth muscle was a result of desensitisation of the contractile apparatus within the cell to calcium.

Noradrenaline exerts a biphasic response in rat aorta (Godfraind & Kaba, 1972), the first phase mediated by an inositol phosphate mediated pathway, causing release of calcium from intracellular storage sites (Streb *et al*, 1983; Berridge & Irvine, 1989) and the second phase due to the influx of extracellular calcium (Benham & Tsien, 1987).

EPA and DHA have previously been shown to inhibit the contraction induced by noradrenaline or other α -adrenoceptor agonists in rat aortic rings, (Juan & Sametz, 1986; Engler *et al*, 1990; Engler, 1992b), so the objectives of the initial experiments described in this chapter was to confirm that EPA caused a reduction of the contractural response to noradrenaline in isolated, endothelium-denuded aortic rings and then to investigate whether the affect of EPA could be blocked by α -adrenoceptor antagonists.

Consistent with previous reports (Engler 1992b; Juan *et al*, 1987), it was shown that EPA decreased noradrenaline-induced contractures. The rightward shift in the concentration-response curve was accompanied by a reduction in the maximal response (E_{max}) of the tissue, consistent with previous studies (Juan *et al*, 1987). It has previously been suggested that the effect of EPA is not due to competition at the α -adrenoceptor level (Juan *et al*, 1987) and in support of this suggestion, examination of the effect of α_1 -adrenoceptor antagonist prazosin and the non-selective α_1 - and α_2 -antagonist phentolamine revealed that EPA enhanced the blocking effect of prazosin or phentolamine, indicated by the pD_2 values shown in table 8.3 which suggested that the relaxatory effect of EPA was not prevented by the antagonists. Therefore, consistent with a previous suggestion (Juan *et al*, 1987), it appeared that the effect of EPA occurred through a different mechanism than the α -adrenoceptor.

In view of the effect of EPA on the transient increase in calcium and also on the effect of inositol phosphate, a study was performed to investigate whether EPA affected the phasic contracture resulting from release of intracellular calcium.

Therefore, the objective of the next part of this study was to investigate the effect of α -adrenoceptor antagonists on the EGTA-resistant response in order to determine the effect of EPA upon the phasic part of the contractural response. Contractures produced in calcium-free PSS containing EGTA (ERR) have been widely used as an indication of the release of intracellular calcium (Cauvin & Malik, 1984) and in the present study, following a 2 min incubation of the aorta in Ca^{2+} -free EGTA PSS, the ERR response to noradrenaline was 34.66 ± 3.12 % of the control response in PSS. Similar results have previously been shown (Criddle *et al*, 1996; Godfraind *et al*, 1982; Cauvin & Malik, 1984), demonstrating that the phasic

component of the contractural response to noradrenaline in the rat aorta was due to the release of intracellular calcium. Also, the ERR elicited by noradrenaline was inhibited by prazosin, consistent with the ERR being mediated *via* the α_1 -adrenoceptor (Berridge & Irvine, 1989). Noradrenaline was still able to elicit an ERR in the presence of EPA, demonstrating that EPA did not affect the component of the contraction arising from the release of intracellular calcium. However, in chapter 5, EPA was shown to cause a transient release of calcium, but the results shown in the present chapter revealed that the transient contraction obtained in the tissue following the release of intracellular calcium, was no greater than the transient contraction obtained in the absence of EPA. This suggested that the transient release of calcium observed in chapter 5 was not associated with the intracellular release of calcium which caused the subsequent contraction of the tissue in the present contractural study. It was considered, however, that the noradrenaline concentration used in the contractural study may have been maximal in eliciting calcium release and subsequent contraction, which could have contributed to the lack of any extra contraction being observed in the presence of EPA. To confirm that the vasorelaxatory effect of EPA was not associated with the phasic contraction in the tissue, α_1 - and α_2 -adrenoceptor antagonists were used to block the ERR. In the event that EPA was not acting on the α -adrenoceptor pathway, then the effects of the α_1 - and α_2 -adrenoceptor antagonists would remain unchanged in either the presence or absence of EPA.

In the presence of EPA, prazosin did not block the noradrenaline-induced ERR as much as it did when EPA was not added. However, this effect was also observed when ethanol was added with the prazosin. There was no effect of EPA on the response to phentolamine. Therefore, these results demonstrated that the transient contraction to noradrenaline which is induced by α_1 -adrenoceptors and mediated by the release of intracellularly stored calcium, was unaffected by EPA.

The results therefore demonstrated that the effect of EPA to transiently increase intracellular calcium through inositol phosphate, as suggested in chapter 6, was unlikely to cause contraction of the tissue. These results were all consistent with the earlier results obtained in this chapter, which demonstrated that the effect of EPA occurred through a different mechanism than the α -adrenoceptor-linked, inositol phosphate system. Therefore, it appeared likely that the EPA-induced transient increase in intracellular calcium was associated with a mechanism which subsequently caused the cells of the tissue to relax.

These results therefore revealed that in the aorta of the rat, inactivation of postjunctional α_1 -adrenoceptors reduced the release of intracellular calcium and the vasorelaxatory effect of EPA was not to decrease the release of intracellular calcium, nor to affect antagonists which were effective in reducing the ERR.

It was previously shown in this report that EPA could cause an increase in inositol phosphate formation (see chapter 6) and also a transient rise in the level of $[Ca^{2+}]_i$ in the cells (see chapter 5) and yet mediate a decreased constrictor response to KCl or noradrenaline (chapter 4) and a decreased contractural response to noradrenaline (figure 8.2). It was considered therefore, that a possible effect of EPA may be linked to a decreased sensitivity of the contractile elements to calcium, as this has been shown to mediate a vasorelaxatory response (Somlyo & Himpens, 1989). Therefore, a final study was performed to determine whether the transient increase in calcium, which appeared not to increase the contractile status of the cells (chapter 4), nor the intact tissue, shown in the results in the present chapter, was due to a decreased sensitivity of the contractile filaments to calcium. To study this, the tissue was depolarised in a nominally calcium-free high potassium solution (KPSS) to ensure that voltage-operated calcium channels were open. Calcium was then added to the solution bathing the cells. If the sensitivity of the contractile machinery to calcium was normal, then the addition of extracellular calcium to the solution (KPSS) should produce a change in the responses obtained. However, the contractile concentration-response curve was not significantly shifted rightward compared to the control response measured in the presence of ethanol, suggesting that the contractile filaments were not desensitised to calcium by EPA.

In conclusion therefore, consistent with previous reports (Engler 1992b; Juan *et al*, 1987), it was shown that EPA decreased noradrenaline-induced contractures. This effect was not due to competition at the α -adrenoceptor level, in support of a previous suggestion (Juan *et al*, 1987) as the relaxatory effect of EPA was not prevented by α -adrenoceptor antagonists. The ERR elicited by noradrenaline was inhibited by prazosin, consistent with the ERR being mediated *via* the α_1 -adrenoceptor (Berridge & Irvine, 1989). Noradrenaline still elicited an ERR in the presence of EPA, demonstrating that EPA did not affect the phasic component of the contraction arising from the release of intracellular calcium. Although EPA was shown to cause a transient release of calcium in chapter 5, the transient contraction obtained in the tissue following the release of intracellular calcium, was no greater than the transient contraction obtained in the absence of EPA, suggesting that the transient release of calcium observed in chapter 5 was not associated with contraction.

The results also demonstrated that the contractile filaments were not desensitised to calcium by EPA, thereby increasing the likelihood that the EPA-induced transient increase in intracellular calcium was associated with a mechanism, possibly that of potassium efflux, to subsequently caused the cells of the tissue to relax.

Chapter 9

General discussion

In the present study, the effect of the n-3 polyunsaturated fatty acid, cis-5,8,11,14,17-eicosapentaenoic acid (EPA) was investigated upon the contractile mechanisms linked to both calcium influx and the mobilisation of intracellular calcium in vascular smooth muscle. To perform this investigation a suitable system of vascular smooth muscle was identified and subsequently developed for use. The vascular smooth muscle used was obtained from the conducting artery, the aorta. Although the *tunica media* of the aorta contains more elastic fibres and less smooth muscle than muscular arteries (Tortora & Anagnostakos, 1991), the contractile mechanisms linked to both calcium influx and the mobilisation of intracellular calcium are present in the aorta.

An attempt can be made to relate cells *in vitro* to their tissue of origin if the cells express their normal functions. Therefore, primary cultured aortic smooth muscle cells (AOCs) which expressed their normal functions were used. The culture was allowed to reach confluency prior to experimentation so that the closest morphological resemblance to the parent tissue was achieved. Furthermore, subject to certain limitations, the cells retained their ability to respond to KCl depolarisation and noradrenaline, demonstrating that the AOCs expressed functional α -adrenoceptors and L-type calcium channels. The main limitation of the system is that there may be differences in the sensitivity of the responses achieved in the isolated cells compared with the aortic tissue *in vivo*. Nevertheless, the system has the potential to allow direct assessment of cellular mechanics and pharmacology and avoids the interpretative complexities associated with multicellular preparations.

Epidemiological studies on Greenland Eskimos since the late 1940s revealed a reduced prevalence of cardiovascular-related diseases in this population (Ehrstrom, 1951; Kromann & Green, 1980) and through experimental and clinical intervention trials, subsequent attempts to elucidate the reasons for this demonstrated that diet, especially fish and other marine animal consumption (Bang *et al*, 1976) is important in lowering the rate of myocardial infarction (Dyerberg & Bjerregaard, 1987; Burr, *et al*, 1989; Albert *et al*, 1998). This fish-related beneficial effect has been attributed in recent clinical investigations (Goode *et al*, 1997; Horrobin, 1993), to a mechanism associated with improving vascular function. In support of these conclusions which describe an effect of n-3 PUFAs on the vascular system, the results in the present study demonstrated that by blocking calcium influx induced by vasoactive agents, EPA could improve

vascular function. Such an effect supports many clinical trials and *in vitro* investigations (Shimokawa & Vanhoutte, 1989; Juan & Sametz, 1986; Juan *et al.*, 1987; Lockette *et al.*, 1982; Bregestovski *et al.*, 1989; Hallaq *et al.*, 1990; 1992; Smith *et al.*, 1992; Engler *et al.*, 1990; Engler, 1990; 1992a; 1992b; 1994; Pepe *et al.*, 1994; Mano *et al.*, 1995; Bretherton *et al.*, 1995; 1996a; 1996b; Asano *et al.*, 1997; Gardener *et al.*, 1998) particularly as the effect described from the present results is endothelium-independent. This does not dispute previous reports which demonstrated an endothelium-dependent effect of n-3 PUFAs (Shimokawa & Vanhoutte, 1989; Shimokawa *et al.*, 1987; Schini *et al.*, 1993; Joly *et al.*, 1995; Furchgott & Vanhoutte, 1989), but merely demonstrates that an intact endothelium is not necessarily a prerequisite for a beneficial effect of n-3 PUFAs on vascular function.

Previously, the beneficial vascular effect was shown to be a relaxatory effect and was attributed to a number of mechanisms. One proposed mechanism was that the n-3 fatty acids decrease vascular reactivity to constrictor substances (Juan & Sametz, 1986; Yin *et al.*, 1991; Mano *et al.*, 1995), leading to a decrease in blood pressure (Lockette *et al.*, 1982; Norris *et al.*, 1986; Knapp & Fitzgerald, 1989; McMillan, 1989; refer to Bønaa *et al.*, 1990; Mano *et al.*, 1995; Joly *et al.*, 1995).

However, investigations on blood pressure have not always demonstrated a beneficial effect of n-3 PUFAs (Chin *et al.*, 1993), leading to the suggestion that the presence of a hypotensive effect of fish oils is only observed in subjects with high blood pressure or hypercholesterolaemia. Contrary to this suggestion, results obtained in the present study suggested that EPA mediated an effect on vascular smooth muscle cells taken from normotensive subjects, since the AOCs used in the present investigation were prepared from the aortae of Wistar rats, a normotensive strain. A vasorelaxatory response of n-3 fatty acids in AOCs from these normotensive animals was demonstrated, as EPA (50 μM) reduced the contraction of AOCs to KCl (60 mM) or noradrenaline (1 μM), clearly suggesting that the hypotensive effect of fish oils is observed in normotensive subjects.

Notwithstanding the evidence that fish oils mediate a hypotensive effect, the mechanism of action remains inconclusive. However, it has been suggested that the effect of n-3 PUFAs on the vasculature is associated with blocking calcium influx into smooth muscle cells (Hallaq *et al.*, 1990; 1992; Pepe *et al.*, 1994; Asano *et al.*, 1997; Gardener *et al.*, 1998). Depolarisation of vascular smooth muscle induced by high concentrations of potassium causes contraction (Droogmans *et al.*, 1977) associated with increased calcium influx (van Breeman *et al.*, 1972), leading to increased intracellular calcium ion concentration ($[\text{Ca}^{2+}]_i$). The KCl-induced contractile response of vascular smooth muscle cells is mediated *via* an increase in $[\text{Ca}^{2+}]_i$ through L-type calcium channels. So, in the present study, the effect of EPA upon the KCl-induced contractile response of vascular smooth muscle cells

was examined to investigate whether the effect of n-3 PUFAs on the vasculature is associated with blocking calcium influx into smooth muscle cells. In chapter 4, it was shown that EPA (50 μM) reduced but did not completely block the change in the shape of AOCs after KCl (60 mM) suggesting an effect of EPA to partly block calcium influx through L-type voltage-dependent calcium channels, in support of the conclusions proposed by Hallaq *et al*, 1990; 1992 and Pepe *et al*, 1994. However, in the present study, the subsequent effect of EPA on calcium influx, shown in chapter 5, revealed that although EPA (50 μM) significantly decreased calcium influx into the AOCs when exposed to low (≤ 10 mM) concentrations of KCl, no such reduction was observed in the higher, depolarising concentrations of KCl (≥ 20 mM). Moreover, a further comparative study in the present investigation demonstrated that the effect of EPA was not consistent with that of an L-type calcium channel blocker, nifedipine on calcium influx. This latter result supported a previous suggestion by Hallaq *et al* (1992) that EPA does not bind at the same subunit site as the L-type dihydropyridine calcium channel blockers. Therefore, the results of the present study showed that the mechanism of action of EPA was associated with blocking calcium influx into smooth muscle cells, but that the effect was not simply to block L-type voltage-dependent calcium channels.

Calcium entry in vascular smooth muscle is also mediated through receptor-operated calcium channels (ROCs) (Bolton, 1979; van Breeman, 1989; Somlyo & Somlyo, 1970). Noradrenaline mediates an increase in $[\text{Ca}^{2+}]_i$ through ROCs, leading to the tonic part of the noradrenaline-induced contracture in vascular smooth muscle. So, in the present study, the effect of EPA upon the noradrenaline-induced contractile response of vascular smooth muscle cells was examined to investigate whether the effect of n-3 PUFAs on the vasculature is associated with blocking calcium influx into smooth muscle cells through ROCs, as proposed by Asano *et al* (1997). EPA (50 μM) reduced but did not completely block cell contraction after noradrenaline (1 μM) (chapter 4) and furthermore, inhibited noradrenaline-induced calcium influx (chapter 5), suggesting an effect of EPA to block calcium influx through ROCs. Moreover, the observed effect of EPA to block calcium influx was consistent with the subsequent results shown in the present study, whereupon EPA decreased noradrenaline-induced $[\text{Ca}^{2+}]_i$ (chapter 5). This provided further evidence for the likelihood that EPA blocked calcium influx through ROCs. However, final confirmation of this mechanism of action of EPA was provided (chapter 8) when EPA was shown to reduce the noradrenaline-induced contractures of intact aortic tissue (Juan & Sametz, 1986; Engler *et al*, 1990; Engler, 1992a). Overall, therefore, these results demonstrated that the mechanism of action of EPA was, at least in part, consistent with an action to block calcium influx through ROC, supporting a recent suggestion by Asano *et al* (1997).

However, it appeared unlikely that the mechanism of action of EPA could simply be attributed to blocking calcium influx through ROC as this effect does not explain some previously reported observations.

In 1992, Engler examined whether the reduction of noradrenaline-induced contractions in rat aortic tissues by DHA or EPA was related to the inhibition of intracellular calcium mobilisation (Engler, 1992b). Engler reported that DHA or EPA were able to reduce the noradrenaline-induced contractile response in calcium-free, EGTA containing buffer, an effect which cannot be attributed to blocking calcium influx through calcium channels as there was no calcium present to be blocked from influxing. Noradrenaline induces a biphasic contractural response in aortic tissue, in which the slower, tonic phase is due to influx of calcium through ROC, but the fast, phasic part of the response arises from the release of intracellularly stored calcium. Engler (1992b) therefore suggested a role of n-3 fatty acids in intracellular calcium regulation and in particular, that DHA or EPA decreased $[Ca^{2+}]_i$ by an effect on intracellular calcium mobilisation. Therefore, in the present study, the effect of EPA on intracellular calcium regulation was investigated to determine whether the mechanism of action of EPA was indeed multifactorial. It was shown that EPA caused a transient increase in $[Ca^{2+}]_i$, apparent in the absence of extracellular calcium, consistent with a previous report (Sumida *et al*, 1993). This suggested that the mechanism of action of EPA on vascular smooth muscle may indeed be multifactorial.

To elucidate the mechanism by which EPA caused the transient increase in $[Ca^{2+}]_i$, it was considered that a transient increase in $[Ca^{2+}]_i$ can occur following inositol 1,4,5-trisphosphate-induced releases of calcium from intracellular storage sites including the sarcoplasmic reticulum (Berridge & Irvine, 1989). However, an increase in $[Ca^{2+}]_i$ is usually associated with subsequent cell contraction and not the relaxation normally assigned to the effect of n-3 PUFAs. Furthermore, in chapter 4 of the present study, the effect of EPA upon the KCl- and noradrenaline-induced contractile response of vascular smooth muscle cells did not reveal an effect of EPA to cause contraction of the AOCs. However, a review by Van Breeman & Saida (1989) reported that a portion of the sarcoplasmic reticulum is located close to the inner surface of the plasmalemma, referred to as *superficial* sarcoplasmic reticulum. Sarcoplasmic reticulum lying in the interior of the smooth muscle cells was referred to as *deep* sarcoplasmic reticulum. They described this two-compartment model to explain the differences in the release of stored calcium in response to various stimuli. Consistently, it has previously been shown that $[Ca^{2+}]_i$ is not always effectively utilised to phosphorylate the myosin light chain and so mediate cell contraction in the event that $[Ca^{2+}]_i$ is released from the *superficial* sarcoplasmic reticulum (Kitajima *et al*, 1996; Abe *et al*, 1995).

It can be concluded therefore, that calcium released from the *deep* sarcoplasmic reticulum is likely to cause cell contraction, whereas calcium released from the *superficial* sarcoplasmic reticulum is not. It is suggested therefore, that the results in the present study which showed that EPA caused a transient increase in $[Ca^{2+}]_i$ arise from EPA-induced calcium release from the *superficial* sarcoplasmic reticulum. This would explain the results obtained in chapter 4 of the present study, whereby EPA did not induce contraction of the AOCs. Furthermore, the phasic contraction obtained in the tissue following the release of intracellular calcium (chapter 8) was no greater than the phasic contraction obtained in the absence of EPA, confirming that the transient release of calcium observed in chapter 5 was not associated with contraction.

Recently, Berridge (1993b) reported that there are indeed different storage compartments for calcium and these can be identified on the basis of the receptor-coupled channel they possess, *i.e.* *myo*-inositol-1,4,5-trisphosphate or ryanodine receptor channels. The *superficial* sarcoplasmic reticulum possess receptors for *myo*-inositol-1,4,5-trisphosphate (Berridge; 1993b).

Therefore, to elucidate the mechanism by which EPA caused the transient increase in $[Ca^{2+}]_i$, the effect of EPA upon inositol 1,4,5-trisphosphate-induced releases of calcium from intracellular storage sites was investigated. In chapter 6, it was shown that EPA increased inositol phosphate levels, therefore, it is suggested that in the present study, the transient increase in $[Ca^{2+}]_i$ was associated with calcium release from the *superficial* sarcoplasmic reticulum. This effect confirms, therefore, that the mechanism of action of EPA on calcium regulation is indeed multifactorial.

It has previously been reported, however, that the lack of contraction following an increase in $[Ca^{2+}]_i$ may occur if the contractile filaments are desensitised to calcium. This can be investigated by bathing the tissue in a calcium-free solution containing a high concentration of potassium to activate the voltage-dependent calcium channels. Upon addition of calcium to the bathing solution, the tissue would be expected to contract due to the influx of calcium into the tissue. However, in the event that the contractile filaments were desensitised, then the addition of calcium would not produce the resultant contraction. The investigation was performed in the present study (chapter 8) and the results showed that EPA did not cause a rightward shift in the concentration-response curve to calcium, suggesting that EPA did not desensitise the contractile filaments to calcium. However, EPA decreased noradrenaline-induced contractures in aortic tissue, shown in chapter 8, which supports the suggestion that the action of EPA is associated with a vasorelaxatory response to agonist-induced effects in the aorta (Juan & Sametz, 1986; Engler, 1989; Yin *et al.*, 1991; Lervang *et al.*, 1993).

It was shown that the contractile filaments were not desensitised to calcium

by EPA (chapter 8), so it is suggested that the transient increase in $[Ca^{2+}]_i$ is associated with calcium release from an internal binding site, possible that of the *superficial* sarcoplasmic reticulum and that this increase in $[Ca^{2+}]_i$ is associated with the "down-regulation" of membrane-bound receptor-signalling pathways. The release of compartmentalised calcium in this way may locally activate membrane ion channels, ion pumps, exchange carriers and enzymes (Abe *et al*, 1995). Thus, calcium influx may be prevented in the longer term, by an action on receptor-operated calcium channels, consistent with the results shown in chapter 5, to subsequently block further cell contraction to noradrenaline, consistent with a previous report by Asano *et al* (1997) or by an action on voltage-dependent calcium channels, to subsequently block further cell contraction to KCl, as shown in chapter 5.

Calcium entry through the voltage-dependent calcium channels may be inhibited by the activation of membrane-associated potassium channels (K^+) which hyperpolarise the plasma membrane, moving the membrane potential farther from the activation range for calcium channels. In so doing, calcium influx could subsequently be reduced, so this was proposed as a possible explanation of the reduced calcium influx results obtained to EPA, in chapter 5. Consistently, it was demonstrated in chapter 7 that EPA (50 μ M) induced ^{86}Rb efflux from the AOCs, suggesting that the vasorelaxatory effect of EPA was mediated partly, by an effect on K^+ channels. In support of this result, it has previously been shown that n-6 fatty acids (Ordway *et al*, 1989; Kim & Clapham, 1989) and n-3 fatty acids (Bregestovski *et al*, 1989; Smith *et al*, 1992, Asano *et al*, 1997; Gardener *et al*, 1998) have a direct effect on potassium ion channels situated in vascular membranes, activation of which results in membrane stabilisation towards the non-contractile state, by drawing the membrane potential closer to the potassium equilibrium potential (Halliday *et al*, 1995). It is suggested therefore, that this could explain the effect of EPA to reduce calcium influx induced by low concentrations of KCl in chapter 5 and to block cell contraction to KCl in chapter 4.

Conclusions

In conclusion, therefore, it was shown in this thesis, that EPA partially blocked the contraction of cells to the vasoactive agents, KCl and noradrenaline (chapter 4). This effect was subsequently found to be associated not simply with a block of calcium influx through L-type channels (chapter 5), consistent with a previous suggestion by Hallaq *et al* (1992) but with a reduction of calcium influx through receptor-operated channels (chapter 5), supporting a recent suggestion by Asano *et al* (1997). This could account for the results observed with EPA to partially block the contraction of cells to noradrenaline (chapter 4) and to decrease noradrenaline-induced contractures (chapter 8).

To elucidate the observed effect of EPA on cell contractions to KCl (chapter 4) it was found that EPA caused a transient increase in $[Ca^{2+}]_i$ in the absence of extracellular calcium (chapter 5). This was resolved in chapter 6 when it was shown that EPA increased inositol phosphate formation which, it is suggested, caused the release of calcium from an inositol phosphate-dependent internal binding site, possibly that of an intracellular membrane or *superficial* sarcoplasmic reticulum, producing the transient increase in $[Ca^{2+}]_i$. It was shown that the contractile filaments were not desensitised to calcium by EPA (chapter 8), so it is suggested that the transient increase in $[Ca^{2+}]_i$ subsequently blocks further cell contraction to noradrenaline or KCl, by activating membrane-associated K^+ channels (chapter 7). Activation of K^+ channels induces the cellular efflux of potassium ions, thereby hyperpolarising the plasma membrane and moving the membrane potential farther from the activation range for calcium channels. This would prevent calcium influx in the longer term and could explain the effect of EPA to block cell contraction to KCl, shown in chapter 4.

Although the investigations in this thesis allowed for conclusions to be drawn regarding the mechanism of action of EPA, and showed that a multidisciplinary strategy can be used to obtain an integrative study, it should be noted that the design of a study in this way often leads to conflicting results, which may occur from differences in the methodologies utilised. It therefore can become difficult to compare the results accurately. On this basis, therefore, it is recommended that any future investigations into the effect of n-3 fatty acids upon the vasculature, be undertaken using a strategy which allows for only the minimum number of techniques to be used. In this way, variables could be increased to include wider choice of fatty acids, increased range of fatty acid concentrations and incubation times and increased choice of positive and negative controls.

Future Work

It is suggested that future work could be undertaken to investigate other fatty acids, an increased range of fatty acid concentrations, a greater range of experimental incubation times and an increased choice of positive and negative controls but perhaps to limit the techniques utilised in the studies. This should make the resultant conclusions more easily identifiable.

It is also suggested that future work could be undertaken to demonstrate clearly, the effect of EPA and other n-3 fatty acids upon membrane potassium permeability as the results in the present study which examined ^{86}Rb efflux should be confirmed with patch-clamp studies. This technique has previously been used to demonstrate that fatty acids directly activate potassium channels in smooth muscle cells (Ordway *et al* 1989) although the type of channel activated is not always clarified (Asano *et al*;1997), but has been suggested to be that of the K_{ATP} channel in rat cardiac myocytes (Muller *et al*, 1992;Gardener *et al*; 1998) or a voltage-sensitive, delayed rectifier K^+ current (Gardener *et al*; 1998), or a large conductance Ca^{2+} -activated K^+ channel (Gardener *et al*; 1998).

Further work could also be undertaken to investigate the intracellular source of the transient increase in $[\text{Ca}^{2+}]_i$. Improvements in imaging technology could identify the calcium source and then identify whether the increase was localised near to the plasma membrane, clarifying whether the activation of K^+ channels was likely.

It is also suggested that further work could extend to comparing the effect of endothelium-dependent and endothelium-independent effects of the n-3 fatty acids to establish whether the mechanism of action can be generalised or whether the reported therapeutic benefits in cells and tissues with and without endothelium are summative.

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Appendix

Appendix 1

Composition of Minimum Essential Medium D-val Medium

<i>Component</i>	<i>mg l⁻¹</i>
Inorganic salts	
CaCl ₂ •2H ₂ O	264.00
KCl	400.00
MgSO ₄ •7H ₂ O	200.00
NaCl	6800.00
NaHCO ₃	2200.00
NaH ₂ PO ₄ •2H ₂ O	158.00
Other components	
D-Glucose	1000.00
Phenol Red	10.00
Amino acids	
L-arginine•HCl	126.40
L-cystine	24.00
L-glutamine	292.00
L-histidine HCl•H ₂ O	41.90
L-isoleucine	52.40
L-leucine	52.50
L-lysine•HCl	73.10
L-methionine	14.90
L-phenylalanine	33.00
L-threonine	47.60
L-tryptophan	10.20
L-tyrosine	36.20
D-valine	92.00
Vitamins	
D-Ca pantothenate	1.00
Choline chloride	1.00
Folic acid	1.00
i-inositol	2.00
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.10
Thiamine HCl	1.00

Appendix 2

Calcium influx into cells was calculated according to the volume of solution which bathed the cells throughout the experiment, the concentration of calcium contained in this solution and the activity of the tracer added to the solution.

In each experiment, 1 ml PSS containing 1 mM CaCl₂ was added to each well of cells. 2 μCi ⁴⁵Ca was added to each well, unless otherwise specified. Therefore, it was considered appropriate that in each well, 2 μCi ⁴⁵Ca represented 1 μmol calcium. The radioactivity in standards of 1 ml PSS containing 2 μCi ⁴⁵Ca was measured. Radioactivity in the cell lysate from well n was measured as counts per minute (cpm) and expressed as moles calcium. Values were corrected for cell protein, determined using the methodology described in 2.23.2 and calcium influx was expressed according to equation 7 as nmol mg⁻¹ protein.

$\text{Calcium influx}_n = \frac{\text{radioactivity in cell lysate}_n \times \frac{\text{specific radioactivity of incubation solution}}{\text{average radioactivity for standards}}}{\text{cell protein}_n}$	<i>Equation 7</i>
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Appendix 3

The ⁸⁶Rb efflux curves were obtained by plotting the efflux rate coefficients against time. The rate coefficient, a measure of ⁸⁶Rb efflux from aortic cells, was estimated according to equation 8, described by Lorenzi (1994):

$\text{Rate Coefficient}_i = \frac{\text{count in interval}_i / \text{count remaining}}{\text{interval duration}}$	<i>Equation 8</i>
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where *i* refers to the interval number. Interval duration was in mins.

Appendix 4

The author is associated with the following list of publications:

BRETHERTON, N., SMITH, J.W., WILSON, K.A. (1996). The effect of eicosapentaenoic acid upon contractures of isolated rat aortic rings pre-treated with α -adrenoceptor antagonists. *Br. J. Pharmacol.*, **119**, 91P.

BRETHERTON, N., RICHARDSON, A.G., SMITH, J.W., WILSON, K.A. (1996). The effect of eicosapentaenoic acid upon contractures of isolated aortic rings and inositol phosphate production in cultured aortic cells from the rat. *Br. J. Pharmacol.*, **118**, 89P.

BRETHERTON, N., RICHARDSON, A.G., SMITH, J.W., WILSON, K.A. (1995). The effect of eicosapentaenoic acid upon contractures of isolated aortic rings and calcium influx into cultured aortic cells from the rat. *Br. J. Pharmacol.*, **116**, 150P.

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