# α-ADRENOCEPTORS AND CALCIUM ACTIVATION MECHANISMS IN VASCULAR MUSCLE

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# α-Adrenoceptors and Calcium Activation Mechanisms in Vascular Muscle.

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## SUMMARY.

A pharmacological study was made of α-adrenoceptor mediated calcium activation mechanisms in the vascular muscle of the rat. The portal vein was examined for the presence of postjunctional α-adrenoceptors. The results did not provide evidence for the existence of two subtypes of α-adrenoceptor in this vessel.

In the pithed rat, pressor responses to continuous infusions of phenylephrine were resistant to diltiazem while those to UK-14,304 were attenuated. Pretreatment with prazosin or with corynanthine revealed a diltiazem sensitive component of the pressor response of the pithed rat to cirazoline. Yohimbine had no significant effect on the pressor response to cirazoline following treatment with phenoxybenzamine, but, after prazosin, diltiazem attenuated the response to the highest three doses of cirazoline tested.

α-adrenoceptor mediated vasoconstrictor responses of the rat aorta were examined. Pretreatment with either phenoxybenzamine, benextramine, prazosin or corynanthine, at concentrations that had little effect on the response in the presence of calcium, significantly reduced the EGTA resistant responses. Prazosin had a greater effect on the initial, transient peak than on the second, sustained phase of the EGTA resistant response to noradrenaline. Following treatment with noncompetitive antagonists, the maximum slow, sustained phase of the calcium present response to phenylephrine was reduced significantly more than the fast phase of the response. EGTA resistant responses of the rat aorta to a range of agonists were compared with the parameters which defined the agonists. No conclusive association could be found between EGTA resistant responses and agonist affinity or efficacy. The maximum for the fast phase of the response to UK-14,304 and to amidephrine was significantly greater in endothelium denuded tissues compared with tissues with intact endothelia. Similarly, the $EC_{50}$ of the curve for the fast phase of the response to phenylephrine was significantly shifted to the left.

The results were discussed in relation to drug-receptor interactions.

Key words: α-adrenoceptors vascular muscle calcium α-adrenoceptor agonist

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Finally, I would like to begin this account of my work with a quotation from Sherlock Holmes, in 'A study in scarlet' by Sir Arthur Conan Doyle: "In solving a problem, the grand thing is to be able to reason backward."

## 2. CONTENTS

## 3. LIST OF FIGURES.

## 4. LIST OF TABLES.

## 5.INTRODUCTION TO THE THESIS.

α-adrenoceptors on vascular muscle have been subdivided into postsynaptic, $\alpha_1$-, which have been said to be excitatory, and presynaptic, $\alpha_2$-, which have been considered autoinhibitory (Langer 1974). In 1979, Drew and Whiting provided evidence that the pressor responses of the pithed rat and the anaesthetised cat to noradrenaline, and to phenylephrine, could be described in terms of a mixed population of postjunctional α-adrenoceptors. Timmermans and Van Zwieten (1980a) later concluded that the postjunctional α-adrenoceptors in the pithed rat could be subclassified as $\alpha_1$- and $\alpha_2$-. In 1983, Hicks reported that the portal vein of the rat supports a mixed population of postjunctional α-adrenoceptors whereby the $\alpha_2$- subtype were responsible for changes in phasic activity and were particularly sensitive to changes in the extracellular calcium concentration, whereas the $\alpha_1$-adrenoceptor mediated response effected changes in tone (Hicks 1983). It was considered that the portal vein of the rat could be a convenient model with which to examine the functional differences between the receptor subtypes, and was examined for the presence of postjunctional $\alpha_2$-adrenoceptors with this in mind.

There has been much interest in the difference in sensitivity to calcium channel antagonists of the two postjunctional α-adrenoceptor subtypes (e.g. Van Meel, DeJonge, Kalkman, Wilffert, Timmermans and Van Zwieten 1981a and b, Langer and Shepperson 1981, Cauvin, Loutzenhiser, Hwang and Van Breemen 1982). It was therefore considered worthwhile to examine the pressor responses of the pithed rat to α-adreno-

ceptor agonists with or without calcium channel antagonists, firstly to examine a difference in the time course of the $\alpha_1$- and $\alpha_2$-adrenoceptor mediated responses. The reason for this was based on *in vitro* observations, that the response of the rabbit aorta to noradrenaline is biphasic, whereby there is an initial, rapid phase that may be attributed to the release of intracellular calcium (Godfraind and Kaba 1972). The pressor responses of the pithed rat to $\alpha_1$-adrenoceptor agonists have been shown (Timmermans and Van Zwieten 1980) to be rapid and transient, while the pressor responses to $\alpha_2$-adrenoceptor agonists occur more slowly. It was considered that if there were any relationship between the *in vitro* and the *in vivo* responses to α-adrenoceptor agonists, as described above, then it is possible that the pressor response of the pithed rat to $\alpha_1$-adrenoceptor agonists could be insensitive to calcium channel antagonism because of the time course of the response; rather than the α-adrenoceptor subtype activated.

The second question addressed in the pithed rat concerned the receptor reserve of the preparation to $\alpha_1$-adrenoceptor agonists. Ruffolo and co-workers (1984) demonstrated that the pressor response of the pithed rat to cirazoline is resistant to the calcium channel antagonist diltiazem, but following pretreatment with phenoxybenzamine, a diltiazem sensitive component of the response was exposed. These workers suggested that their observations could be explained in terms of a receptor reserve of the pithed rat to cirazoline, whereby the receptor reserve could "buffer" the effect of a calcium channel antagonist; an effect not

possible in a system lacking spare receptors (Ruffolo, Morgan and Messick 1984). It was considered worthwhile to repeat the observations of Ruffolo *et al* (1984), that phenoxybenzamine exposes a diltiazem sensitive component of the pithed rat to cirazoline, and extend them to consider the competitive antagonists prazosin and corynanthine. The rationale behind this was that previous work has shown that prazosin selectively inhibits the fast phase of the response of the isolated rat aorta to noradrenaline; an effect not seen with corynanthine (Downing, Wilson and Wilson 1983). The fast phase may be attributed to the release of intracellular calcium (Godfraind and Kaba 1972). It was suggested that the selective effect of prazosin may have been due to the slow rate of dissociation of the antagonist from adrenoceptors, thereby reducing the rate of rise of receptor occupancy of the agonist (Downing, Wilson and Wilson 1985). Therefore, it was considered that phenoxybenzamine could have had a selective effect on the intracellular calcium release component of the pressor response of the pithed rat to cirazoline, due to its slow rate of dissociation from the receptors. If this were true, then it would also be expected to be true of prazosin, but not of corynanthine.

The next step in the investigation was to examine in more detail the influence of agonists and antagonists on $\alpha$-adrenoceptor mediated responses. It was considered that this would best be achieved using an *in vitro* system, but it followed that $\alpha_2$-adrenoceptor mediated responses could no longer be studied, for practical reasons. The

vasoconstrictor responses of the rat aorta to $\alpha_1$-adrenoceptor agonists in the presence and absence of extracellular calcium were examined for the effect of the non-competitive antagonists phenoxybenzamine and benextramine, and the competitive antagonists prazosin and corynanthine on the α-adrenoceptor mediated intracellular and extracellular calcium components of the response. The inter-relationship between the component of the $\alpha_1$-adrenoceptor mediated response that may be attributed to intracellular calcium release, and the sensitivity of the total response to calcium channel antagonists was also examined. The purpose of the *in vitro* experiments was to look for a possible explanation for the *in vivo* observation that both prazosin and corynanthine, as well as phenoxybenzamine exposed a diltiazem sensitive component of the pithed rat to cirazoline.

To examine further the hypothesis of Downing *et al* (1985) that the ability of an antagonist to inhibit α-adrenoceptor mediated intracellular calcium release in vascular muscle is related to the dissociation rate of the antagonist, it was considered that if this is true, then it should also be true that the ability of an agonist to cause the release of intracellular calcium is related to the rate of rise of agonist receptor occupation. Therefore, the *in vitro* investigations were extended to evaluate the relationship between agonist affinity, efficacy, potency, and intrinsic activity, and the component of the agonist mediated response that may be attributed to intracellular calcium release.

Finally, since the response of a tissue to an agonist is

as much dependent on the ability of the tissue to translate the stimulus into a response, as it is on the properties of the stimulant, an investigation into the effect of the vascular endothelium on the responsiveness of the rat aorta to α-adrenoceptor agonists was conducted. The purpose of the study was to ascertain whether a tissue related property could influence the ability of an agonist to initiate as well as to sustain a response to an agonist.

The results have been discussed in terms of the composite nature of drug-receptor interactions, and the consequent mobilisation of calcium sources for contraction.

## 6. GENERAL INTRODUCTION

### 6.1. Historical background to receptor subclassification.

The early work of Dale on neurotransmitters (Dale 1906) laid the foundation for the concept of receptors. In a series of experiments studying the effect of ergot alkaloids on the effects of adrenaline and sympathetic nervous stimulation on a range of organs, he proposed that different effects of the alkaloids could be explained in terms of different active principles on the tissues. This concept was developed further by Langley who, in 1909 described two types of "atom-groups"; receptive and fundamental. The receptive atom-groups were said to be localised in the nerve endings of differentiated cells. He suggested that combination of a chemical substance with such an atom-group could cause changes in the whole molecule that were associated with its function, or alternatively, that combination of a chemical substance with a fundamental atom-group could cause a serious alteration of the molecule; presumably an inhibitory combination (Langley 1909).

Barger and Dale (1910) questioned Langley's concept of specific chemical receptors. While they recognised the existence of pairs of bases eg. atropine and pilocarpine where one stimulates and the other paralyses, they felt that preferential stimulation by a certain group of substances may have reflected the ease with which such substances could reach the site of action. However the concept of receptors remained and in 1948, Ahlquist classified adrenotropic receptors into two types. He tested a series of six sympathomimetic amines on various physiological systems and

suggested the existence of two types of receptor, based on the differential sensitivity of the preparations to the agonists tested. The two distinct types of receptor were termed α and β.

The same method of classification was applied in 1967 by Lands, Arnold, McAuliff, Luduena and Brown. Considering the relative potency of a series of sympathomimetic amines in several experimental systems, it was concluded that there must exist two subtypes of β-adrenoceptor, which were named $\beta_1$- and $\beta_2$-.

The subclassification of α-adrenoceptors was not made until 1974 when Langer, in a review of neuronal and extraneuronal uptake mechanisms, described an hypothesis in which the noradrenaline released by stimulation, once it reaches a threshold concentration in the synaptic gap, would activate presynaptic α-adrenoceptors triggering a negative feedback mechanism that would inhibit further release of the transmitter. He went on to suggest that the postsynaptic α-receptor that mediates the response of the effector organ should be referred to as $\alpha_1$- while the presynaptic α-receptor that regulates transmitter release should be called $\alpha_2$- (Langer 1974). This negative feedback hypothesis is generally accepted, but has been questioned recently by Kalsner and Quillan (1984). They studied the effect of the $\alpha_2$-adrenoceptor antagonist yohimbine on stimulation induced tritium overflow of the guinea-pig, rabbit and rat atria, guinea-pig vas deferens and ureter, rabbit ear artery and rat spleen. They reasoned that if yohimbine interrupted a negative feedback system, then yohimbine-induced increases

in the transmitter efflux associated with field stimulation should show a predictable pattern. Moreover, increases in efflux should be proportional to the level of ongoing feedback in the absence of yohimbine. However, these workers presented data that did not support the negative feedback hypothesis. They suggested that presynaptic antagonists interferred with the process of inactivation of the calcium gating mechanism mediated by the outward conductance of potassium ions (Kalsner and Quillan 1984). Thus it was shown that prejunctional $\alpha_2$-adrenoceptors were not in themselves inhibitory, but that the end product of prejunctional $\alpha_2$-adrenoceptor stimulation was to effect an inhibition of transmitter release. More recently, the concept of a heterogeneous population of postjunctional α-adrenoceptors has been indicated.

## 6.2. The Subdivision Of Postjunctional α-Adrenoceptors

Evidence for a mixed population of postjunctional α-adrenoceptors in the vasculature of the pithed rat and anaesthetised cat was first provided by Drew and Whiting in 1979. Using the selective $\alpha_1$- and $\alpha_2$-adrenoceptor antagonists prazosin and yohimbine (respectively) on the pressor responses of these preparations to phenylephrine and noradrenaline, they demonstrated a prazosin resistant response to noradrenaline. Yohimbine was not a potent antagonist of this response and so at this time it was not concluded that postjunctional $\alpha_2$-adrenoceptors were present in these preparations. Further evidence for a mixed population of postjunctional α-adrenoceptors was obtained in

the pithed rat by, for example, Docherty and McGrath (1980b), Flavahan and McGrath (1980) and Timmermans and Van Zwieten (1980a). Based on the selectivity of B-HT 933 and yohimbine as an agonist and an antagonist of $\alpha_2$-adrenoceptors respectively, Timmermans and Van Zwieten (1980a) concluded that postjunctional α-adrenoceptors in the pithed rat could be divided into $\alpha_1$- and $\alpha_2$- subtypes.

There have since been many attempts to isolate vessels with postjunctional $\alpha_2$-adrenoceptors, but these have met with limited success. In the isolated perfused hindquarters of the rat, pretreatment with reserpine revealed a population of postjunctional α-adrenoceptors, whereby yohimbine antagonised vasoconstriction due to B-HT 920 (Kobinger and Pichler 1981). There is evidence for the presence of postjunctional $\alpha_2$-adrenoceptors in the resistance vessels of the rat (Gerold and Haeusler 1983). Medgett and co-workers have reported a significant population of postjunctional $\alpha_2$-adrenoceptors in the tail arteries of spontaneously hypertensive rats (Medgett, Hicks and Langer 1984, Hicks, Medgett and Langer 1984). Langer, Massingham and Shepperson (1980) have demonstrated the presence of postjunctional $\alpha_2$-adrenoceptors in the autoperfused hindlimb of the dog. It has also been suggested that these receptors may be located in the human forearm (Jie, Van Brummelen, Vermey, Timmermans and Van Zwieten 1984) although this is disputed (Thom, Calvete, Hayes, Martin and Sever 1985).

Attempts to demonstrate postjunctional $\alpha_2$-adrenoceptors in isolated vessels has met with limited success. A

significant population of postjunctional $\alpha_2$-adrenoceptors in vitro have been shown in the canine saphenous vein (DeMey and Van Houtte 1981, Shoji, Tsuru and Shigei 1983 and Ruffolo and Zeid 1985). Other reports of vascular postjunctional $\alpha_2$-adrenoceptors in vitro have been made in canine mesenteric vein (Kou, Ibengwe and Suzuki 1984), human saphenous vein (Steen, Sjöberg, Skärby, Norgren and Andersson 1984) and rabbit vena cava (Palluk, Hoefke and Gaida 1985).

Following on from demonstrations of the existence of postjunctional $\alpha_2$-adrenoceptors, many investigations have been carried out which attempt to highlight differences between postjunctional $\alpha_1$- and $\alpha_2$-adrenoceptors. De Mey and Vanhoutte (1981) studied the subtype characteristics of postjunctional α-adrenoceptors in isolated blood vessels of the dog. Using a range of α-adrenoceptor agonists and antagonists with varying selectivities for $\alpha_1$- and $\alpha_2$-adrenoceptors, these workers concluded that venous smooth muscle cells possessed both $\alpha_1$- and $\alpha_2$-adrenoceptors, whereas arterial smooth muscle cells contained mainly post-junctional $\alpha_1$-.

Investigations into the location of receptor subtypes in the region of the synapse have been examined. It has been demonstrated that in both the pithed rat and in the canine hindlimb, stimulation of the sympathetic outflow activates predominantly $\alpha_1$-adrenoceptors, while exogenously administered noradrenaline activates mostly $\alpha_2$- (Docherty and McGrath 1980b ,Yamaguchi and Kopin 1980 and Langer, Massingham and Shepperson 1980 and 1981). It was suggested

by Langer et al (1981) that postjunctional $\alpha_1$-adrenoceptors are located mainly within the synapse while postjunctional $\alpha_2$-adrenoceptors are found extrasynaptically. Furthermore, it was suggested by Ariëns and Simonis (1983) that $\alpha_2$- and $\beta_2$-adrenoceptors are adrenergic in nature and would therefore be located in close proximity to the circulating catecholamine adrenaline, while $\alpha_1$- and $\beta_1$-adrenoceptors are noradrenergic and located within the synapse where they would be activated by the neurotransmitter noradrenaline. Further evidence that postjunctional $\alpha_2$-adrenoceptors may be located extrasynaptically is provided by the observations of several workers, that low doses of catecholamines in the pithed rat stimulate mainly $\alpha_2$-adrenoceptors while high doses stimulate $\alpha_1$- (Drew and Whiting 1979, DeMey and Vanhoutte 1981 and McGrath, Flavahan and McKean 1982). It was suggested by Docherty and McGrath (1980b) that the proportions of the two types of α-adrenoceptor may vary according to their proximity to the adrenergic nerve endings. It is reasonable to explain the observations of the above workers by assuming a primarily synaptic location for $\alpha_1$-adrenoceptors and an extrasynaptic location for $\alpha_2$- because if $\alpha_2$-adrenoceptors are more readily accessible to circulating catecholamines then they will be more easily stimulated, hence more readily responsive. Conversely, circulating catecholamines may undergo enzymatic degradation or neuronal uptake thus preventing the catecholamines from stimulating $\alpha_1$-adrenoceptors, except when the agonists are present in high concentrations.

A difference in the time course of responses of the

pithed rat to $\alpha_1$- and $\alpha_2$-adrenoceptor agonists has been described (Timmermans and Van Zwieten 1980b), whereby responses to $\alpha_2$- adrenoceptor agonists have a slower onset of action and slower decline than those to $\alpha_1$- agonists. This observation was also used as evidence in favour of two distinct types of postjunctional α-adrenoceptor in vascular muscle.

There have been several reports that angiotensin II potentiates the responsiveness of postjunctional $\alpha_2$-adrenoceptors. In the pithed rat, inhibition of angiotensin converting enzyme significantly attenuated the pressor response to B-HT 920 but had little effect on the response to cirazoline. This effect was shown to be reversible (De Jonge, Knape, Van Meel, Kalkman, Wilffert, Thoolen, Timmermans and Van Zwieten 1982). In vitro , it has been shown that contractile responses of the rabbit saphenous vein to B-HT 920, are significantly augmented by the presence of 5nM angiotensin II (Schümann and Lues 1983). In the rabbit aorta, subcontractile concentrations of angiotensin II potentiated responses to B-HT 920 but it was concluded that B-HT 920 was acting at $\alpha_1$-adrenoceptors (Lues and Schümann 1984a).

There have been efforts to differentiate biochemically between the two types of postjunctional α-adrenoceptor. A link has been found between the stimulation of $\alpha_1$-adrenoceptors and the activation of phosphatidylinositol turnover in vascular muscle, which in turn leads to the mobilisation of intracellular calcium ions (Jakobs and Schultz 1982, Berridge 1984, and Legan, Chernow, Parrillow

and Roth 1985). No such link has been found between the phospholipid and $\alpha_2$- adrenoceptors, although whether this is because $\alpha_2$-adrenoceptors do not cause the release of intracellular calcium or whether the tissues studied do not have a significant population of postjunctional $\alpha_2$-adrenoceptors has not been discussed. However a causal link between a fall in cyclic AMP concentration and $\alpha_2$-adrenoceptor stimulation has been suggested (Jakobs and Schultz 1982). Thus there appears to be a difference in the post receptor coupling mechanisms of $\alpha_1$- and $\alpha_2$-adrenoceptors.

A facilitation of $\alpha_2$- adrenoceptor mediated responses has been reported during acidosis (McGrath, Flavahan and McKean 1982) and during moderate cooling (Flavahan, Linblad, Verbeuren, Shepherd and Vanhoutte 1985). It was suggested by Flavahan et al (1985) that cooling may represent a functional differentiation of adrenoceptors i.e. regulation of the local vasculature by circulating catecholamines under conditions of a cold environment. It is reasonable to conclude that it may be physiologically advantageous to divert blood away from peripheral vessels under conditions of a cold environment in order to prevent excessive heat loss.

In addition to the subclassification of α-adrenoceptors into the $\alpha_1$- and $\alpha_2$- subtype, there have been efforts to further subdivide adrenoceptors. For example, two further subpopulations of both $\alpha_1$- and $\alpha_2$-adrenoceptor have been suggested (Wilffert, Gouw, Timmermans and Van Zwieten 1983a and b) and even an $\alpha_{1S}$ receptor to explain the action of

the synthetic agonist Sgd 101/75 (Timmermans, Thoolen, Mathy, Wilffert, DeJonge and Van Zwieten 1983). There has also been the suggestion of a new class of junctional adrenoceptors called $\gamma$-receptors (Hirst, Neil and Silverberg 1982). However Sneddon and Burnstock (1985) disputed the existence of a third class of adrenoceptor on the grounds that noradrenaline is not the only neurotransmitter released from sympathetic nerves. They suggested that the hypothesis of Hirst et al (1982) depended on the assumption that noradrenaline is the only transmitter released from sympathetic nerves and showed that ATP may be responsible for excitatory junction potentials in the rat tail artery (Sneddon and Burnstock 1985).

An apparent difference in the ability of $\alpha$-adrenoceptor subtypes to mobilise different pools of calcium has aroused much interest, and is considered, in detail, below. However, it is first necessary to establish the role of calcium in receptor mediated vascular muscle contraction.

## 6.3. Calcium And Smooth Muscle Contraction

The observation that calcium is necessary in the fluid perfusing isolated organs was made as early as 1883 by Ringer. In 1962, Edman and Schild suggested that calcium is necessary for the acetylcholine mediated contraction of the rat uterus, and for the contraction mediated by depolarisation with potassium. In addition, they suggested that calcium is released from a bound site at the moment of excitation of the muscle cell (Edman and Schild 1962).

The means by which free calcium ions are utilised for the

contractile process has been summarised by Rüegg (1982). That is, free calcium ions in the cytoplasm bind with calmodulin to form a complex that activates myosin light chain kinase. Conformational changes in the cross bridges between myosin and actin ensue and the muscle fibre develops force and/or shortens. Enzymatic splitting of the cross bridges restores the filaments to their former dormant state. This interaction occurs in a cyclic fashion involving attachment, power stroke and detachment of crossbridges and the splitting of one molecule of ATP in each cyclic interaction. (For reviews see Rüegg 1982 and Baum 1984).

However, the mechanisms by which agonists increase the cytoplasmic concentration of free calcium ions is largely unexplained. Godfraind and Kaba (1969) demonstrated that the rabbit mesenteric artery contracted in the presence of adrenaline in both calcium containing and calcium free medium. These results suggest two sources of calcium available for the contractile process; one dependent on the presence of extracellular calcium ions and one independent. These workers later went on to examine the nature of the calcium pools involved in the action of drugs on the arterial smooth muscle of the rabbit and the rat. They demonstrated that the contraction of the rat aorta to adrenaline was biphasic, whereby the tonic component of the contraction was dependent on the calcium concentration of the medium, and the phasic component was depressed only after prolonged incubation in calcium free medium containing EDTA (Godfraind and Kaba 1972). Again the results indicate an extracellular and an intracellular source of calcium for

contraction mediated by α-adrenergic activation in vascular muscle.

The observations by Godfraind and Kaba (1969 and 1972) were confirmed by Van Breemen, Farinas, Casteels, Gerba, Wuytack and Deth (1973). Using lanthanum to displace extracellularly bound calcium and to block calcium channels, these workers provided additional data to show that the initial rapid phase of contraction of the rabbit aorta to noradrenaline is due to intracellular calcium release and that the slow tonic phase is due to calcium influx. In addition, they showed that relaxation of the smooth muscle is brought about by intracellular calcium binding. Intracellular stores of calcium have been suggested to occur in the cell membrane, sarcoplasmic reticulum, microvesicles, mitochondria and the nucleus membrane of the pig coronary artery (Jonas and Zelck 1974).

Deth and Van Breemen (1977) studied the noradrenaline, angiotensin and histamine mediated release of intracellular calcium in the rabbit aorta. They provided evidence to suggest that the agonists tested utilised the same intracellular calcium source. In particular, the stimulation of $^{45}Ca^{++}$ efflux by each agonist may be reduced (or eliminated) by prior stimulation with another agonist. Thus the release of intracellular calcium in vascular muscle is not exclusive to α-adrenoceptor activation, but the means by which a common source of calcium could be released by different receptor mechanisms was not discussed (Deth and Van Breemen 1977).

However, the intracellular release of calcium in the rat

aorta has been suggested to arise from two independent sources. In a study of the EGTA resistant contractions of the rat aorta to noradrenaline, it was shown by Heaslip and Rahwan (1982) that the response is biphasic. There is an initial, transient peak which cannot be repeated unless the tissue is re-exposed to extracellular calcium, and a second, sustained phase that does not require refilling from an extracellular source. (Heaslip and Rahwan 1982). It was further shown by these workers that the two phases of the response were not inhibited by calcium channel antagonists (Heaslip and Rahwan 1983b) but were influenced by the α-adrenoceptor antagonist phentolamine (Heaslip and Rahwan 1983a). These observations suggest firstly, that extracellular calcium is not necessary for either the initiation or the maintenance of the EGTA resistant response, and secondly, that the EGTA resistant response of the rat aorta to noradrenaline is mediated by α-adrenoceptors.

The ability of α-adrenoceptor agonists to cause the release of intracellular calcium is not exclusive to responses on the rat and rabbit aorta. Den Hertog (1981) demonstrated a biphasic response of the guinea-pig taenia caeci to adrenaline, and suggested that the two components of the response were linked to intracellular and extracellular calcium.

The different pools of calcium that may be mobilised by α-adrenoceptor activation in vascular muscle, has aroused much interest in the subdivision of postjunctional α-adrenoceptors. It was first suggested in 1981 by Van Meel and co-

workers , that postjunctional α-adrenoceptors may be differentiated according to the pools of calcium utilised by the receptor subtypes. Using calcium channel antagonists to determine the dependence of *in vivo* responses of the pithed rat to α-adrenoceptor agonists, they showed that $\alpha_2$-adrenoceptor responses were susceptible to calcium channel antagonism while $\alpha_1$-adrenoceptor responses were not (Van Meel, DeJonge, Kalkman, Wilffert, Timmermans and Van Zwieten 1981a and b). They went on to show that the response of the pithed rat to $\alpha_2$-adrenoceptor agonists was invariably susceptible to blockade by a series of both organic and inorganic calcium channel antagonists (Van Zwieten, Van Meel and Timmermans 1982 and 1983).

Support for the observation that $\alpha_2$- mediated responses of vascular muscle are sensitive to calcium channel antagonists while $\alpha_1$- responses are resistant, has been provided *in vitro* in the canine saphenous vein (Langer and Shepperson 1981), the canine autoperfused hindlimb (Llenas and Massingham 1983) and the tail artery of spontaneously hypertensive rats (Hicks, Tierney and Langer 1985). However, it has also been suggested that in rings of isolated rabbit aorta, $\alpha_1$-adrenoceptor activation may induce an influx of extracellular calcium as well as intracellular calcium release (Cauvin, Loutzenhiser, Hwang and Van Breemen 1982) while $\alpha_2$-adrenoceptor activation in the canine saphenous vein (an *in vitro* system with both $\alpha_1$- and $\alpha_2$-adrenoceptors) has been suggested to mobilise stores of cellular calcium (DeMey and Vanhoutte 1981). Also, Jim, DeMarinis and Matthews (1985) have shown that in the canine saphenous

vein, contraction mediated by $\alpha_1$-adrenoceptor activation was susceptible to calcium channel antagonism. Therefore, the *in vitro* evidence does not suggest that intracellular calcium release is a property linked to $\alpha_1$-adrenoceptor activation.

In order to consider further the relative contribution of intracellular and extracellular calcium to α-adrenoceptor mediated vascular muscle contraction by either subtype, consideration must be given to the properties of the agonists which may describe their ability to initiate the mobilisation of calcium.

## 6.4. Agonists And Receptor Activation

The first quantitative treatment of receptor theory was presented in 1926 by Clarke. He postulated that the interaction of drugs with receptors adhered to Langmuirs adsorption isotherm. This has become known as the occupation theory. Ariëns, in 1954, introduced the terms affinity and intrinsic activity to describe the ability of a drug to bind to a receptor and to elicit a response subsequent to receptor binding. The affinity is the ability of a drug to bind to the receptor while the intrinsic activity is independent of the affinity and describes the ability of the drug to induce an effect following receptor occupation. He defined the intrinsic activity of a potent agonist as unity and that of a weak agonist as some positive value between zero and unity.

However, both Clarke and Ariëns made several assumptions which have since been criticised. The first assumption was that the response of a tissue to a full agonist was

proportional to the number of receptors occupied. This suggested that the index $ED_{50}$, which is the concentration of agonist required to elicit 50% of maximum response, was also the concentration necessary to occupy 50% of the available receptor population. It was also assumed that one drug molecule would combine with one receptor molecule (Clarke 1926, and Ariens 1954).

Stephenson (1956) criticised the work by Clarke on the grounds that it had involved the use of acetylcholine in systems which contained cholinesterases. Stephenson broadened receptor theory by the following hypotheses which, in brief are that

(i) A maximum effect can be produced by an agonist when occupying only a small proportion of the receptors.

(ii) The response is not linearly proportional to the number of receptors occupied.

(iii) Different drugs may have varying capacities to initiate a response and consequently occupy different proportions of the receptors when producing equal responses.

The last term he defined as efficacy (Stephenson 1956) and this replaced the intrinsic activity term as defined by Ariens (1954). He went on to test his hypothesis using an homologous series of alkyltrimethylammonium ions acting on the muscarinic receptors of the guinea-pig ileum. The series of agonists occupied different positions on the log agonist concentration axis. In order to describe the effects observed, he introduced the term "partial agonist" and showed how the maximal effects produced by partial agonists varied with their efficacy. He also introduced a quantity s

which was defined as the product of the efficacy of the agonist and the proportion of receptors occupied. The convention s=1 for 50% of maximum response was adopted. These alterations to the existing theory made it unnecessary to assume that the percentage of receptors occupied was equal to the percentage response.

At the same time, support for Stephenson's modification of receptor theory came from work on antagonists by Furchgott (1955) and by Nickerson (1956). It was independently demonstrated by these workers that non competitive antagonists produced parallel rightward displacement of the dose response curves to agonists before reduction of the slope and maximum were observed. It followed from this that the assumption by Clarke could not be true, because if it was correct then blockade of a given fraction of the total receptors with an irreversible competitve antagonist would be expected to decrease both the maximum and the slope by the same fraction, without a shift to the right. The observation that the active receptor population could be reduced without necessarily causing a decrease in the maximum response of a given tissue to an agonist also invalidated the assumption that the concentration of agonist necessary to produce a half maximal response is that required to occupy 50% of the receptors.

The concept of "spare receptors" was introduced by Nickerson in 1956 to explain the parallel rightward displacement of the response of isolated strips of guinea-pig ileum to histamine following irreversible inactivation of part of the receptor population with GD-121. He explained

his observations by assuming that receptor occupancy was not the limiting factor in tissue activation and that occupancy of only about 1% of the histamine receptors was adequate to produce a maximal response.

Shortly after this work, Paton introduced his rate theory of drug receptor interactions. He criticised the occupation theory on the grounds that it made no prediction of desensitisation and that occupation theory predicts that a test dose of agonist added before complete washout of a previous dose should summate with that part of the original dose not yet dissociated from the receptors, which does not occur. With rate theory, excitation is attributed to the process of occupation. The response of a tissue to a drug is proportional to the number of drug-receptor interactions per unit time. Whether a drug is a full or partial agonist or a competitive antagonist is dependent on the rate at which that drug dissociates from the receptors so freeing them for further occupation (Paton 1961).

However a number of questions arose from the rate theory. Firstly, it predicted that antagonists would have an initial stimulant action when they first combined with the receptor, and secondly that there would be an initial peak followed by a reduction to a plateau as the drug initially combined rapidly with the receptors and equilibrated to a steady state. Neither of these effects are seen generally and occupation theory remained the most widely used description of drug-receptor interactions.

The occupation and rate theories attempted to relate pharmacological response to the interaction of drugs with

their receptors. However, Belleau (1964) put forward a macromolecular perturbation theory which attempted to explain, at the molecular level, the events which lead to an observed biological response. In brief, Belleau described two conformational changes which could occur when receptors were acted on by drugs; a specific conformational perturbation, which was proposed to lead to a response, and a non-specific conformational perturbation, which was suggested to be ineffective at initiating a response (Belleau 1964). There have been other theories put forward to suggest the means by which agonist-receptor combinations lead to a response, but for the purpose of the present study, the theories which deal with the interactions of drugs with receptors i.e. the occupation and the rate theory are the most relevant.

Attempts have been made to quantify drug-receptor interactions. In 1965, Mackay argued that Stephenson's method of determining efficacy depended on the assumption that a given stimulus applied to a tissue always produces the same response. He therefore eliminated all assumptions about stimulus and response and derived a method for determining the relative order of intrinsic efficacies of agonists. This method gives a positive or a negative value which indicates the order of relative intrinsic efficacy. However, this method becomes technically difficult if the dose response curves for the agonists are parallel.

However, the most reliable method of quantifying drug-receptor interactions was provided by Furchgott (1966). Seeking a quantitative measure of affinity, Furchgott

derived a method for obtaining the dissociation constant (which is the reciprocal of the affinity constant) from dose response data (Furchgott 1966). The assumptions made for using this method are, in brief, as follows:-

(a) That the agonist interacts with only one type of receptor.

(b) That the population of this type of receptor is uniform with respect to the agonist dissociation constant.

(c) That the measured response to the agonist is the same function of the stimulus both before and after irreversible receptor inactivation.

(d) That, when the response is measured, the concentration of agonist in the region of the receptors is essentially equal to that bathing the tissue.

(e) That, when the response is measured, a steady state exists between the agonist and receptors.

(f) That the formation of the receptor-agonist complex does not lead to inactivation of the receptors.

(g) That inactivation of receptors is completely irreversible.

The stimulus produced by two agonists is defined by the product of receptor occupancy, the concentration of receptors and the intrinsic efficacy.

For equieffective concentrations of agonist before and after irreversible receptor inactivation

$$\frac{E_A}{E_m} = f\,e.\,\frac{[A]}{K_A + [A]} \quad \text{and} \quad \frac{E'_A}{E'_m} = f\,q.e.\,\frac{[A']}{K_A + [A']}$$

Assuming that equal responses arise from equal stimuli, the unknown terms f and e cancel out of the calculation and

$$\frac{1}{[A]} = \frac{1}{q}.[A'] + \frac{(1-q)}{q.K_A}$$

Where

$E_A$ :Measured response of the tissue to an agonist A.

$E_m$ :Potential maximal response of the tissue.

e :Efficacy according to Stephenson (1956).

[A]:Concentration of free agonist in region of receptors.

$K_A$ :Dissociation constant of receptor-agonist complex with dimension of [A].

q :Fraction of initial receptors remaining active after treatment of the tissue with an irreversible receptor antagonist.

$E'_A$, $E'_m$ and [A'] :Equivalent to $E_A$, $E_m$ and [A] respectively following reduction of $[R_t]$ to $q.[R_t]$ (see below).

The $K_A$ is represented by the (slope-1)/intercept of the line through a plot of equi-effective concentrations of agonist before and after irreversible receptor inactivation (Furchgott 1966).

The method for determining the relative efficacy of agonists was devised by Furchgott and Bursztyn in 1967. The dissociation constant for the agonist was determined by the method of Furchgott (1966) and all concentration data converted to the fractional receptor occupancy ($[RA]/[R_t]$) for any given concentration of agonist,

Where

[RA] :Concentration of receptor-agonist complex.

[$R_t$] :Initial concentration of total active receptors.

This was plotted against response and the antilog of the displacement between two curves along the log [RA]/[$R_t$] axis was taken as the ratio of equiactive concentrations of agonist. This was therefore a measure of the efficacy of one agonist relative to the other since the more powerful the agonist, the lower the receptor occupancy necessary to generate a response.

The work of Furchgott (1966) was extended by Waud in 1969 to calculate the dissociation constant of partial agonists. One method Waud used was to replace the assumption that irreversible receptor antagonists inactivate receptors and are then washed away, with the assumption that full agonists have a spare receptor capacity. Thus a double reciprocal plot of equieffective concentrations of a full and a partial agonist are plotted, as described above, and the dissociation constant derived from the line through the points; where

$K_p$=(slope/intercept).

Another method for the determination of agonist dissociation constants derived by Furchgott and Bursztyn (1967) and tested by Waud (1969), was to occlude a large enough fraction of the receptor population that the partial agonist no longer produced a response. The partial agonist could then be used as a competitive antagonist to the full

agonist and the dissociation constant calculated using the method for competitive antagonists by Arunlakshana and Schild (1959).

These methods have been validated for a range of α-adrenoceptor agonists on the rat aorta by Ruffolo, Rosing and Waddell (1979), who found that dissociation constants for different agonists in this system, derived by the methods described above, were in close agreement. The methods of Furchgott (1966) and Furchgott and Bursztyn (1967) described above are currently in general use to determine the dissociation constant and efficacy of agonists.

With the availability of a means of determining the efficacy and affinity of agonists, there have been studies to compare the agonist parameters with the ability of an agonist to cause the release of intracellular calcium. Chiu and co-workers (1986) studied the ability of a range of full and partial α-adrenoceptor agonists to mobilise calcium in the isolated rat aorta. They considered both the contractile response and $^{45}Ca^{++}$ efflux, and suggested that it is the interaction of a particular α-adrenoceptor agonist with the $\alpha_1$-adrenoceptor that determines whether intracellular or extracellular calcium is triggered, and to what extent. Further, that the partial agonist Sgd 101/75 lacked some critical property that would be necessary to stimulate the release of intracellular calcium (Chiu, McCall, Thoolen and Timmermans 1986).

A study by Jim and co-workers (1985) attempted to correlate the calculated efficacy of $\alpha_1$-adrenoceptor

agonists with their ability to initiate lanthanum resistant responses, and responses in calcium free medium, in the canine saphenous vein. These workers were not able to demonstrate a direct relationship between efficacy and the ability to release internal calcium (Jim, Macia and Matthews 1985).

However, it should be noted that the ability of an agonist to cause the release of intracellular calcium, will also be dependent on the ability of the tissue to translate the stimulus into a response. Therefore, it was considered of interest to study a property of the tissue that could alter the responsiveness of that tissue to α-adrenoceptor activation; hence the effect of vascular endothelium was examined for its influence on α-adrenoceptor mediated vascular muscle contractility.

### 6.5. The Endothelium On Vascular Muscle.

It was first noted, by Furchgott and Zawadzki (1980) that the endothelium is necessary for acetylcholine induced relaxation of vascular muscle. These workers showed that rings or strips of rabbit thoracic aorta, precontracted with noradrenaline, relaxed in a concentration dependent manner following addition of acetylcholine. Removal of the endothelial cell layer by mechanical rubbing prevented the relaxation to acetylcholine and in some cases caused a further contraction of the tissue. The presence or absence of endothelium had no effect on the relaxation caused by some directly acting vasodilators, and it was found that the acetylcholine induced relaxation was mediated via muscarinic

receptors since it was highly sensitive to blockade by atropine. It was postulated that acetylcholine was acting on muscarinic receptors to stimulate endothelial cells to release a substance (or substances) which activated the relaxation mechanism. Such a substance(s) appeared to be derived from the lipoxygenase pathway (Furchgott and Zawadzki 1980), and has been termed the endothelial derived relaxant factor(s) or EDRF. Since this report, all of these original observations and postulae remain unrefuted.

An early report that calcium is necessary for the release of EDRF was made by Singer and Peach in 1982. Rabbit aortic rings precontracted with phenylephrine, showed an endothelial dependent relaxation to the calcium ionophore A23187, or the muscarinic agonist methacholine. Calcium free media, or the calcium channel antagonists verapamil and nifedipine inhibited this relaxation. The data is consistent with a role of calcium in regulating either the production or the release of EDRF. That the release of EDRF is calcium dependent has since been confirmed by Long and Stone (1985).

It was later found that relaxation induced by EDRF occurs by the same biochemical mechanism as the directly acting vasodilators. It has been suggested that both endothelial dependent vasodilators and nitrovasodilators activate guanylate cyclase which brings about the formation of cGMP which in turn causes dephosphorylation of myosin light chain kinase thereby effecting relaxation (Rapoport, Draznin and Murad 1983). More recently, Popescu, Panoiu, Hinescu and Nutu (1985) provided evidence that cGMP does not act directly but is mediated by cGMP protein kinase which

phosphorylates and ultimately activates the sarcolemmal $Ca^{++}$ extrusion pump. This has been taken a step further by Rapoport, Schwartz and Murad (1985) who have suggested a complex interaction between α-adrenoceptors, cGMP and the $Na^{+}$-$K^{+}$ pump. Recently, Palmer et al (1987) examined the relationship between nitric oxide and EDRF in cultured porcine aortic endothelial cells. They reported that nitric oxide was released from vascular endothelial cells, but not from smooth muscle cells in amounts sufficient to account for the relaxation attributed to EDRF. Further, that nitric oxide has the same biological activity and chemical stability as EDRF and that its action was as susceptible to inhibition by haemoglobin, and to potentiation by superoxide dismutase. These workers concluded that EDRF and nitric oxide are identical but did not suggest whether nitric oxide is released from vascular endothelial cells as nitric oxide itself, or as a precursor which gives rise to it following release (Palmer, Ferrige and Moncada 1987).

However, Bigaud, Schoeffter, Stoclet and Miller (1984), while confirming that smooth muscle contraction in the presence of endothelium is associated with an increase in tissue levels of cGMP compared with those in its absence, were unable to correlate these levels with the differential effect of EDRF on the response of the rat aorta to clonidine or methoxamine.

Species differences have been reported for EDRF. Förstermann, Trogisch and Busse (1985) measured the half life of EDRF in the rabbit aorta and the canine femoral artery and found that for the rabbit to be approximately 24

seconds while the half life of canine EDRF was 49 seconds. They found EDRF to be rapidly oxidised and felt that high $pO_2$ levels may explain the shorter half life of EDRF in the rabbit of approximately 6 seconds reported by Griffith, Edwards, Lewis, Newby and Henderson (1984).

It might be assumed that agonists most inhibited by the endothelium bring about the release of EDRF which would modify the response of the smooth muscle to the direct effect of an agonist. Upon investigating this point, Miller, Mony, Schini, Schoeffter and Stoclet (1984) measured a significant decrease in the basal tissue level of cGMP upon removal of the endothelium in the rat aorta. They concluded that the endothelium of this tissue continuously releases a product that influences the smooth muscle and modulates the contractile response of the tissue to α-adrenoceptor agonists. This conclusion was supported by Martin, Furchgott, Villani and Jothianandan (1986) who reported that addition of haemoglobin to an endothelium containing preparation exhibiting little tone in the presence of clonidine, immediately elicited a strong contraction, comparable in magnitude to a contraction by clonidine in an endothelium denuded preparation.

It was first noted by Egleme, Godfraind and Miller (1984) that removal of endothelium from the rat aorta increased the responsiveness to clonidine and noradrenaline. They concluded that these agonists activated receptors present in endothelial cells which were highly sensitive to clonidine. Miller et al (1984) classified these receptors as $\alpha_2$-adrenoceptors since $\alpha_2$-adrenoceptor agonists were most

affected by endothelium removal. However Martin et al (1986) were unable to detect a clonidine or phenylephrine induced rise in the cGMP content of rings of rat aorta, whereas a rise in the cyclic nucleotide content was found to occur in response to acetylcholine. Furthermore, Lues and Schümann (1984b) reasoned that if the release of EDRF may be triggered by $\alpha_2$-adrenoceptors on the endothelium then a selective $\alpha_2$-adrenoceptor antagonist should mimic the removal of the endothelium. They failed to observe this effect after challenging the response of the rat aorta to B-HT 920 with rauwolscine. These two factors are compelling evidence against the existence of $\alpha_2$-adrenoceptors on the endothelium of the rat aorta which may stimulate additional release of EDRF.

The removal of the endothelium has a dramatic effect on the responsiveness of the rat aorta to α-adrenoceptor agonists. Several studies have examined the responsiveness of the tissue to a range of agonists before and after endothelium removal (e.g. Eglème et al 1986, Lues and Schümann 1984b) and Martin et al 1986). All responses were larger in the absence of endothelium than in its presence. This was shown to be most effective for agonists that have a low intrinsic activity in the presence of endothelium (Lues and Schümann 1984b). That this effect is probably due to the release of EDRF is evidenced by the fact that haemoglobin, which inhibits EDRF (Martin, Villani, Jothianandan and Furchgott 1985) reversibly mimics endothelial removal (Martin et al 1986).

Since the removal of the endothelium has been shown

(above) to increase the maximum, sustained phase of the response of the rat aorta to α-adrenoceptor agonists, it was considered that the ability of an agonist to cause the release of intracellular calcium may also be increased. This concept is examined in the final results section of the present study.

# 7. MATERIALS AND METHODS

## 7.1. Preparation Of The Tissues.

### 7.1.1. Longitudinal Portal Vein.

Male Wistar rats (180-220g) were killed by stunning followed by cervical dislocation. The hepatic portal vein, 10-15mm in length, was exposed and cleaned *in situ* of fat. Cotton was tied firmly around each end of the vessel, which was then cut away from surrounding fat and connective tissue. The cotton attached to the mesenteric end of the vessel was used to attach the portal vein to a tissue holder while the hepatic end was connected to a force-displacement transducer and maintained under a resting tension of 0.5g in a 25ml organ bath.

### 7.1.2. Aorta

Rats were killed (as above) and the thoracic aorta was removed and placed in Krebs' solution. Fat and connective tissue were cut away taking care not to stretch the tissue. Two rings 2-3mm in length were cut from one aorta, and each ring suspended under a resting tension of 2g from triangular tissue holders made from stainless steel wires (1mm O.D. Coopers Needleworks), in a 15ml organ bath.

All isolated tissues were connected to force-displacement transducers (type UF1 PIODIN). These were coupled to a pre-amplifier (ORMED 3559) which in turn had two outputs; one to a chart recorder (ORMED MX4) and the other to a BBC microcomputer.

Tissues were bathed in Krebs' bicarbonate solution of the following composition (in mMol):- NaCl 118.3; KCl 4.7; $CaCl_2$ 2.5; $KH_2PO_4$ 1.2; $NaHCO_3$ 25; $MgSO_4.7H_20$ 1.2 and dextrose 11.7. The solution was maintained at 37°C, gassed with 95% $O_2$/ 5% $CO_2$ and routinely contained the following:- ascorbic acid (50μM) and EDTA (10μM) to inhibit the oxidation of catecholamines (Furchgott 1955), propranolol (1μM) to inhibit β-adrenoceptor mediated relaxation (Furchgott 1976) and cocaine (10μM) to prevent neuronal uptake of catecholamines by the $Uptake_1$ mechanism (Starke, Montel and Schumann 1971).

Tissues were allowed to equilibrate for one hour with washing every 20 minutes. Then a maximum 'conditioning' concentration of the agonist to be used in the experiment was added to the bathing medium, and the tissues allowed to develop a sustained contraction. For agonists with low efficacy, noradrenaline (30μM) was used to evoke the initial conditioning response. The tissues were then allowed to rest at baseline tension for at least thirty minutes before reproducible responses were obtained to submaximal concentrations of agonist. This conditioning procedure enabled consistent responses to agonists to be obtained throughout the experiment. This method is similar to that of Ruffolo, Rosing and Waddell (1979).

Throughout the experiments, resting baseline tension was adjusted if necessary to maintain portal veins at 0.5g and aortae at 2g.

7.1.3. Pithed Rat.

Male Wistar rats, weight range 180-220g were anaesthetised with pentobarbitone sodium (Sagatal) 60mg/Kg i.p.. The tracheae were cannulated and the animals pithed by inserting a steel rod, 1.5mm in diameter, through the orbit and foramen magnum and along the spinal canal. Immediately after pithing, the tracheal cannula was attached to an SRI respiratory pump and the animals were artificially respired with room air at a frequency of 70 cycles/minute and a volume of 2ml/100g. Systemic arterial blood pressure was recorded via a Bell and Howell pressure transducer connected via a saline filled polyethylene cannula to the right carotid artery. Heart rate was measured by a Devices Instantaneous Heart Rate Meter, triggered by the pulse pressure. Blood pressure and heart rate were recorded on an ORMED MX4 chart recorder. The left jugular vein and the left femoral vein were cannulated for administration of drugs. Heparin (1000 i.u./Kg) and atropine (1µg/Kg) were administered i.v. when the cannulae were sited.

Animals to receive phenylephrine were given propranolol (750µg/Kg) 15 minutes before commencing the experiment. This was to inhibit β-adrenoceptor stimulated increase in heart rate and blood pressure.

Rectal temperature was continuously monitored with an electrical thermometer and maintained at approximately 37°C by manually adjusting the height of a 60W lamp positioned above the chest. A twenty minute equilibration period was allowed between setting up the preparation and commencement of the experiment.

## 7.2. Experimental Methods

### 7.2.1. Response of the portal vein to α-adrenoceptor agonists and the effect of antagonists.

The spontaneous activity of the portal vein was measured over a period of five minutes using a BBC microcomputer to assess the integral of the mechanical contraction. A BBC microcomputer was used to sample the tension dependent voltage output from the pre-amplifier. Tension dependent voltage was applied to the analogue input on a BBC microcomputer and converted to a digital value. The analogue digital converter in the computer accepted four inputs and measured each over a 10msec. period at 20csec. intervals. The cumulative digital input for each channel was recorded over a variable period controlled by keyboard entry, and an average value was computed for the digital input per minute. The AD converter in the BBC microcomputer gave 12 bit resolution over an input range of 0-1.8V / digital range 0 to 65520. The numerical value obtained from the computer for this time period was defined as the base. Agonists were added to the organ bath in a constant injection volume of 250µl. For all agonists except cirazoline, tissues were allowed to respond for one minute, prior to measurement of the responses over a two minute period. With cirazoline, two minutes was required before a consistent response was achieved, and this was measured over a further two minute period. The base value for the spontaneous activity was then subtracted from the induced phasic response value to give a measure of the increase in activity induced by the agonist. Following washing, at least five minutes after return to

baseline values was allowed before addition of subsequent concentrations of agonist.

Using this protocol, the response of the portal vein to increasing bolus additions of agonist were determined. Where preliminary results indicated that changes in sensitivity of the tissue to the agonist under test was likely, concentrations of agonist were given in random order.

Following construction of a complete control concentration response curve, the greatest numerical value for the response of a tissue to the agonist obtained from the microcomputer (as described above) was considered the control maximum, and defined as 100%. All other responses were calculated as a percentage of this maximum. In this way, each tissue acted as its own control.

Antagonists were added to the Krebs' solution and remained in contact with the tissues for thirty minutes before construction of subsequent concentration response curves.

### 7.2.2. Response of the pithed rat to α-adrenoceptor agonists and the effect of antagonists.

Dose response curves were constructed for the pressor responses of the pithed rat to phenylephrine and to UK-14,304, administered by continuous infusion. An SRI slow infusion pump administered the drugs at a rate of 30µl/minute until a constant pressor response was achieved (between ten and fifteen minutes). The infusion was then stopped and the blood pressure allowed to return to pre-infusion values. After a fifteen minute interval,

succeeding concentrations of agonist were infused.

Dose response curves to cirazoline were constructed by cumulative administration of bolus doses of agonist.

The effect of α-adrenoceptor antagonists were assessed by 15 minutes i.v. pretreatment of the animals with saline (0.5ml/Kg-control), prazosin (0.3 and 1.0mg/Kg), yohimbine (0.3mg/Kg), corynanthine (10mg/Kg) or phenoxybenzamine (0.2mg/Kg). The calcium channel antagonist, diltiazem (25µg/Kg/min) was given as a continuous infusion commencing fifteen minutes prior to administration of agonist. It was considered necessary to infuse an agent where the duration of its antagonist effect was shorter than the time required to complete the dose response curve to the agonist under test (Cavero, Shepperson, Lefevre-Borg and Langer 1983).

All bolus injections were given in the volume 0.5ml/Kg and drugs were washed through the cannulae using 0.2ml of normal saline.

Only one dose response curve was constructed per animal and results were expressed as the increase in diastolic blood pressure induced by the agonist with or without pretreatment with an antagonist.

### 7.2.3. Response of the rat aorta to agonists, and the effect of antagonists.

Agonists were added to the organ bath in a consistent injection volume of 150µl and the response allowed to develop. The displacement of the trace at the inflection point of the response was considered the fast response. The inflection point was taken to be the point at which the

initial, rising phase of the response, as represented on the trace, deviated from linearity. The slow response was measured either as the maximum sustained contraction or as the mean integral value obtained from the microcomputer for the total response. At least five minutes was allowed after return to baseline before subsequent addition of agonist.

Cumulative concentration response curves were constructed by bolus administration of agonist according to the method of Van Rossum (1963), using 50µl aliquots of solution.

When the effects of α-adrenoceptor antagonists or calcium channel antagonists were reversible by washing, these were present in the Krebs' solution. Thirty minutes equilibration was allowed before subsequent addition of agonists.

The irreversible α-adrenoceptor antagonists phenoxybenzamine (10nM) and benextramine (1µM) were added to the organ baths for fifteen minutes while the tissues were resting at baseline tension. The antagonists were then washed out for one hour, with washing every fifteen minutes, before construction of subsequent concentration response curves. Sodium thiosulphate (100µM) was present for the first thirty minutes of washing after phenoxybenzamine to prevent further alkylation of α-adrenoceptors by newly formed aziridinium ions from residual phenoxybenzamine (Graham 1957, Kenakin 1984).

All responses have been expressed as a percentage of the maximum response of the tissue to agonist before receptor inactivation. Except for experiments designed to evaluate the effect of endothelium removal on the response of the rat aorta to agonists, all experiments were conducted on

endothelium denuded preparations.

### 7.2.4. The response of the rat aorta to agonists in the presence and absence of endothelium.

Following the construction of a concentration response curve in the presence of endothelium, the endothelial cell layer was removed by placing one tip of a pair of fine forceps through the lumen of the aorta and rolling the tissue back and forth over filter paper moistened with Krebs' solution. This method was found to successfully remove the endothelium without damaging the muscle.

The presence or absence of endothelium was determined by testing the ability of 1μM acetylcholine to relax the tissue pre-contracted with 0.1μM noradrenaline (Furchgott 1984). The method was further validated histologically using a modified version of the method by Poole, Sanders and Florey (1958) in which the tissues were bathed in 1% silver nitrate solution for two minutes, followed by a further two minutes in a solution of 3% cobalt bromide plus 3% ammonium bromide. Tissues were rinsed in Krebs' solution before mounting, and examined under a light microscope.

### 7.2.5. EGTA-resistant responses of the rat aorta to agonists.

EGTA-resistant responses were elicited by bathing the tissues with calcium free Krebs' solution containing 0.5mMol EGTA for five minutes, (with one wash after two minutes) prior to administration of agonist. Responses were measured for a minimum of twenty minutes. Preliminary experiments

revealed that there is a small, time-related decrease in repeated EGTA-resistant responses, therefore concentrations of agonist were tested in random order. Tissues were allowed to rest at baseline tension for twenty minutes in Krebs' solution containing calcium between subsequent contractions, to allow refilling of loosely bound stores of intracellular calcium (Heaslip and Rahwan 1982).

Results have been expressed in terms of the percentage of the maximum sustained response of the tissue to 3μM noradrenaline in the presence of calcium, or in terms of the tension developed (mg).

## 7.3. Calculation Of Results And Statistical Analysis.

Results of isolated tissue experiments were expressed either as the percentage of the control maximum response (i.e. in the absence of antagonist or endothelium, where appropriate) or as the tension developed (mg). Pressor responses in the pithed rat experiments were expressed as the increase in diastolic blood pressure above the resting pressure.

All results have been presented as the mean ± standard error of the mean. The differences between concentration response curves have been evaluated by estimating the $EC_{50}$ for each curve and calculating the mean, then estimating the log shift. Least squares analysis was used where possible to fit the best straight line through the linear portion of each curve, and $EC_{50}$ values were estimated from this line. Curves were considered parallel if the 95% confidence limits

for the slopes of these lines overlapped.

Statistical significance for the difference between the means of two groups of points was assessed using a two tailed Student's t-test; paired for within tissue comparisons and unpaired for between tissue or animal comparisons.

Dissociation constants for full agonists ($K_A$) were determined using the method of Furchgott and Bursztyn (1967). Double reciprocal plots of equieffective concentrations of agonist before and after partial irreversible blockade of adrenoceptors with benextramine or phenoxybenzamine were made for each tissue.

From this:-

$$K_A = \frac{(\text{slope}-1)}{\text{intercept}}$$

A modified version of this method (Waud, 1969) was used to determine the dissociation constant of partial agonists ($K_p$) whereby double reciprocal plots of equieffective concentrations of the partial agonist compared with a full agonist (i.e. noradrenaline) were made. Then

$$K_p = \frac{\text{slope}}{\text{intercept}}$$

and

$$\frac{[RA]}{[R_t]} = \frac{[A]}{K_A + [A]}$$

where $[RA]/[R_t]$ = fraction of receptors occupied by the agonist.

and $[A]$ = concentration of agonist in the region of the receptors.

From these values, receptor reserve was estimated by determining the fraction of receptors occupied to produce 95% of the maximum response (Flavahan and Vanhoutte 1986). Also, efficacy (e) was estimated as the reciprocal of $[RA]/[R_t]$ at the $EC_{50}$ (Furchgott 1966). Since efficacy is a dimensionless term with no theoretical limit, relative efficacies ($e_r$) have been assigned where the $e_r$ of a reference agonist (usually a full agonist) is unity (Furchgott and Bursztyn 1967, Ruffolo 1982).

Finally, intrinsic activity ($I_A$) was assessed as the ratio of the maximum response of a test agonist to the maximum response of a reference (usually full) agonist (Ariëns 1954).

## 7.4.Drugs And Solutions

| | |
|---|---|
| Acetylcholine chloride | Sigma |
| Amidephrine mesylate | *Mead & Johnson |
| Ascorbic acid | BDH |
| Atropine sulphate | BDH |
| Benextramine tetrahydrochloride | Sigma |
| Caffeine (anhydrous) | Sigma |
| Cirazoline hydrochloride | Synthelabo |
| Cocaine hydrochloride | McCarthys |
| Corynanthine hydrochloride | Sigma |
| Diltiazem hydrochloride | Synthelabo |
| EDTA | BDH |
| EGTA | Sigma |
| Guanfacine hydrochloride | *Sandoz |
| Heparin sodium | Evans |
| Idazoxan (RX781094) | *Reckitt & Colman |
| α-Methylnoradrenaline (free base) | Sigma |
| (-) Noradrenaline bitartrate | Sigma |
| Pentobarbitone sodium (Sagatal) | May & Baker |
| (-) Phenylephrine hydrochloride | Sigma |
| Phenoxybenzamine hydrochloride | *S.K.&F. |
| Prazosin hydrochloride | *Pfizer |
| Propranolol hydrochloride | Sigma |
| Rauwolscine hydrochloride | ROTH |
| Sodium thiosulphate | BDH |
| UK-14,304 tartrate | *Pfizer |
| Yohimbine hydrochloride | BDH |

*-indicates drugs generously donated as gifts.

Idazoxan was dissolved in dilute hydrochloric acid; prazosin was dissolved in distilled water containing 5% glucose and 5% glycerine; solutions of catecholamines (including dilutions) contained 100μM ascorbic acid, and phenoxybenzamine was dissolved in absolute ethanol.

Solutions were stored frozen and were freshly prepared each week. Dilutions were made when required. Solutions for addition to organ baths were diluted with distilled water, those for administration to pithed rats were diluted with normal saline.

Caffeine and EGTA were dissolved in the Krebs' solution when required.

# 8. RESULTS

## 8.1. PORTAL VEIN

### 8.1.1. Introduction

Many workers have demonstrated a mixed population of postjunctional α-adrenoceptors *in vivo* (e.g. Flavahan and McGrath 1980, Kobinger and Pichler 1980 and Timmermans and Van Zwieten 1980). It has been suggested by Van Meel, De Jonge, Kalkman, Wilffert, Timmermans and Van Zwieten (1981a and b) that the α-adrenoceptor subtypes utilise different calcium activator mechanisms in initiating contraction of vascular muscle. Contraction initiated by the $\alpha_1$-subtype, activates the release of calcium from intracellular stores and is not directly dependent on the influx of extracellular calcium ions, while the $\alpha_2$-subtype initiates vascular muscle contraction primarily through an influx of extracellular calcium. Support for this hypothesis has been provided in pithed rats and isolated canine saphenous vein strips by Cavero, Shepperson, Lefevre-Borg and Langer (1983) and in the canine autoperfused hindlimb by Llenas and Massingham (1983), but the results of several other workers have failed to confirm it e.g. in canine saphenous vein (Janssens and Verhaeghe 1984) or canine splenic artery (DeMey and Vanhoutte 1981)

The pharmacological classification of receptors is dependent upon the existence of equilibrium conditions, which cannot be established in experiments on the intact animal (Furchgott 1972). Therefore an isolated vascular preparation was sought in which a mixed population of

postjunctional $\alpha_1$- and $\alpha_2$-adrenoceptors could be studied. Furthermore, if an hypothesis is advanced regarding receptors *in vivo* , and if the hypothesis is valid for the receptors themselves and not a unique property of the test system, it should be possible to reproduce the results *in vitro* where steady state conditions may be attained.

At the commencement of this study, the existence of a mixed population of postjunctional α-adrenoceptors was reported in the portal vein of the rat (Hicks 1983). It was concluded that postjuctional $\alpha_2$-adrenoceptors were responsible for changes in phasic activity and were particularly sensitive to changes in extracellular calcium, whereas the $\alpha_1$-adrenoceptor mediated response effected changes in tone.

The portal vein of the rat is a suitable vessel for the study of venous smooth muscle (Golenhofen 1972). The advantages are that it is convenient to dissect, and it undergoes minimal changes in sensitivity to potent agonists during experimentation. Among the disadvantages are that it is spontaneously active and so measurement of responses to agonists are complicated. In addition, it is dependent upon extracellular calcium for phasic activity (Axelsson, Wahlstrom, Johansson and Jonsson 1967).

Most studies have shown the presence of only the $\alpha_1$-subtype of adrenoceptor in this vessel (Ruffolo, Waddell and Yaden 1981, McGrath 1982 and Digges and Summers 1983). However, it seemed worthwhile to investigate whether the presence of postjunctional $\alpha_2$-adrenoceptors could be confirmed in this vessel, since it might then provide a

useful model with which to investigate the excitation - contraction coupling processes for the two receptor subtypes.

Consequently, initial experiments on the portal vein of the rat were carried out with the objective of confirming or denying the presence of a mixed population of postjunctional α-adrenoceptors.

8.1.2. Results

8.1.2.1. The effect of idazoxan on the response of the portal vein of the rat to UK-14,304 and to phenylephrine.

Initial experiments were performed in an attempt to ascertain whether the portal vein would contract in response to stimulation by an $\alpha_2$-adrenoceptor agonist. The portal vein of the rat is spontaneously active. Spontaneous phasic contractions arose at a frequency of 2.6 ± 0.2 per minute. The interval between peaks was 25.3±1.8 seconds and the size of the contractions was 891±112mg (mean values from ten random samples).

UK-14,304 is a potent, selective agonist at $\alpha_2$-adrenoceptors (Cambridge 1981). In the present study, UK-14,304 caused an increase in the size of contraction and the frequency of the spontaneous phasic activity with little or no change in the basal tone of the vessel (figure 1A).

The selective $\alpha_2$-adrenoceptor antagonist idazoxan (Doxey, Roach and Smith 1983), caused a parallel rightward displacement of the concentration response curve of the portal vein to UK-14,304 (figure 3A). The mean log shift by 0.1 and 0.5μM idazoxan measured at the $EC_{50}$ was 0.45±0.06 and 0.79±0.09 respectively (n=8-16).

Phenylephrine is a potent, preferential $\alpha_1$-adrenoceptor agonist (Timmermans and Van Zwieten 1982). Low concentrations (0.1-0.3μM) produced a response which was qualitatively similar to the response to UK-14,304 (figure 1B); but higher concentrations (1-30μM) caused a sustained increase in tone, superimposed on the phasic activity

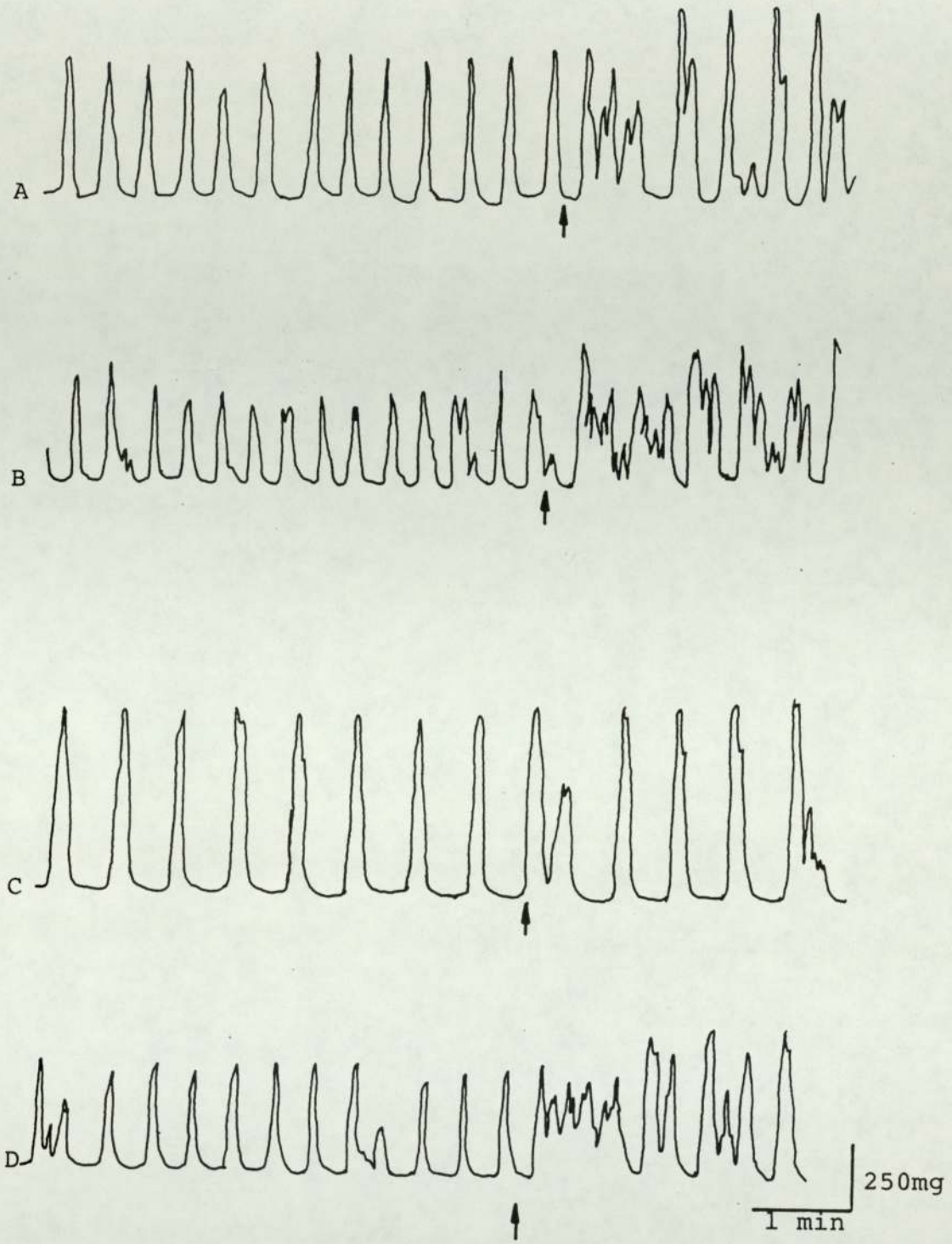



Figure 1: Representative traces of the response of the portal vein of the rat to A. UK-14,304 (10μM), B. phenylephrine (0.1μM), C. cirazoline (0.01μM) and D. α-methylnoradrenaline (0.3μM).
Arrows indicate addition of agonist.

(figure 2A). At the highest concentrations used, a tonic response only was observed to phenylephrine (figure 2A).

Idazoxan caused a parallel rightward shift of the concentration response curve to phenylephrine, with no suppression of the maximum (figure 3B). At the $EC_{50}$ the log shifts by 0.1 and 0.5μM idazoxan were respectively 0.23±0.04 and 0.42±0.04 (n=12-16). For both concentrations of idazoxan tested, the log shift at the $EC_{50}$ of the phenylephrine curve was significantly less than the log shift of the UK-14,304 curve ($p<0.01$).

If there were postjunctional $\alpha_2$-adrenoceptors in this vessel, it would have been expected that idazoxan would have caused a rightward displacement of the concentration response curve to an $\alpha_2$-adrenoceptor agonist that was clearly greater than that to an $\alpha_1$-adrenoceptor agonist. Since idazoxan had a small, but significant effect on the response to UK-14,304, further investigation was indicated.

8.1.2.2. The effect of prazosin on the response of the portal vein of the rat to UK-14,304

The objective of these experiments was to determine whether UK-14,304 was acting on $\alpha_1$-adrenoceptors. Prazosin is a potent, selective competitive antagonist of $\alpha_1$-adrenoceptors (Cambridge, Davey and Massingham 1977). In figure 4 it has been shown that low concentrations of prazosin (5-125nM) caused progressive shifts of the UK-14,304 concentration response curve (n=8-24). The slope of the linear portion of the curve in the presence of 25nM prazosin was significantly less than the slope of the

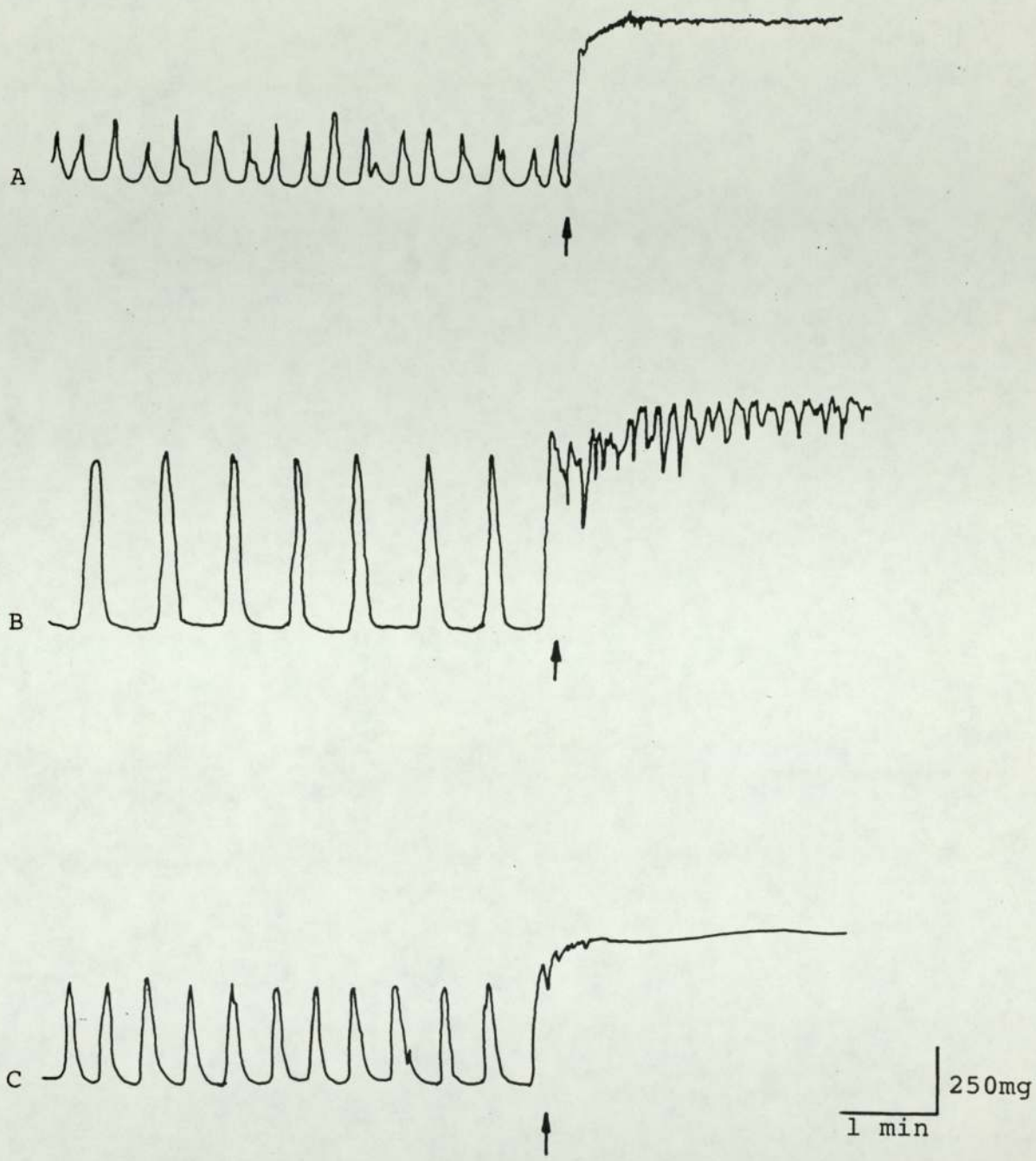



Figure 2: Representative traces of the response of the portal vein of the rat to A. phenylephrine (30μM), B. cirazoline (1μM) and C. α-methylnoradrenaline (30μM). Arrows indicate addition of agonist.

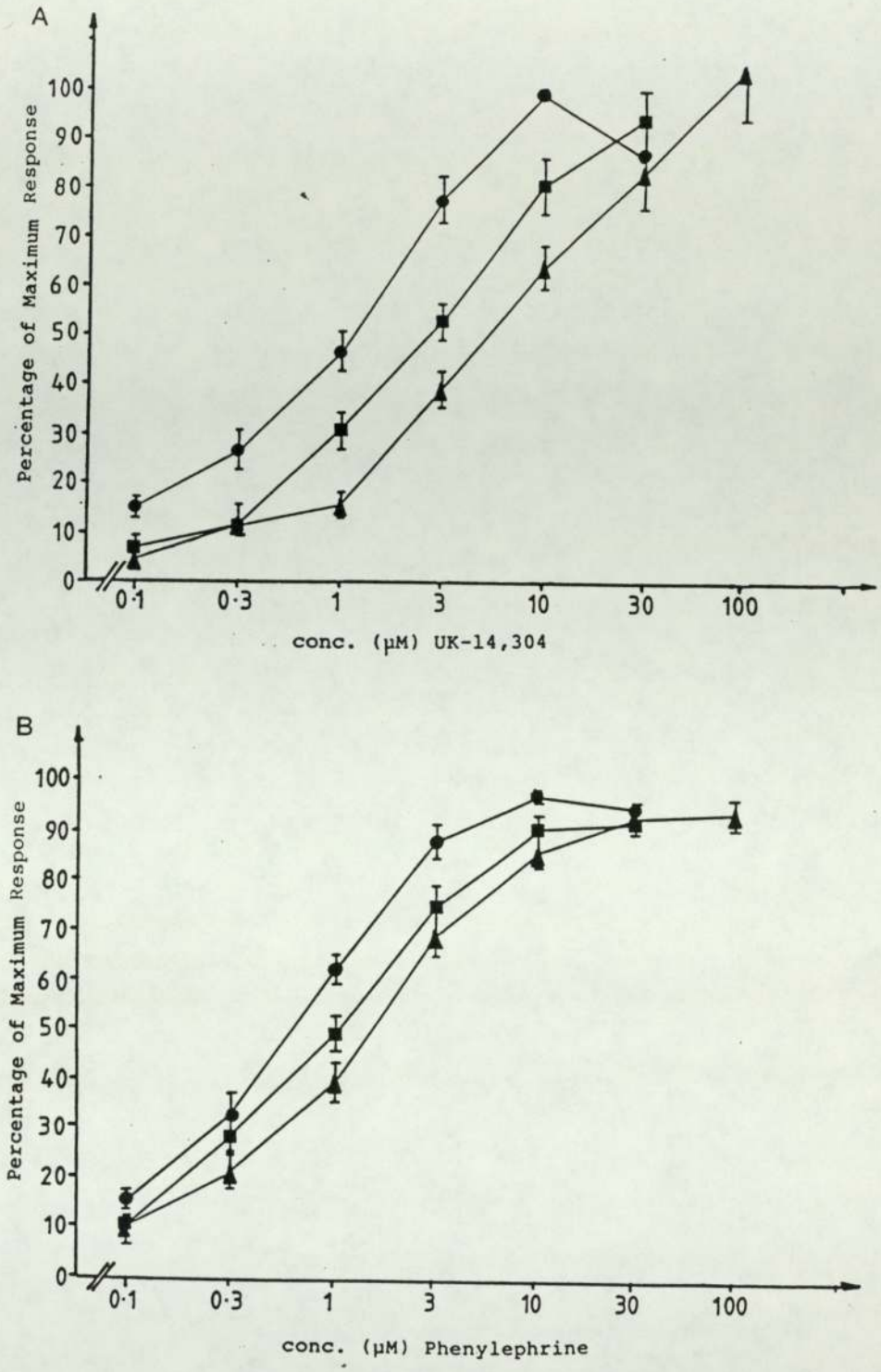



Figure 3: The effect of idazoxan on the response of the portal vein of the rat to A. UK-14,304 (n=8-16) and B. phenylephrine (n=12-16).
(●) control; (■) 0.1μM idazoxan; (▲) 0.5μM idazoxan.

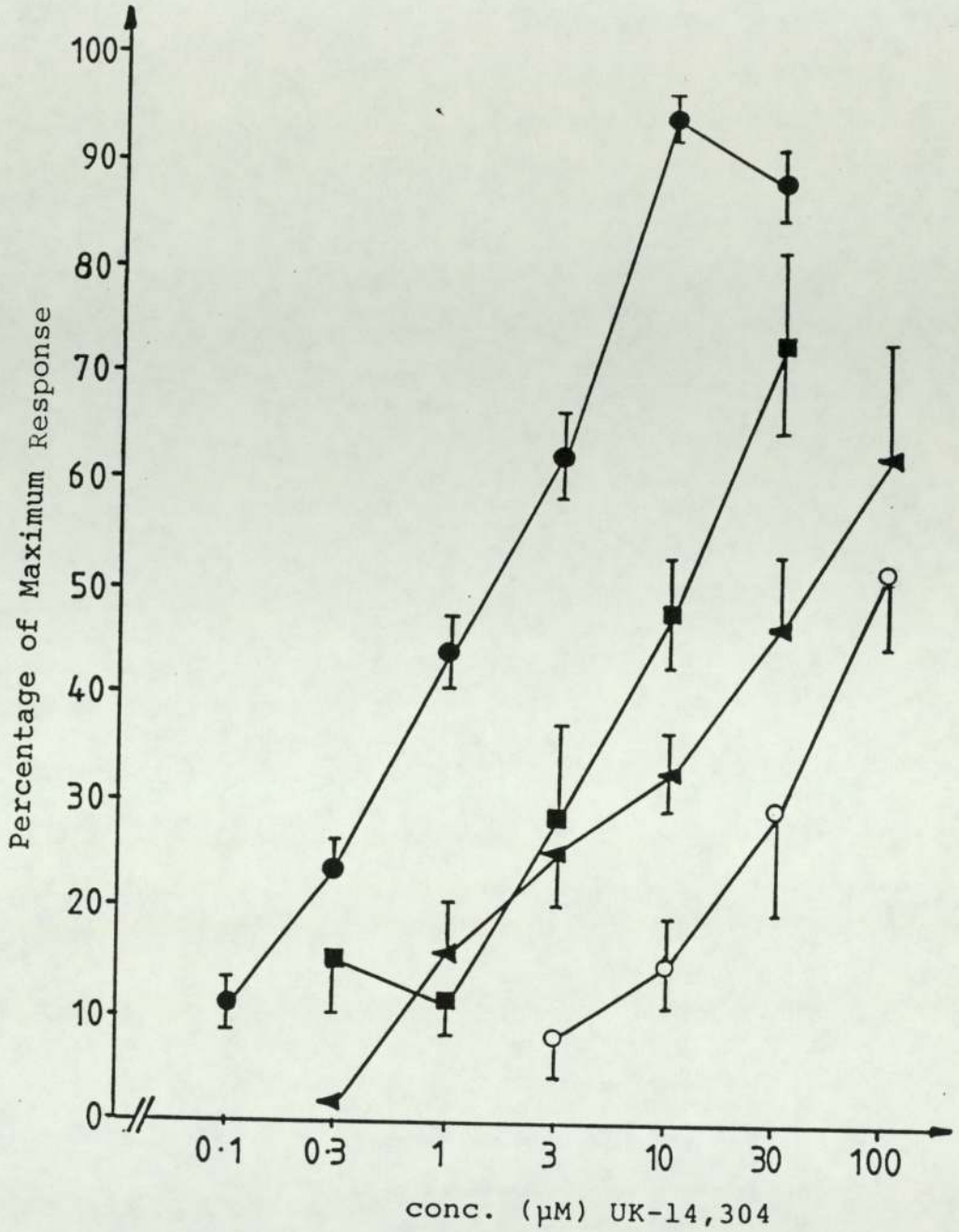


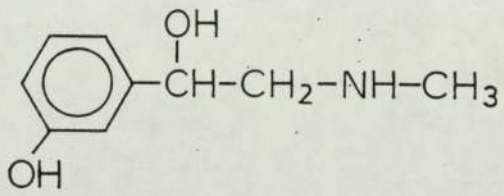

Figure 4: The effect of prazosin on the response of the portal vein of the rat to UK-14,304 (n=8-24).
(●) control; (■) 5nM prazosin; (◀) 25nM prazosin;
(○) 125nM prazosin.

control curve. However, since prazosin caused a parallel shift of the curve at 5 and 125nM the response in the presence of 25nM prazosin may be anomolous. Responses of the portal vein to UK-14,304, in the presence of prazosin, were not determined for concentrations of UK-14,304 exceeding 100μM due to the poor solubility of the agonist.

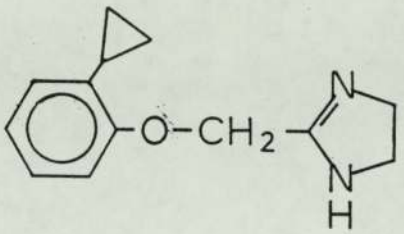
Nevertheless it has been shown that the potent, selective $\alpha_1$-adrenoceptor antagonist prazosin caused progressive rightward displacement of the control concentration response curve to UK-14,304. This implies that the response of the portal vein to UK-14,304 is mediated, at least in part, through $\alpha_1$-adrenoceptors.

8.1.2.3. The effect of idazoxan on the response of the portal vein of the rat to cirazoline and to α-methylnoradrenaline

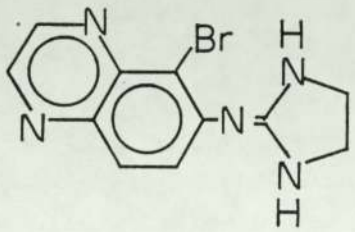
Ruffolo and Waddell (1983) explored the structure activity relationships of imidazolines and phenethylamines on α- adrenoceptors, and suggested that these two classes of agent interacted differently with the receptors. Since UK-14,304 is an imidazoline and phenylephrine is a phenethylamine, two other agents were investigated namely α-methylnoradrenaline, an $\alpha_2$- selective phenethylamine (Timmermans and Van Zwieten 1982) and cirazoline, an $\alpha_1$-selective imidazoline (Van Meel, Timmermans and Van Zwieten 1982 - see figure 5). The purpose of this investigation was to determine whether any of the differences in effects observed between UK-14,304 and phenylephrine could have been due to physico-chemical differences.



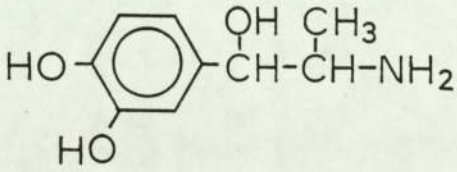
Phenylephrine



Cirazoline



UK-14,304



α-methylnoradrenaline

Figure 5: Schematic representation of the structural formula of phenylephrine, cirazoline, UK-14,304 and of α-methyl-noradrenaline.

The representative trace shown in figure 2B illustrates that high concentrations of cirazoline (1-3μM) caused an increase in tone of the vessel which overlayed the spontaneous phasic activity. This appeared to be qualitatively similar to the response of this tissue to phenylephrine. α-methylnoradrenaline (10μM) also caused an increase in tone which did not resemble the response to UK-14,304 (figure 2C). This is in contrast to the suggestion of Hicks (1983) that $\alpha_2$-adrenoceptor mediated responses in the rat portal vein give rise to changes in phasic activity while responses mediated by $\alpha_1$-adrenoceptors cause changes in tone.

The selective $\alpha_2$-adrenoceptor antagonist idazoxan (0.1 and 0.5μM) caused parallel rightward displacement of concentration response curves to both cirazoline (n=8) and α-methylnoradrenaline (n=8-16) with no suppression of the maximum responses to either agonist. (figures 6A and B). At the $EC_{50}$ the log shift by 0.1μM idazoxan of the cirazoline and of the α-methylnoradrenaline concentration response curves were 0.55±0.21 and 0.42±0.10 respectively. There was no significant difference between these values ($p>0.05$).

It was concluded from these experiments that the structural origin of the agonists used could not be considered an important factor in determining the sensitivity of α-adrenoceptor mediated responses of the portal vein to idazoxan. As an alternative to the use of different agonists, the use of antagonists other than idazoxan and prazosin was investigated.

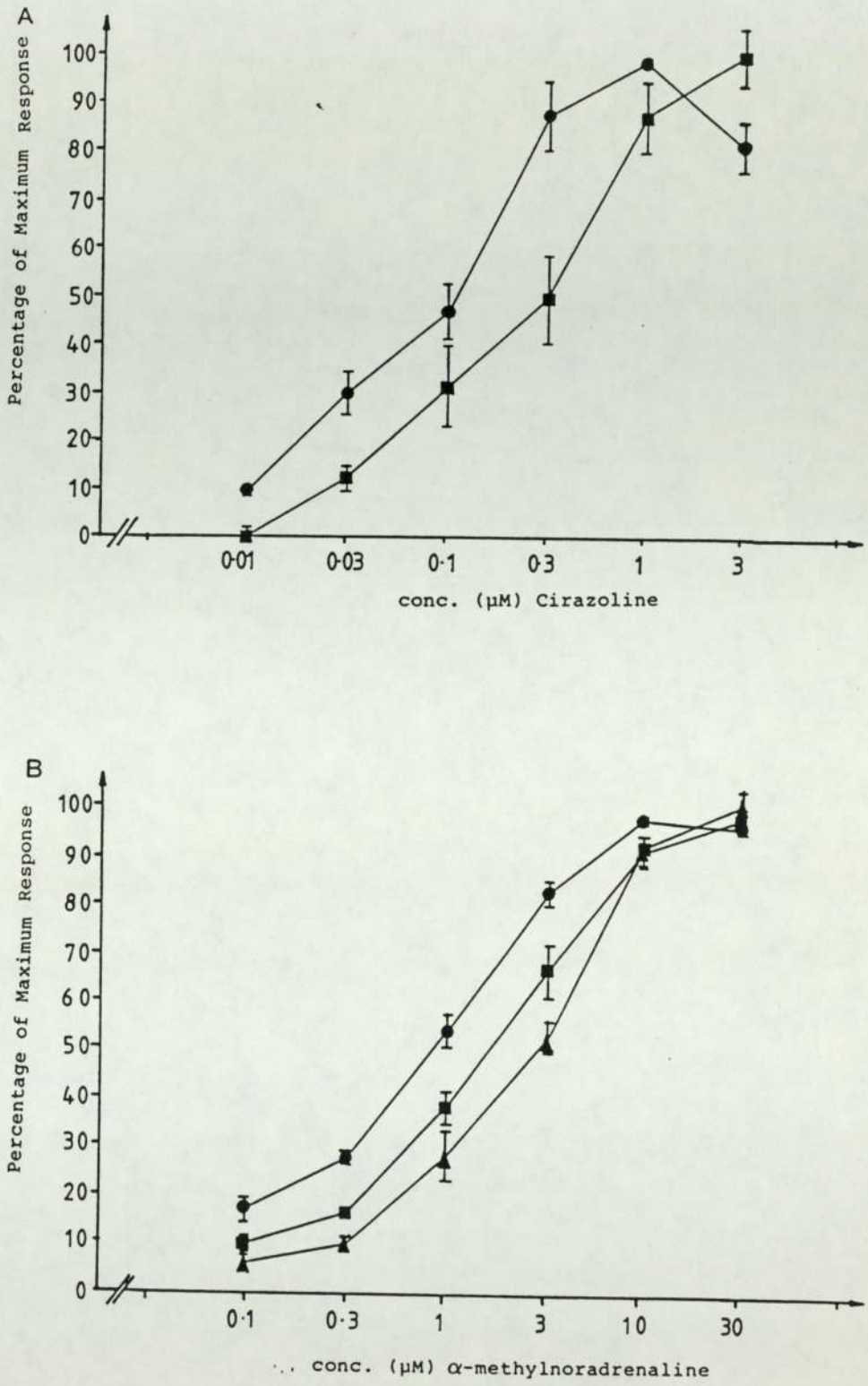



Figure 6: The effect of idazoxan on the response of the portal vein of the rat to A. cirazoline (n=8-16) and to B. α-methylnoradrenaline (n=8-16).
(●) control; (■) 0.1μM idazoxan; (▲) 0.5μM idazoxan.

8.1.2.4. The effect of corynanthine and rauwolscine on the response of the portal vein of the rat to UK-14,304.

Corynanthine and rauwolscine are stereoisomers and are respectively, relatively selective antagonists of $\alpha_1$- and $\alpha_2$-adrenoceptors (McGrath 1982). If the portal vein of the rat has a population of postjunctional $\alpha_2$-adrenoceptors, and UK-14,304 was acting through these receptors, then it would be expected that rauwolscine would cause a greater rightward displacement of the concentration response curve to UK-14,304 than corynanthine.

Figures 7A and B show that both corynanthine and rauwolscine caused parallel rightward displacement of the UK-14,304 concentration response curve with no suppression of the maximum (n=4-8). There was no significant difference between the log shift at the $EC_{50}$ caused by 1μM corynanthine (0.85±0.1) and that caused by the same concentration of rauwolscine (0.9±0.12, $p>0.05$).

Owing to the relative selectivity of these antagonists, it is possible that 1μM corynanthine may have inhibited the $\alpha_1$- component of the response to UK-14,304 to the same extent as 1μM rauwolscine inhibited the $\alpha_2$- component. However, if this were the case, then it would suggest that it is not possible to meet the overall objective of defining a working population of postjunctional $\alpha_2$-adrenoceptors in the portal vein, that could be studied separately from a population of $\alpha_1$-. In a final attempt to define the $\alpha_2$-component of responses of the portal vein, the effect of idazoxan on the response of the portal vein to UK-14,304 in the presence of prazosin was studied.

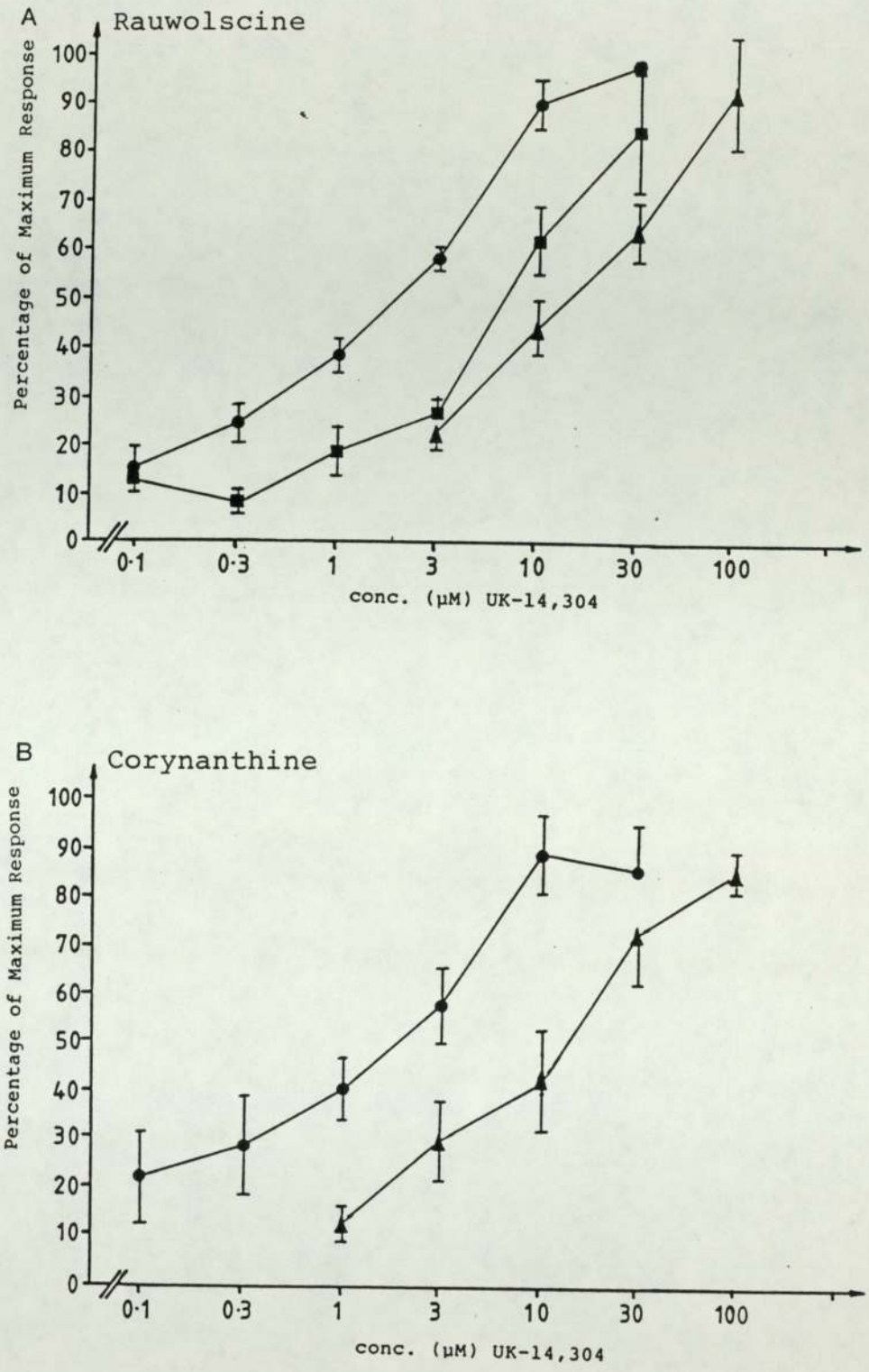



Figure 7: The effect of A. rauwolscine (n=4-8) and B. of corynanthine (n=4) on the response of the portal vein of the rat to UK-14,304.
(●) control; (■) 0.1μM antagonist; (▲) 0.5μM antagonist.

8.1.2.5. The combined effect of idazoxan and prazosin on the response of the portal vein of the rat to UK-14,304.

It has already been established that the selective $\alpha_1$-adrenoceptor antagonist prazosin reduced the response of the portal vein of the rat to the selective $\alpha_2$-adrenoceptor agonist UK-14,304 (figure 4). It was considered worthwhile to investigate whether this response to UK-14,304 was due entirely to an action on $\alpha_1$-adrenoceptors, or whether some component of the response might have been due to $\alpha_2$-adrenoceptor activation. Therefore the effect of idazoxan was investigated on the response to UK-14,304 in the presence of 125nM prazosin.

Figure 8 shows that idazoxan, at a concentration which had previously been shown to inhibit the response of the portal vein to UK-14,304 (figure 3A), caused no inhibition of the response when prazosin was present ($p>0.05$, $n=6-12$). Therefore it was not possible to demonstrate a component of the response to UK-14,304 that was sensitive to the $\alpha_2$-adrenoceptor antagonist idazoxan when prazosin was used to mask that part of the response due to $\alpha_1$-adrenoceptor activation.

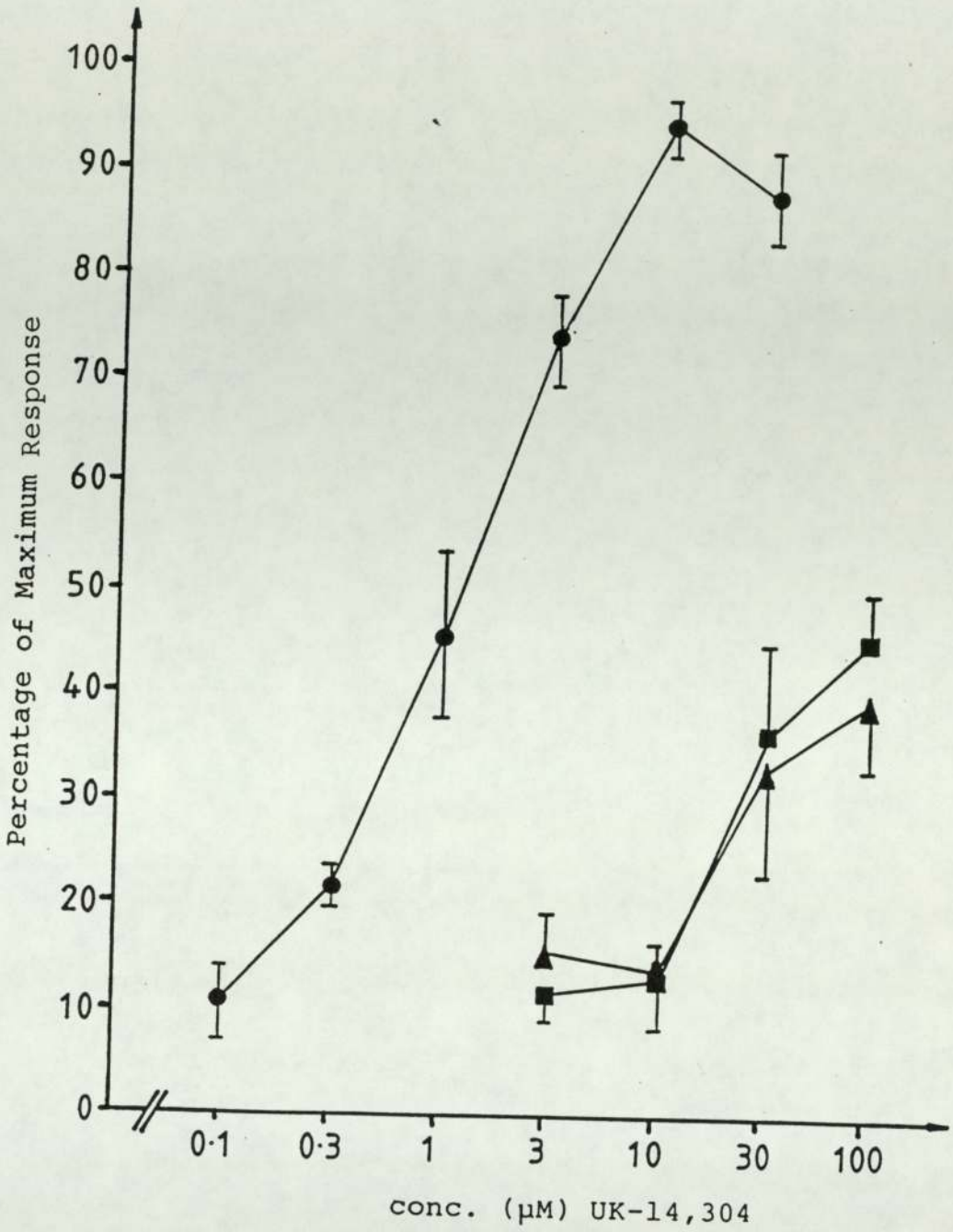



Figure 8: The effect of prazosin and of prazosin plus idazoxan on the response of the portal vein of the rat to UK-14,304 (n=6-12).
(●) control; (■) 125nM prazosin; (▲) 125nM prazosin plus 0.1μM idazoxan.

8.1.3. Discussion

The portal vein of the rat was examined for the presence of a population of postjunctional $\alpha_2$-adrenoceptors. The objective of this study was to examine the excitation - contraction coupling processes for the two subtypes of adrenoceptor. Immediately prior to the beginning of this study, Hicks (1983) reported a mixed population of postjunctional α-adrenoceptors in the portal vein of the rat. Further, he suggested that responses mediated by $\alpha_2$-adrenoceptors effected changes in the phasic activity, while $\alpha_1$-adrenoceptor mediated responses affected the tone of the vessel. In contrast, investigations by Ruffolo, Waddel and Yaden (1981), McGrath (1982) and Digges and Summers (1983) had failed to reveal a subpopulation of postjunctional $\alpha_2$-adrenoceptors in the portal vein of the rat. But in view of the overall aim of the study, it was considered worthwhile to investigate this preparation for the presence of postjunctional $\alpha_2$-adrenoceptors.

Initially, the portal vein was tested to determine whether it would respond to a selective $\alpha_2$-adrenoceptor agonist, and whether such a response could be inhibited by a selective $\alpha_2$-adrenoceptor antagonist. It has been shown in section 8.1.2.1. that the portal vein responded to the selective $\alpha_2$-adrenoceptor agonist UK-14,304 and that the responses were competitively inhibited by the selective $\alpha_2$-adrenoceptor antagonist idazoxan at concentrations (0.1 and 0.5μM) which are considered to be selective for $\alpha_2$-adrenoceptors (Doxey, Roach and Smith 1983). This might be indicative of the presence of a small population of

postjunctional $\alpha_2$-adrenoceptors in the portal vein of the rat, although it should be emphasised that the maximum response of this tissue to UK-14,304 was only approximately 30% of the maximum response of the tissue to the full agonist phenylephrine.

However, it was also possibile that UK-14,304 might be acting through $\alpha_1$-adrenoceptors and that idazoxan might similarly be less selective than expected. Therefore the effect of idazoxan was also investigated on the response to the relatively selective $\alpha_1$-adrenoceptor agonist phenylephrine (Timmermans and Van Zwieten 1982). It has been shown (figure 3B) that idazoxan (0.1 and 0.5μM) caused a rightward shift of the concentration response curve for contraction of the portal vein to phenylephrine, although the log shift at the $EC_{50}$ was less for phenylephrine than for UK-14,304 (section 8.1.2.1.). The possibility remained that UK-14,304 may have been acting through $\alpha_1$-adrenoceptors. Therefore the effect of prazosin on the responses to UK-14,304 was investigated, and it was shown (figure 4) that prazosin (5 and 125nM) caused a parallel rightward displacement of the UK-14,304 concentration response curve. Hence the response of the portal vein of the rat to UK-14,304 may be mediated, at least in part, through $\alpha_1$-adrenoceptors.

The next consideration was that the results described above may have been exclusive to the agents used. It could have been falsely concluded that postjunctional $\alpha_2$-adrenoceptors were not present when the agonists and antagonists used were not sufficiently selective to

discriminate between the receptor subtypes. In addition to this, Ruffolo and Waddell (1983) suggested that imidazolines and phenethylamines interact differently with α-adrenoceptors, which implies that the structure of an agonist may affect its interaction with receptors and hence its ability to produce a response. Therefore the effect of idazoxan was investigated upon responses to the $\alpha_1$- selective imidazoline cirazoline (Van Meel, Timmermans and Van Zwieten 1981) and the $\alpha_2$- selective phenethylamine α-methylnoradrenaline (Timmermans and Van Zwieten 1982). No difference was observed between the rightward displacement by 0.1μM idazoxan of the concentration response curve to either cirazoline or α-methylnoradrenaline. Hence there is no evidence to suggest that these agonists were acting at different subtypes of adrenoceptor.

However, the possibility remained that the results obtained so far were due to poor selectivity of the agents used. Since idazoxan caused a rightward shift of the concentration response curve to cirazoline, it is possible that idazoxan may have had a component of action on $\alpha_1$-adrenoceptors. If this is true, then the effect of idazoxan on UK-14,304 and on α-methylnoradrenaline could be due also to action at $\alpha_1$-adrenoceptors.

In support of the above, the shifts by idazoxan of the concentration response curve to UK-14,304 were parallel. If UK-14,304 was poorly selective, but the portal vein had a population of postjunctional $\alpha_2$-adrenoceptors, then it would be expected that there would be some $\alpha_2$- effect at low concentrations of the agonist. Consequently, the shift by

idazoxan would be expected to vary in accordance with the degree to which the response was mediated by $\alpha_2$-adrenoceptors. However, the shifts of the UK-14,304 concentration response curves by idazoxan were parallel, hence the evidence does not suggest that idazoxan was acting on a mixed receptor population (section 8.1.2.1.).

The next step in these experiments was to consider the effects of antagonists other than prazosin and idazoxan on the response of the portal vein to UK-14,304. Corynanthine and rauwolscine are stereoisomers and are selective antagonists of $\alpha_1$- and $\alpha_2$-adrenoceptors respectively (McGrath 1982). There was no difference between the rightward displacement of the UK-14,304 concentration response curve by 0.1μM corynanthine or 0.1μM rauwolscine (see figure 7). One possibility is that UK-14,304 may have been acting at both $\alpha_1$- and $\alpha_2$-adrenoceptors and that the antagonists used competed with the response to their respective receptor subtypes to the same extent. However, in view of the results obtained so far, it is more likely that the portal vein of the rat does not have a population of postjunctional $\alpha_2$-adrenoceptors. Consider the results of Ruffolo and Zeid (1985). These workers evaluated the response of the canine saphenous vein to cirazoline and to B-HT 933 in the presence of prazosin (10nM) and of rauwolscine (10nM). It was shown that rauwolscine did not affect the response to cirazoline but caused approximately a one and a half log cycle shift of the B-HT 933 concentration response curve at the $EC_{50}$. Similarly, prazosin had no effect on the response to B-HT 933 but shifted the

cirazoline concentration response curve to the right by approximately one log cycle (Ruffolo and Zeid 1985 p638). This is an excellent example of a significant population of postjunctional $\alpha_2$-adrenoceptors that can be considered separately from $\alpha_1$-. There has been no evidence of this kind in the portal vein of the rat.

Nevertheless, a final experiment was performed in which the effect of 0.1μM idazoxan was investigated on the response to UK-14,304 in the presence of 125nM prazosin. Prazosin was used to remove the $\alpha_1$- adrenoceptor mediated component of the response to UK-14,304, which may have allowed exposure of a response sensitive to idazoxan. Results in figure 8 show that idazoxan had no effect additive to that of prazosin. It should be noted that the highest concentration of prazosin tested was 125nM. It is possible that if a higher concentration of prazosin were used, the response to UK-14,304 may have been abolished and the total response could have been said to have been due to activation of $\alpha_1$-adrenoceptors. Alternatively, UK-14,304 could have been activating receptors other than adrenoceptors. Therefore the observations which led to the conclusion that there is a significant population of postjunctional $\alpha_2$-adrenoceptors on the portal vein of the rat, should be examined in more detail.

The results by Hicks (1983) which led to the above conclusion may have been obtained by the unusual method of eliciting responses to agonists, or the way in which this worker measured results. The method used by Hicks for eliciting responses to agonists, was to bathe the tissues in

calcium free medium containing a fixed concentration of agonist. Then, calcium was added to the medium in successively increasing concentrations. The response of the portal vein was then evaluated by summing the vertical height of every rising phase of the phasic activity over a fixed time period. The rationale behind this method of measuring the responses was that increases in tension of the tissue were preceded by a burst of electrical activity, and associated with the mobilisation of calcium (Hicks 1983). However, the conclusions of Hicks would appear to be based on a false premiss; that activation of $\alpha_2$-adrenoceptors causes an increase in phasic activity while $\alpha_1$-adrenoceptor activation causes an increase in tone. There is little evidence to support this distinction of function between the receptors. While it is agreed that responses to UK-14,304 cause changes in phasic activity of the portal vein, while $\alpha_1$-adrenoceptor mediated responses generally cause increases in tone, low concentrations of all the potent agonists tested caused an increase in phasic activity without increasing the tone. This is illustrated in figure 1C by cirazoline, which is a potent and highly selective agonist at $\alpha_1$-adrenoceptors, and at a concentration of 0.01μM increased only the phasic activity of the tissue. In the same context, if Hicks' theory is correct, the relatively selective $\alpha_2$-adrenoceptor agonist α-methylnoradrenaline would not have been expected to produce the observed increase in tone. Again it may be argued that high concentrations of α-methylnoradrenaline were acting at $\alpha_1$-adrenoceptors, but it is unlikely that low concentrations

of cirazoline were acting at the $\alpha_2$- subtype.

Finally, it should be emphasised that the maximum response of the tissue to UK-14,304 represents only 30% of the response to $\alpha_1$-adrenoceptor agonists. Also, that approximately 50% of the response to UK-14,304 was sensitive to inhibition by the $\alpha_1$-adrenoceptor antagonist prazosin.

In summary:

1. The portal vein of the rat was examined for the presence of postjunctional $\alpha_2$-adrenoceptors.
2. Responses mediated by UK-14,304 and by phenylephrine were attenuated by 0.1μM idazoxan, but the magnitude of the response to UK-14,304 was only some 30% of the maximum response to phenylephrine.
3. The response to UK-14,304 was attenuated by low concentrations of prazosin (5-125nM) suggesting that UK-14,304 may be acting at $\alpha_1$-adrenoceptors.
4. There was no significant difference between the shift at the $EC_{50}$ of the concentration response curve to UK-14,304 by either 0.1μM corynanthine or 0.1μM rauwolscine.
5. In conclusion, there is insufficient evidence to suggest that the portal vein of the rat has a significant population of postjunctional $\alpha_2$-adrenoceptors and it follows that this tissue is not a suitable model with which to study α-adrenoceptor subtypes.

## 8.2. PITHED RAT

### 8.2.1. Introduction

In the previous section it was shown that the portal vein of the rat does not have a significant population of post-junctional $\alpha_2$-adrenoceptors. Therefore, an alternative system in which these receptors have been demonstrated was studied. It was considered worthwhile to further studies on the mobilisation of calcium following activation of $\alpha_1$- or $\alpha_2$-adrenoceptor subtypes.

The first demonstration of a mixed population of post-junctional $\alpha_2$-adrenoceptors was in the pithed rat (Drew and Whiting 1979). Since then, it has been shown repeatedly that $\alpha_2$-adrenoceptor mediated pressor responses of the pithed rat are sensitive to calcium channel antagonists while responses mediated by $\alpha_1$-adrenoceptor agonists are, in general only modestly inhibited by these agents (Van Zwieten *et al* 1982 and 1983; Vanhoutte and Rimele 1982; Cavero *et al* 1983 and Timmermans *et al* 1984). It was suggested by Van Zwieten *et al* (1982) that an influx of extracellular calcium is implicated in vasoconstriction initiated by $\alpha_2$-adrenoceptor stimulation. In addition, an influx of extracellular calcium may not play a significant role in the pressor response mediated by $\alpha_1$-adrenoceptor agonists.

It was therefore decided to examine the difference in calcium activation mechanisms for the two α-adrenoceptor subtypes, in the pithed rat. Two points were addressed. The first was to consider whether differences in the time course of $\alpha_1$- and $\alpha_2$-adrenoceptor mediated pressor responses in the pithed rat might account for differences in sensitivity of

the responses to calcium channel antagonists.

Timmermans and Van Zwieten (1980) demonstrated that the pressor response of the pithed rat to intravenously administered $\alpha_1$- and $\alpha_2$-adrenoceptor agonists have a different time course. Thus the maximum response to the $\alpha_2$-adrenoceptor agonist B-HT 933 was achieved one to two minutes after administration, but the peak pressor effect to phenylephrine was achieved in only fifteen seconds. The pressor response of the pithed rat to the relatively selective $\alpha_2$- agonist clonidine has also been shown to have a slow time course (Docherty and McGrath 1980c). These workers compared plasma concentrations of the agonist with the pressor responses and concluded that the effect of clonidine in this preparation should be measured at five minutes after injection for optimum correlation between pre- and postjunctional effects.

In a separate investigation, Docherty and McGrath (1980a) studied the pressor response of the pithed rat to several α-adrenoceptor agonists with varying selectivities for $\alpha_1$- and $\alpha_2$-adrenoceptors. All except phenylephrine were assessed by measuring the pressor responses five minutes after injection. It was found that the response to phenylephrine was of short duration and so the peak pressor effect of this agonist was compared with the effects of the other agonists at five minutes.

In view of these differences in time course of $\alpha_1$- and $\alpha_2$-adrenoceptor mediated pressor responses in the pithed rat, it was considered worthwhile to reinvestigate the sensitivity of $\alpha_1$- and $\alpha_2$-adrenoceptor mediated responses to

calcium channel antagonists, under comparable conditions. This was achieved by administering the α-adrenoceptor agonists by the method of continuous infusion.

The second point concerned the receptor reserve of the agonist in the pithed rat. In 1983, Hamilton, Reid and Sumner demonstrated the presence of a receptor reserve for $\alpha_1$-adrenoceptors but not for $\alpha_2$-adrenoceptors in the vasculature of conscious rabbits. Ruffolo and Yaden (1984) extended this study to pithed rats. They showed that only 20% receptor occupancy was necessary to elicit a maximum pressor response to the selective $\alpha_1$-adrenoceptor agonist cirazoline but that the $\alpha_2$- adrenoceptor agonist B-HT 933 exhibited no receptor reserve. On the assumption that the profile of inhibition described by calcium channel antagonists resembles that of non-competitive antagonists of α-adrenoceptors; they suggested that if a receptor reserve existed for postjunctional $\alpha_1$- but not for postjunctional $\alpha_2$-adrenoceptors, then the calcium channel antagonists would affect $\alpha_2$- mediated vasoconstriction to a greater degree than $\alpha_1$- even if both processes were equally dependent upon calcium translocation.

Subsequently, Ruffolo, Morgan and Messick (1984) concluded that differences in the effect of calcium channel antagonists on vascular $\alpha_1$- and $\alpha_2$-adrenoceptors may result from differences in their respective receptor reserves and may not completely reflect differences between the utilisation of calcium by the receptor subtypes.

In the present study, it was considered worthwhile to extend the observations of Ruffolo et al (1984) to consider

the competitive antagonists prazosin and corynanthine. The reasoning behind this was derived from *in vitro* observations. In 1983, it was shown by Downing and co-workers that prazosin selectively inhibits the fast phase of the response of the isolated rat aorta to noradrenaline; an effect not seen with corynanthine (Downing, Wilson and Wilson 1983). It was later suggested by these workers that the selective effect of prazosin on the fast phase may have been due to the slow rate of dissociation of the antagonist from adrenoceptors, thereby reducing the rate of rise of receptor occupancy of the agonist (Downing, Wilson and Wilson 1985). It has been suggested that in the rabbit aorta, the initial, fast phase of the response to noradrenaline may be attributed to the release of intracelular calcium (Godfraind and Kaba 1972). Timmermans *et al* (1985) have suggested that in the pithed rat, phenoxybenzamine may have a selective effect on the component of the response which is not due to an influx of extracellular calcium. It was therefore considered that this effect of phenoxybenzamine could have been due to its slow dissociation rate. If this were true, then it would also be expected to be true of prazosin, but not of corynanthine. Therefore, investigations were carried out to examine the effect of prazosin and corynanthine, as well as of phenoxybenzamine on the diltiazem sensitivity of the pressor response of the pithed rat to cirazoline.

8.2.2. Results

8.2.2.1. The effect of diltiazem on the response of the pithed rat to infusions of UK-14,304 and phenylephrine.

The pressor responses of the pithed rat to infusions of UK-14,304 and of phenylephrine were evaluated in order to evaluate the responses to the agonists over a comparable time course.

The mean resting diastolic blood pressure of pithed normotensive rats prior to drug treatment was 31.8±0.5mmHg (n=113).The pressor response of the pithed rat to continuous infusions of UK-14,304 is shown in figures 9A and B. The maximum pressor effect to 100µg/Kg/min UK-14,304 was 62.4±5.2mmHg (figure 10A, n=7). Preliminary experiments revealed that it was not possible to construct cumulative dose response curves to infusions of UK-14,304. because such sequential administration of UK-14,304 produced unstable responses, possibly due to desensitisation of receptors. It was also found that using the method of continous infusion of agonist, it was difficult practically, to construct full dose response curves. Consequently it was considered misleading to term the dose of agonist causing a 50% maximum response as an $ED_{50}$ and this has been referred to as the dose for the half maximum response.

Diltiazem (12.5µg/Kg/min) caused a parallel shift of the dose response curve to UK-14,304 to the right with no suppression of the maximum response (figure 10, $p>0.05$, n=7). Infusion of diltiazem (25µg/Kg/min) caused a mean decrease in the resting diastolic blood pressure of 9.7±1.3mmHg (n=12); produced decreases in the slope of the

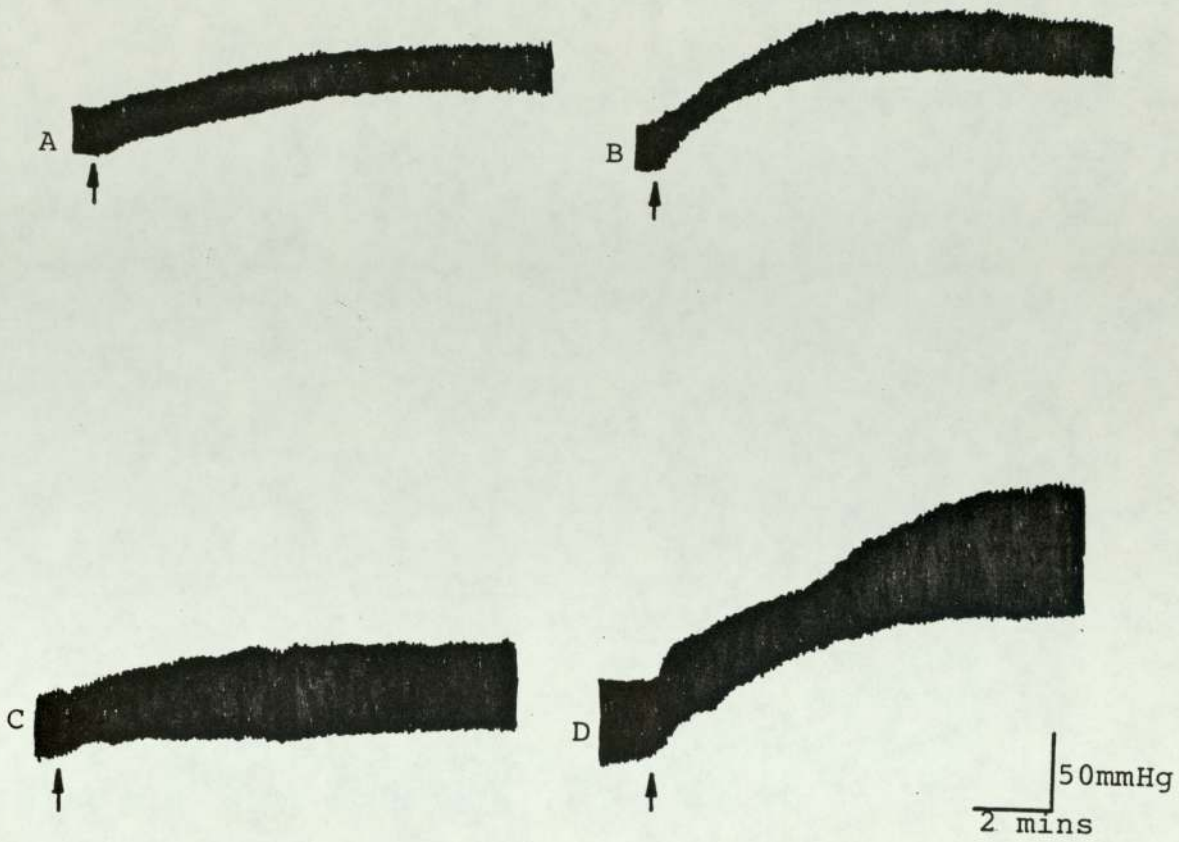



Figure 9: Representative traces showing the response of the pithed rat to infusions of A. 10µg/Kg/min UK-14,304, B. 100µg/Kg/min UK-14,304, C. 1µg/Kg/min phenylephrine and D. 10µg/Kg/min phenylephrine.
Arrows indicate start of infusion.

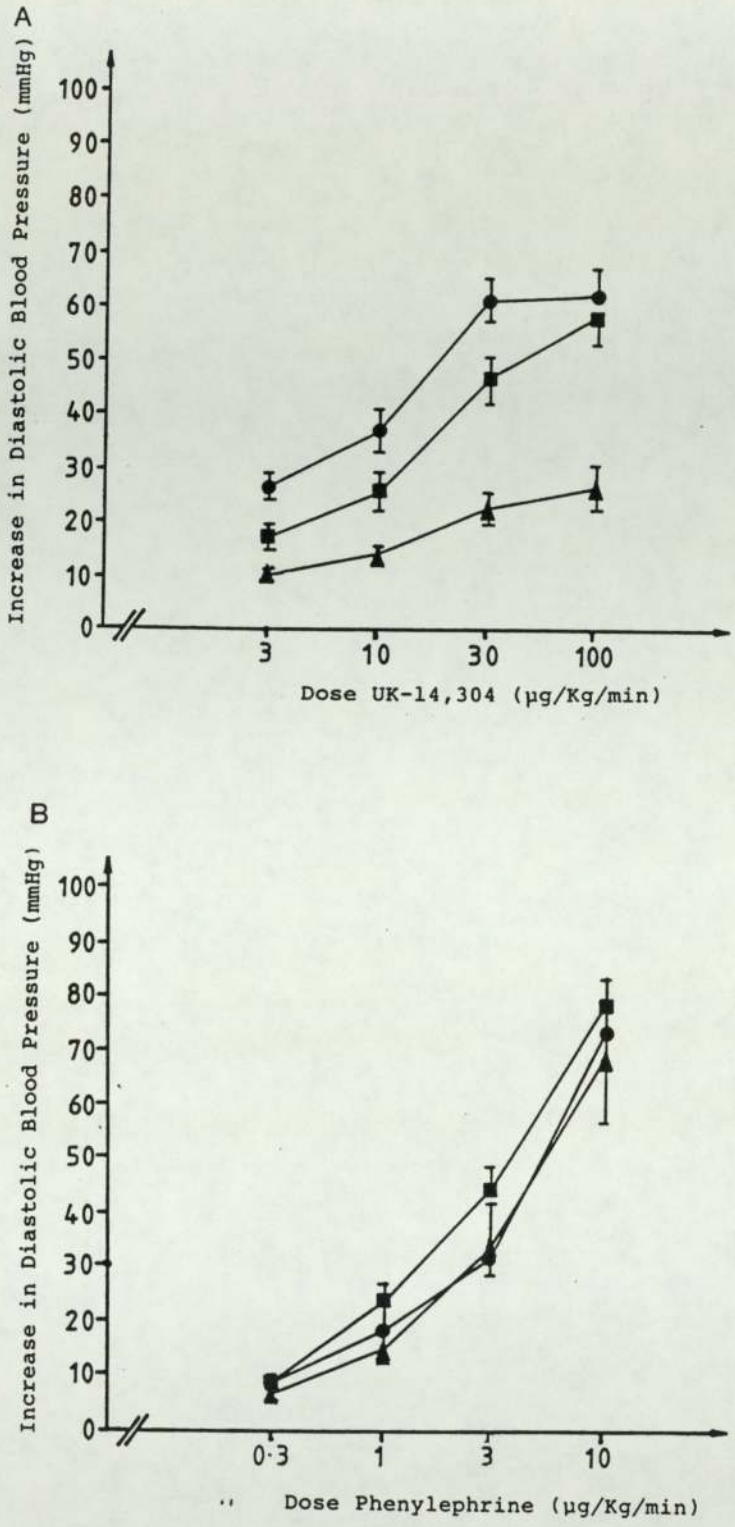



Figure 10: The effect of diltiazem on the pressor response of the pithed rat to A. UK-14,304 (n=7) and to B. phenylephrine (n=7).
(●) control; (■) 12.5µg/Kg/min diltiazem; (▲) 25µg/Kg/min diltiazem.

UK-14,304 dose response curve, and a reduction of the maximum. The increase in diastolic blood pressure induced by the maximum dose of UK-14,304 tested (100µg/Kg/min) was 58.6±4.8mmHg and 26.4±3.8mmHg in the presence of 12.5 and 25µg/Kg/min diltiazem respectively (figure 10A, $p<0.01$, $n=7$).

Continuous infusion of 10µg/Kg/min phenylephrine caused an increase in diastolic blood pressure of 73.9±7.4mmHg (figure 10B, $n=7$) There was also an increase in the pulse pressure at higher concentrations of this agonist (figure 9C and D). The increase in pulse pressure was probably due to the $\beta_1$- agonist effects of phenylephrine (Lefevre, Fenard and Cavero 1977) even though the preparation had been pretreated with propranolol (750µg/Kg - section 7.1.3.).

Diltiazem at concentrations that caused rightward displacement of the UK-14,304 dose response curve, failed to affect the pressor response of the pithed rat to infusions of phenylephrine (figure 10B). Following administration of 12.5 and 25µg/Kg/min diltiazem, the increases in diastolic blood pressure induced by an infusion of phenylephrine of 10µg/Kg/min was 79.3±4.9 and 69.3±11.4 mmHg respectively. These values are not significantly different from the control pressor response in the absence of diltiazem. ($p>0.05$, $n=7$).

Therefore there is no evidence to suggest that the time course of the response is a significant factor in determining the sensitivity of $\alpha_1$- and $\alpha_2$-adrenoceptor mediated pressor responses of the pithed rat to diltiazem.

8.2.2.2. The effect of prazosin on the response of the pithed rat to infusions of phenylephrine with or without diltiazem.

Preliminary experiments were carried out to examine the possibility that the competitive antagonist prazosin could be used to expose a diltiazem sensitive component of the pithed rat to continuous infusions of phenylephrine. A dose of prazosin was selected which would cause parallel rightward displacement of the dose response curve to phenylephrine such that higher doses of the antagonist could have caused further displacement, but with a decrease in slope and suppression of the maximum response. It was intended that these conditions, would be comparable with reduced receptor reserve. Figure 11 illustrates the diastolic pressor response of the pithed rat to infusions of phenylephrine with or without pretreatment with prazosin (1.0mg/Kg) or simultaneous infusion of diltiazem (25µg/Kg/min).

The dose of prazosin selected (1.0mg/Kg) caused a highly significant parallel rightward displacement of the pressor response curve to phenylephrine by approximately 1.6 log cycles measured at 50% of the maximum dose tested (figure 11, $p<0.001$, $n=7$). No suppression of the maximum was observed.

Diltiazem (25µg/Kg/min) caused a significant reduction of the diastolic pressor response to 1000µg/Kg/min phenylephrine in the presence of prazosin (1.0mg/Kg), from 102.4±5.0 to 70.5±6.9mmHg (figure 11, $p<0.01$, $n=7$). The dose of phenylephrine which caused 50% of the maximum response in

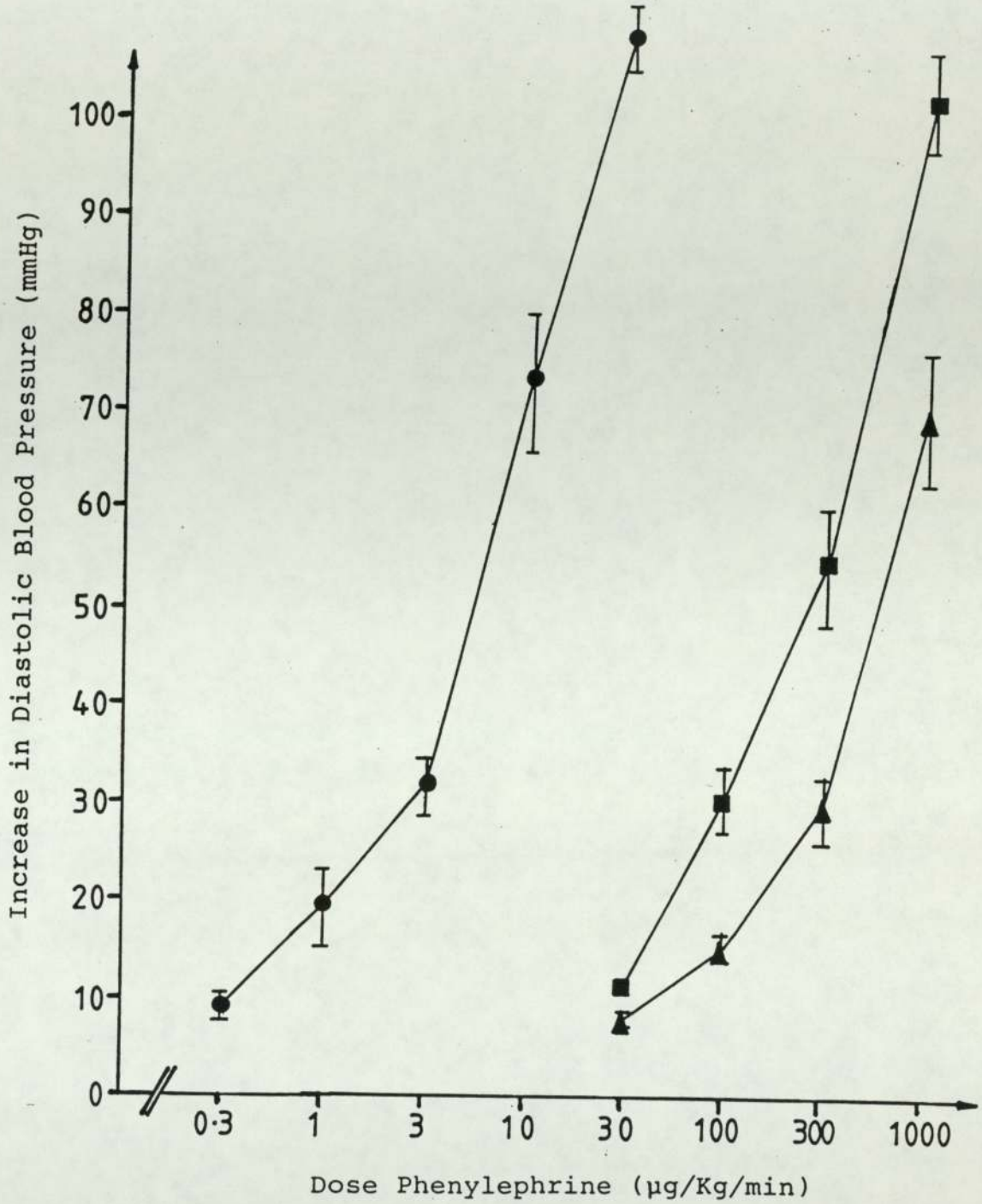



Figure 11: The effect of prazosin and of prazosin plus diltiazem on the pressor response of the pithed rat to phenylephrine (n=7).
(●) control; (■) 1.0mg/Kg prazosin; (▲) 1.0mg/Kg prazosin plus 25µg/Kg/min diltiazem.

the presence of prazosin was significantly increased by concurrent infusion of diltiazem from 219.5±2.8 to 272.4±16.0µg/Kg/min (figure 11, $p<0.05$, n=7). Thus the competitve antagonist prazosin exposed a diltiazem sensitive component of the pressor response of the pithed rat to infusions of phenylephrine.

8.2.2.3. The effect of diltiazem and yohimbine on the pressor response of the pithed rat to cirazoline.

In view of the exposure by prazosin of a diltiazem sensitive component of the pressor response of the pithed rat to phenylephrine, it was considered that the experiments should be extended to include corynanthine. The following experiments were carried out using a similar protocol to that of Ruffolo et al (1984) so that comparisons could be made between their results and those of the present study. For this purpose, cirazoline was administered by cumulative, bolus injection. Preliminary experiments were performed to determine the effect of diltiazem and of yohimbine on the pressor response to cirazoline. In an initial experiment it was established that 0.3mg/Kg yohimbine reduced pressor responses of the pithed rat to UK-14,304 (results not shown).

The dose of cirazoline that produced a 50% of maximum pressor response was 1.1±0.23µg/Kg. The maximum dose tested (10µg/Kg) increased the diastolic blood pressure by 109.4±6.2mmHg (figure 13, n=7). Neither yohimbine (0.3mg/Kg) nor diltiazem (25µg/Kg/min) had any significant effect on

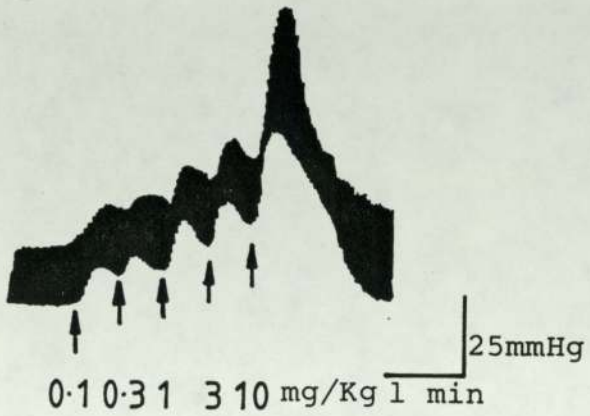



Figure 12: Representative trace showing the pressor response of the pithed rat to cirazoline.

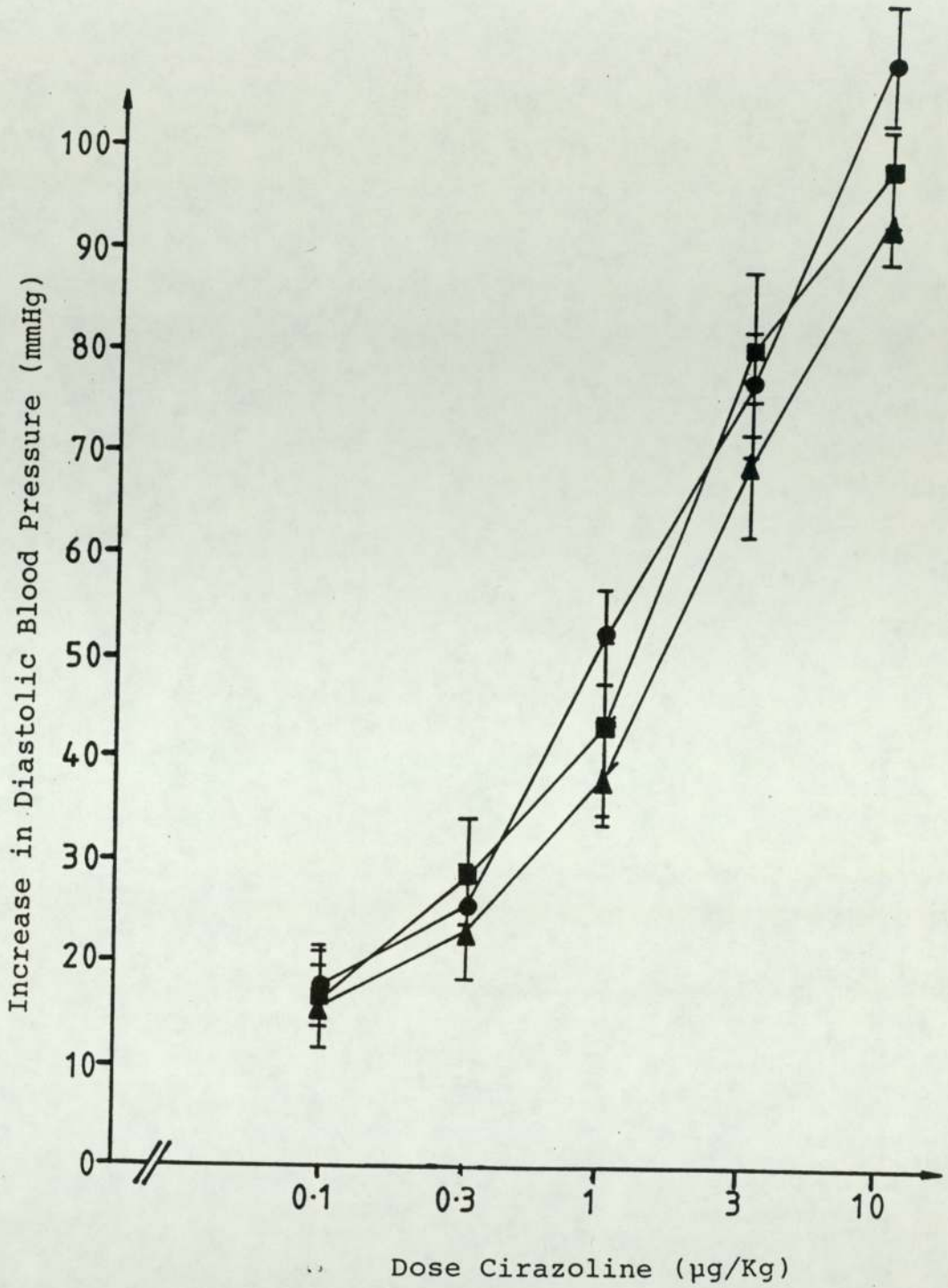



Figure 13: The effect of diltiazem and of yohimbine on the pressor response of the pithed rat to cirazoline (n=5-7). (●) control; (■) 0.3mg/Kg yohimbine; (▲) 25µg/Kg/min diltiazem.

the cirazoline dose response curve. The dose that produced a half maximum response in the presence of yohimbine (10µg/Kg) was 0.9±0.19µg/Kg and the diastolic pressor response to 10µg/Kg cirazoline was 98.2±4.9mmHg (figure 13, n=5). These values are not significantly different from control values for cirazoline in the absence of yohimbine(1.1±0.23µg/Kg, $p>0.05$).

The pressor response of the pithed rat to cirazoline in the presence of diltiazem (25µg/Kg/min) was not significantly different from control values at either the dose that produced the half maximum response (1.1±0.2µg/Kg) or the response to the maximum dose of cirazoline tested (93.8±5.2mmHg; $p>0.05$, n=5).

8.2.2.4. The influence of phenoxybenzamine on the effect of yohimbine and diltiazem on the pressor response of the pithed rat to cirazoline.

Phenoxybenzamine (0.2mg/Kg) caused a significant shift of the cirazoline dose response curve to the right by approximately one log cycle at the dose producing the half maximum response, from 1.1±0.23µg/Kg to 12.1±5.3µg/Kg ($p<0.05$). There was a decrease in the slope, and a suppression of the maximum response, to 81.8±3.3mmHg (figure 14, $p<0.05$, n=6).

Diltiazem (25µg/Kg/min), at a concentration that alone did not affect the control curve to cirazoline (figure 12), in the presence of phenoxybenzamine (0.2mg/Kg) caused a further reduction of both the slope and of the maximum response to cirazoline to 36.2±7.8mmHg (figure 14, $p<0.01$,

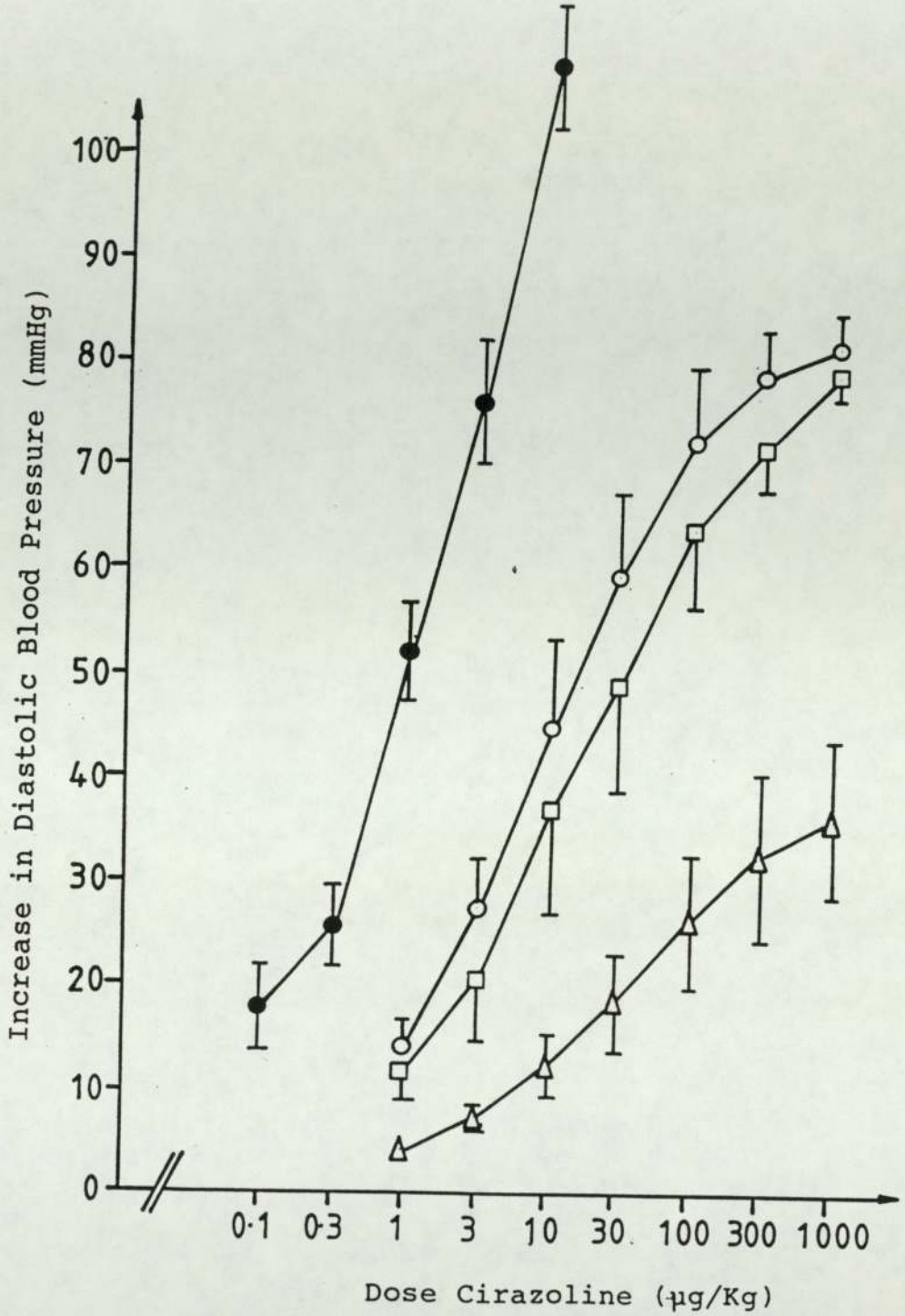



Figure 14: The effect on the pressor response of the pithed rat to cirazoline of pretreatment with phenoxybenzamine alone and of phenoxybenzamine followed by yohimbine or by diltiazem.(n=5-7).
(●) control; (○)0.2mg/Kg phenoxybenzamine (PBZ);
(□) PBZ plus 0.3mg/Kg yohimbine; (Δ) PBZ plus 25µg/Kg/min diltiazem.

n=5). However, the dose for the half maximum response to cirazoline in the presence of both phenoxybenzamine and diltiazem was 30.0±7.9µg/Kg, which is not significantly different from the half maximum response to cirazoline in the presence of phenoxybenzamine alone ($p>0.05$).

Yohimbine (0.3mg/Kg) had no effect on the cirazoline dose response curve following treatment with phenoxybenzamine (figure 14). The dose for the half maximum response in the presence of yohimbine was 23.6±11.1µg/Kg and the maximum was 79.6±2.2 mmHg ($p>0.05$, n=5).

Therefore, the observation by Ruffolo et al (1984) that phenoxybenzamine exposes a diltiazem sensitive component of the pressor response of the pithed rat, is supported by the results presented here. Furthermore, it has been shown that there is no effect of yohimbine (0.3mg/Kg) on the pressor response to cirazoline in the presence of phenoxybenzamine. Thus the diltiazem sensitive component of the pressor response of the pithed rat to cirazoline is not due to cirazoline acting non-selectively at $\alpha_2$-adrenoceptors.

### 8.2.2.5. The effect of prazosin on the cirazoline dose pressor response curve and the effect of diltiazem and yohimbine.

For the further study of the effect of a competitive antagonist on the diltiazem sensitivity of the pressor response of the pithed rat to cirazoline, prazosin was chosen for reasons of its potency and selectivity at $\alpha_1$-adrenoceptors (Cambridge et al 1977).

A dose of prazosin was chosen (0.3mg/Kg) which shifted the cirazoline dose response curve to the right by approximately 1.9 log cycles at the the dose for the half maximum response, from 1.1±0.23µg/Kg to 88.4±18.3µg/Kg. There was no change in the slope or suppression of the response to the maximum dose of cirazoline tested (figure 15, $p>0.05$, $n=6$). When administered after prazosin, diltiazem (25 µg/Kg/min) caused a significant further displacement of the dose response curve to cirazoline by approximately 0.3 log cycles, measured at the dose for the half maximum response, to a new half maximum of 166.3±26.1µg/Kg (figure 15, $p<0.05$, $n=5$), There was no significant change of slope.

Yohimbine (0.3mg/Kg) in the presence of prazosin (0.3mg/Kg), caused a significant reduction of the pressor responses of the pithed rat to cirazoline over the dose range 100-1000µg/Kg ($p<0.05$), but the rightward shift of the dose response curve was not significant. There was no significant difference between the effect of diltiazem or yohimbine on the pressor responses of the pithed rat to cirazoline following pretreatment with prazosin. Thus the results of this section demonstrate the exposure of a diltiazem sensitive component of the pressor response of the pithed rat to cirazoline by the competitive antagonist prazosin. However, in contrast to the results with phenoxybenzamine, prazosin revealed a yohimbine sensitive component of the response to cirazoline at the highest three doses of cirazoline tested.

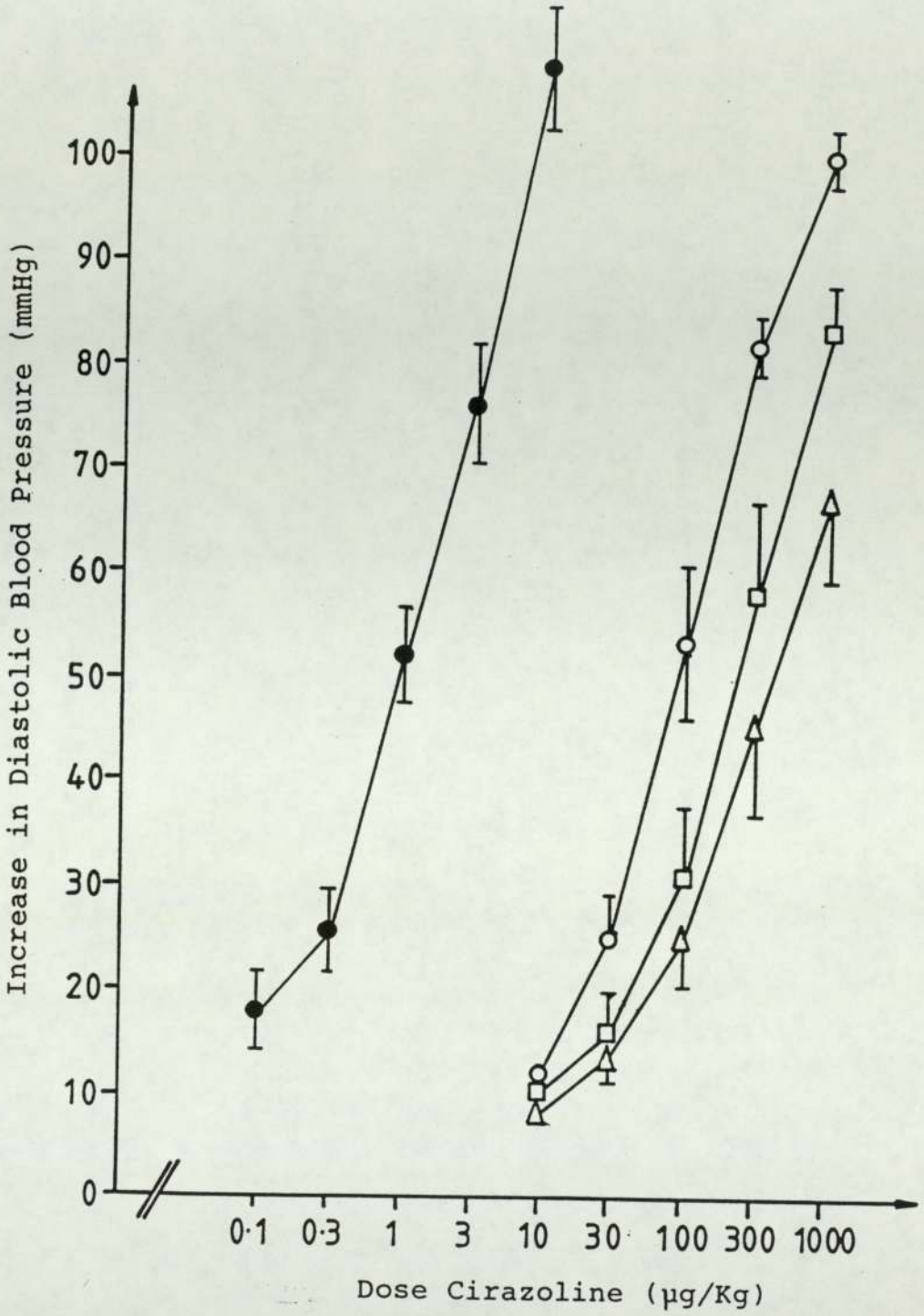



Figure 15: The effect of prazosin alone and of prazosin plus yohimbine or diltiazem on the pressor response of the pithed rat to cirazoline (n=5-7).
(●) control; (○) 0.3mg/Kg prazosin (PZ); (□) PZ plus 0.3mg/Kg yohimbine; (Δ) PZ plus 25µg/Kg/min diltiazem.

8.2.2.6. The effect of corynanthine on the cirazoline dose pressor response curve of the pithed rat, and the effect of diltiazem

To ensure that the results described in section 8.2.2.5. (above) were not unique to prazosin, the effect of corynanthine was also examined on the dose pressor response curve to cirazoline.

Figure 16 illustrates the effect of corynanthine on the pressor response of the pithed rat to cirazoline. At a concentration of 10mg/Kg, the antagonist caused significant rightward displacement of the cirazoline dose pressor response curve by approximately 1.5 log cycles measured at the dose for the half maximum response, which represents a change from 1.1±0.23µg/Kg (n=7) to 33.4±5.38µg/Kg (n=4, $p<0.01$, figure 16). When diltiazem (25µg/Kg/min) was infused in addition to corynanthine, there was a further shift to the right of the cirazoline dose response curve of approximately 0.25 log cycles, which represented a significant change in the dose for the half maximum response to 58.8±6.8µg/Kg ($p<0.05$, n=5). All curves in this experiment were parallel but the maximum response to 1000µg/Kg cirazoline in the presence of corynanthine (10mg/Kg) was depressed by approximately 10mmHg in the additional presence of diltiazem (figure 16, $p<0.05$, n=5).

The results show that, like prazosin, corynanthine (10mg/Kg) also exposes a diltiazem sensitive component of the pressor response of the pithed rat to cirazoline.

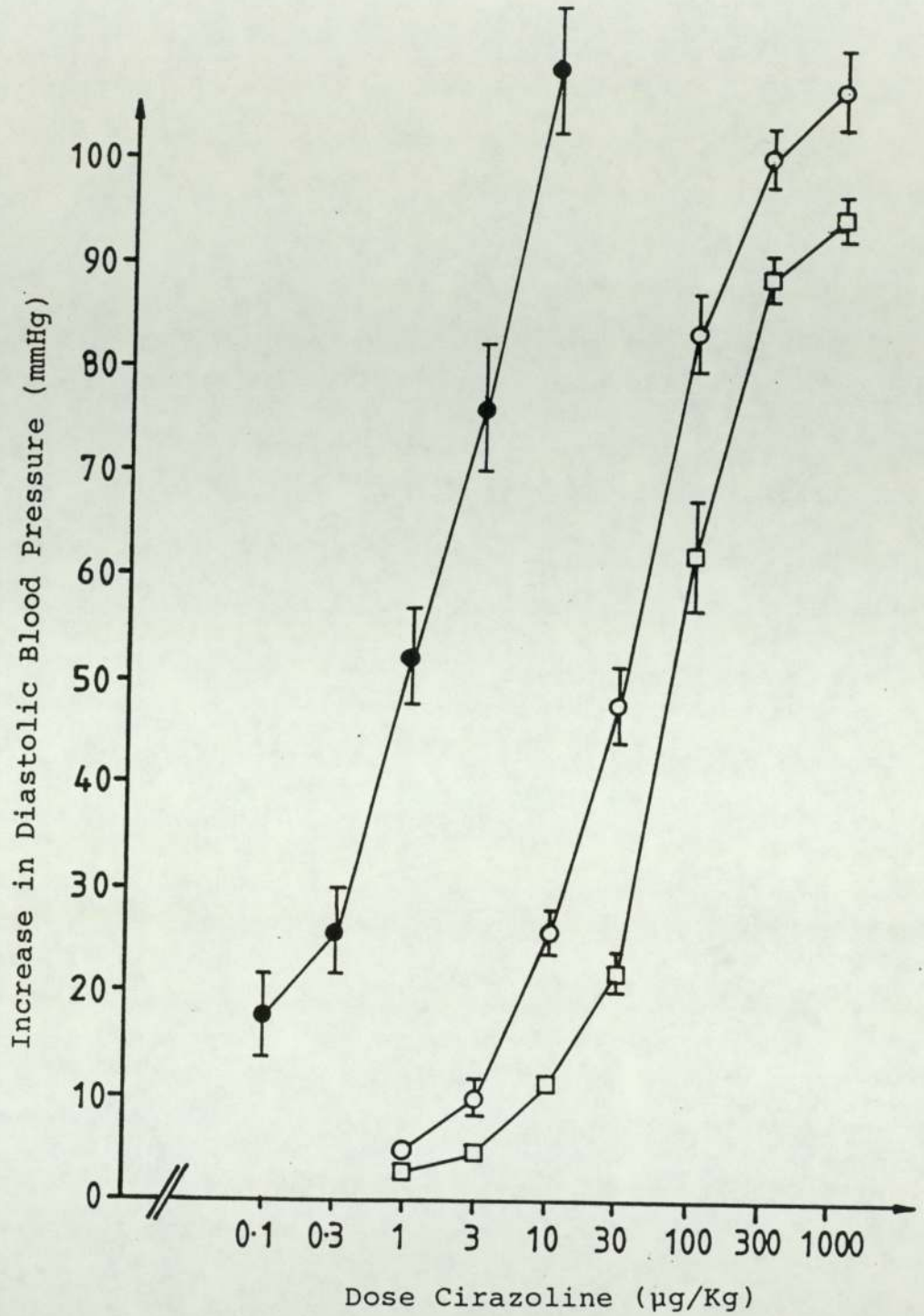



Figure 16: The effect of corynanthine or diltiazem on the pressor response of the pithed rat to cirazoline (n=4-7). (●) control; (○) 10mg/Kg corynanthine (CORY); (□) CORY plus 25µg/Kg/min diltiazem.

8.2.3.Discussion

In this section, two questions were adressed. Firstly, whether the time course of the pressor responses of the pithed rat to $\alpha_1$- and $\alpha_2$-adrenoceptor agonists were important in determining their sensitivity to calcium channel antagonists, and secondly, whether prazosin and/or corynanthine could expose a diltiazem sensitive component of the pressor response of the pithed rat to cirazoline.

In adressing the first question, it was considered whether the rapid rise and fall of the pressor response of the pithed rat to $\alpha_1$-adrenoceptor agonists could have been due to a release of intracellular calcium, in a way analogous to that seen in vascular smooth muscle *in vitro* where the initial, rapid rise in tension may be attributed to calcium release (Godfraind and Kaba 1972). It therefore seemed pertinent to ask whether the rapid rise in pressor response seen with $\alpha_1$- agonists could similarly reflect intracellular calcium release. The pressor response of the pithed rat to $\alpha_2$-adrenoceptor agonists is slower than to $\alpha_1$-, and it may be that the faster $\alpha_1$- response is completed before the calcium influx occurs.

The reason for a difference in the time course of $\alpha_1$- compared with $\alpha_2$-adrenoceptor mediated pressor responses may not be pharmacological but pharmacokinetic. The time course of the plasma concentration of a drug in the whole animal is dependent upon many different factors such as lipophilicity or the ionic charge of the molecule, the detoxification pathway and the means of excretion. When an agonist is given as a bolus injection, maximum plasma levels are achieved

within a few seconds and then decline (Docherty and McGrath 1980c). It is possible that the difference between the time course of the pressor response of the pithed rat to selective $\alpha_1$- and $\alpha_2$-adrenoceptor agonists, as reported by Timmermans and Van Zwieten (1980), may be due to a difference in the way the drugs are (pharmacokinetically) distributed within the animal.

Godfraind and Kaba (1972) described experiments in the rabbit aorta in which noradrenaline elicited a biphasic response. They attributed the initial fast phase of the response to the release of intracellular calcium, and the slow, sustained phase to an influx of extracellular calcium. If the same were true in vivo as in vitro then if $\alpha_1$-adrenoceptor agonists were able to combine rapidly with $\alpha_1$-adrenoceptors and cause the release of intracellular calcium, then the response would not be expected to be sensitive to calcium channel antagonists. Alternatively, if rapid association with adrenoceptors is not possible for $\alpha_2$-adrenoceptor agonists but the pharmacokinetics determine that the agonists remain in the region of the receptors for a long time, then the response may be analogous with the slow phase of the response of the rabbit aorta and may be sensitive to calcium channel antagonism.

In the pithed rat it was not possible to achieve a rapid response to $\alpha_2$-adrenoceptor stimulation and so mimic the time course of the $\alpha_1$- response. However it was possible to administer $\alpha_1$- agonists by continuous infusion which might have achieved a response which resembled that of the $\alpha_2$-adrenoceptor mediated stimulation. The method of

continuous infusion has the additional advantage that equilibrium conditions between drug and receptor could be approached.

The results described in section 8.2.2.1. clearly support the hypothesis of Van Zwieten et al (1982), and extend it to include conditions of continuous infusion. The pressor responses to the $\alpha_2$-adrenoceptor agonist UK-14,304, were sensitive to diltiazem; whereas the responses to the preferential $\alpha_1$-adrenoceptor agonist phenylephrine, were resistant. Therefore the results shown are in accord with the hypothesis that $\alpha_1$-adrenoceptor mediated responses of the pithed rat are resistant to calcium channel antagonism.

An alternative explanation for the differential sensitivity of $\alpha_1$- and $\alpha_2$-adrenoceptor mediated pressor responses to calcium channel antagonists was provided by Ruffolo, Morgan and Messick (1984). They used phenoxybenzamine to reduce $\alpha_1$-adrenoceptor reserve in the vascular system of the pithed rat. As a result of this treatment, a calcium influx dependent component of the pressor response to cirazoline was exposed. Therefore, in the second part of this section of the present study, it was considered worthwhile to re-examine the hypothesis of Ruffolo et al (1984), to determine whether a diltiazem sensitive component of the pressor response of the pithed rat to cirazoline could be uncovered with prazosin or with corynanthine. The reasoning behind this was that prazosin has a slow dissociation rate and may have selectively reduced the component of the response due to intracellular calcium release (Downing et al 1985). The same would not

be expected to be true of corynanthine.

Phenoxybenzamine (0.2mg/Kg) was shown to cause approximately a one and a half log cycle shift to the right of the cirazoline dose-pressor response curve, with a decrease in slope and suppression of the maximum (section 8.2.2.4.). The change in the dose response curve to cirazoline suggests that this agonist does not have a receptor reserve in the pithed rat in the presence of 0.2mg/Kg phenoxybenzamine. Prazosin was chosen for its potency and selectivity as an antagonist of $\alpha_1$-adrenoceptors (Cambridge et al 1977) and for its slow dissociation rate (Downing et al 1983 and 1985). In order to compare the effect of prazosin with that of phenoxybenzamine, a dose of prazosin was selected which caused a parallel two log cycle shift to the right of the cirazoline dose pressor response curve. With a two log cycle shift, it was thought that the competitive antagonist was present at a dose sufficient to displace the response to cirazoline to the limit of its receptor reserve. It was shown in section 8.2.2.5. that 0.3mg/Kg prazosin exposes a diltiazem sensitive component of the pressor response of the pithed rat to cirazoline.

At a dose of 10mg/Kg, corynanthine caused a one and a half log cycle shift of the control cirazoline pressor response curve. This also exposed a diltiazem sensitive component not seen in the absence of $\alpha_1$-adrenoceptor antagonism. Since prazosin caused a two log cycle shift with no reduction of the slope or suppression of the maximum response, it is probable that following treatment with corynanthine (which caused a one and a half log cycle shift)

receptor reserve to cirazoline was still present. Therefore, elimination of receptor reserve was not necessary in order to expose a diltiazem sensitive component of the pressor response of the pithed rat to cirazoline. Furthermore, exposure of this component cannot be explained in terms of the slow dissociation of either prazosin or phenoxybenzamine from adrenoceptors.

It was considered that the inhibitory effect of diltiazem might have been due, at least in part, to exposure of an $\alpha_2$-component of the cirazoline response. The reasoning behind this is that diltiazem, in common with other calcium channel antagonists has been shown to reduce the pressor response of pithed rat mediated by $\alpha_2$-adrenoceptor agonists (Van Zwieten, Van Meel and Timmermans 1982 and 1983, Cavero et al 1983). To investigate this possibility, the effect of yohimbine (0.3mg/Kg) on the dose-pressor response curve of the pithed rat to cirazoline, with or without pretreatment with phenoxybenzamine (0.2mg/Kg) or prazosin (0.3mg/Kg) was investigated. The results failed to show a yohimbine sensitive component of the pressor response of the pithed rat to cirazoline, except for the highest three concentrations of cirazoline (100-1000µg/Kg) following pretreatment with prazosin. Therefore, following treatment with phenoxybenzamine, the evidence does not suggest that cirazoline has been acting non-selectively at postjunctional $\alpha_2$-adrenoceptors. In addition, the pressor response of the pithed rat to the single dose of cirazoline (10µg/Kg) should be considered. In the absence of corynanthine, this pressor response was not susceptible to inhibition by yohimbine

(0.3mg/Kg), or diltiazem (25µg/Kg/min, figure 13). However, in the presence of corynanthine (10mg/Kg) the response was inhibited by diltiazem (figure 16). Therefore at this dose (10µg/Kg) cirazoline was not acting non-selectively at $\alpha_2$-adrenoceptors. If it were, it would be expected that an inhibitory effect of yohimbine, in the absence of corynanthine, would have been observed. Yet a diltiazem sensitive component had apparently been uncovered.

Additional support that reduction of receptor reserve in the pithed rat does not expose an $\alpha_2$- component of the pressor response to an $\alpha_1$-adrenoceptor agonist has been given recently by Timmermans et al (1987). In a study of the effect of nifedipine on the pressor response of the pithed rat to phenylephrine, they showed that following treatment with phenoxybenzamine, phenylephrine remained refractory to reduction by 1mg/Kg yohimbine (Timmermans, Beckeringh, Van Zwieten and Thoolen 1987).

In summary:

1. Two questions were addressed concerning the α-adrenoceptor mediated calcium activation mechanisms in the pithed rat. Firstly, whether the difference in sensitivity between $\alpha_1$- and $\alpha_2$-adrenoceptor mediated pressor responses to diltiazem were due to differences in the time course of the responses. Secondly, whether exposure of a diltiazem sensitive component of the pressor response of the pithed rat to cirazoline could be uncovered other than by reduction of receptor reserve.

2. The pressor response of the pithed rat to the preferential $\alpha_1$-adrenoceptor agonist phenylephrine was insensitive to diltiazem (12.5 and 25µg/Kg/min) when the agonist was administered by continuous infusion.

3. Prazosin (0.3mg/Kg) and corynanthine (10mg/Kg) exposed a diltiazem sensitive component of the pressor response of the pithed rat to cirazoline.

4. It is concluded that insensitivity of $\alpha_1$-adrenoceptor mediated pressor responses of the pithed rat to calcium channel antagonism cannot be explained in terms of the time course of the response. Furthermore, elimination of receptor reserve is not necessary in order to expose a diltiazem sensitive component of the pressor response of the pithed rat to cirazoline. The effect is not exclusive to antagonists which dissociate slowly from adrenoceptors. The evidence does not suggest that diltiazem sensitivity is due to exposure of an $\alpha_2$- component of the cirazoline response.

## 8.3. Rat Aorta - α-adrenoceptor mediated calcium activation.

### 8.3.1. Introduction

In 1984, Ruffolo and co-workers suggested that the reason $\alpha_2$-adrenoceptor mediated pressor responses of the pithed rat are sensitive to calcium channel antagonists, yet $\alpha_1$-adrenoceptor mediated responses are generally resistant is due to a receptor reserve for the $\alpha_1$- subtype but not for the $\alpha_2$-. Thus, pretreatment with a non-competitive antagonist (phenoxybenzamine) could reveal a calcium channel antagonist sensitive component of the $\alpha_1$-adrenoceptor mediated pressor response (Ruffolo, Morgan and Messick 1984). However, it has been shown in the present study (sections 8.2.2.5. and .6.) that exposure of a diltiazem sensitive component of the pithed rat to cirazoline occurred with competitive antagonists, hence receptor reserve could not be an adequate explanation of the results obtained. An alternative explanation of these findings was therefore necessary.

Recently, the hypothesis by Ruffolo, Waddell and Yaden (1984) (section 8.2.1.) has also been tested, by Timmermans and co-workers (1985). They investigated the effect of nifedipine on the response of the pithed rat to a range of agonists before and after treatment with either of two chemically unrelated, irreversible α-adrenoceptor antagonists (see figure 17). They found that phenoxybenzamine, but not benextramine, enhanced the effectiveness of nifedipine at antagonising vasoconstriction to selective $\alpha_1$-adrenoceptor antagonists. They concluded that phenoxybenzamine selectively blocks that part of the

response that is independent of the influx of extracellular calcium and therefore, increases the relative contribution of the calcium influx dependent component. As a consequence, the response becomes more sensitive to nifedipine. Conversely, benextramine was said not to affect the calcium influx independent component of the response and so the sensitivity of $\alpha_1$-adrenoceptor agonist responses to calcium channel antagonists was not increased (Timmermans, Thoolen, Mathy, Wilffert, DeJonge and Van Zwieten 1985).

In 1984, Cauvin, Saida and Van Breemen investigated the sensitivity of rabbit aorta and mesenteric resistance vessels to diltiazem. The study revealed that the greater the noradrenaline induced release of intracellular calcium, the less the susceptibility to inhibition by diltiazem. If this is generally true, then the possibility exists that adrenoceptor antagonists may indirectly inhibit the release of calcium from intracellular stores thereby increasing the component of the response due to calcium influx hence increase the susceptibility to calcium channel antagonism. It was considered worthwhile to pursue this line of investigation in vitro .

In order to study further the relationship between $\alpha_1$-adrenoceptor mediated intracellular calcium release and calcium entry, a preparation was sought which would provide a reliable system for the study of $\alpha_1$-adrenoceptor mediated contraction of vascular muscle. The rat aorta was considered to be suitable for this study because $\alpha_1$-adrenoceptor mediated responses are reproducible (Ruffolo et al 1979) and $\alpha$-adrenoceptor agonists may be assumed to be acting at the

$\alpha_1$- subtype (Beckeringh *et al* 1984) which would eliminate the difficulties which may have arisen as a consequence of loss of agonist selectivity (see section 8.1.3.).

Heaslip and Rahwan (1982) have shown that EGTA resistant responses of the rat aorta to noradrenaline have two distinct phases. They attributed these two phases to the mobilisation of two distinct pools of intracellular calcium. Further, they demonstrated that the initial peak of the EGTA resistant response could not be repeated unless the tissue was re-exposed to extracellular calcium, whereas the second, sustained phase did not require this re-exposure. Presumably in the presence of extracellular calcium, both pools may be repeatedly released as a result of receptor activation. If the two pools of intracellular calcium are strictly independent of one another, then investigations into the release of intracellular calcium must consider both pools.

A study was made of α-adrenoceptor mediated vasoconstrictor responses of the rat aorta with the objective of examining the influence of the α-adrenoceptor antagonists used in the previous section, plus benextramine, on α-adrenoceptor mediated calcium activation *in vitro* .

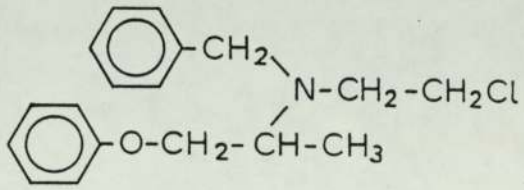
8.3.2. Results

8.3.2.1.The effect of receptor reserve on the diltiazem sensitivity of the response of the rat aorta to phenylephrine.

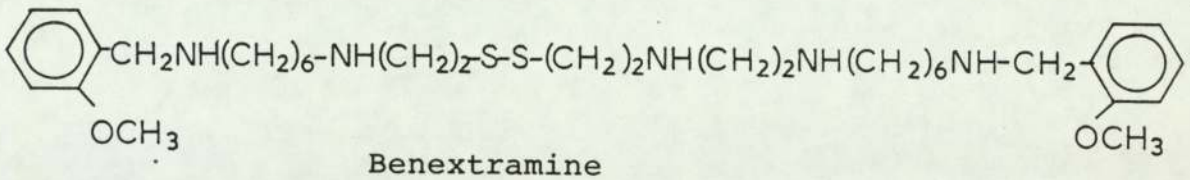
To examine further the effect of non-competitive antagonists on the sensitivity of $\alpha_1$-adrenoceptor mediated responses to calcium channel antagonism, the effect of diltiazem (1μM) was investigated on the response of the rat aorta to phenylephrine, before and after treatment with phenoxybenzamine (10nM). Initially, phenylephrine was used in preference to noradrenaline because it was one of the agonists used in the experiments on the pithed rat (sections 8.2.2.1. and .2.). Although cirazoline may have been considered the better choice of agonist for comparison with the *in vivo* experiments, it was found to be difficult to wash away from an isolated tissue and it was felt that this property would compromise the experiment because of the time required for its completion.

In figure 18A it can be seen that 1μM diltiazem had no significant effect upon the fast phase of the response of the rat aorta to phenylephrine (1nM-0.3μM). There was no shift of the control curve at the $EC_{50}$ although there was a small, but significant suppression of the maximum ($p<0.05$, n=5-10). Diltiazem caused a significant rightward shift of the concentration response curve for the slow phase with a change in the $EC_{50}$ from $(1.8\pm0.64)\times10^{-8}$M to $(5.4\pm2.3)\times10^{-8}$M figure 18B. The slope was not significantly changed but the maximum was depressed by 22.9±5.4%.

Following exposure to phenoxybenzamine (10nM), diltiazem



Phenoxybenzamine



Benextramine

Figure 17: Schematic representation of the structural formula of phenoxybenzamine and of benextramine.

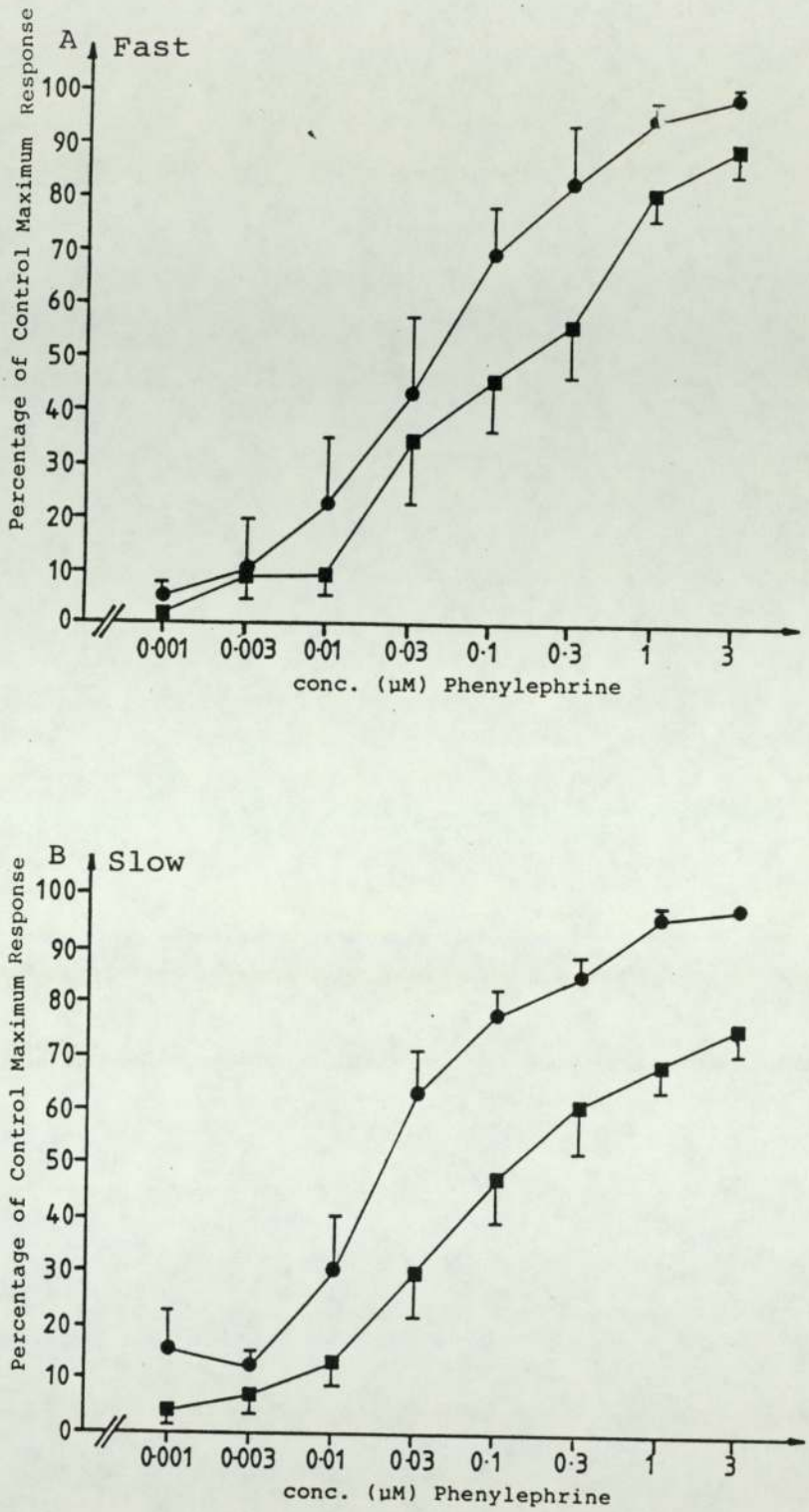



Figure 18: The effect of diltiazem on A. the fast and B. the slow phase of the response of the rat aorta to phenylephrine (n=5-10).
(●) control; (■) 1μM diltiazem.

(1μM) had no effect on the $EC_{50}$ or the slope of the concentration response curve for the fast phase of the response, at the lower concentrations of phenylephrine (10nM-3μM); but significantly reduced the fast phase observed at the two highest concentrations tested (10 and 30μM, figure 19A, $p<0.05$, $n=9$). Also following treatment with phenoxybenzamine, diltiazem reduced the slow phase of the responses for all except the lowest two concentrations of phenylephrine (10 and 30nM), with a further reduction from control of the maximum response by 34.0±12.8%. These effects are illustrated in figures 19 and 20 where it can be seen that pretreatment of the rat aorta with 10nm phenoxybenzamine caused a reduction of the slow sustained phase of the response to 3μM phenylephrine. Diltiazem (1μM) caused a further reduction of this response. Therefore, pretreatment of the isolated rat aorta with 10nM phenoxybenzamine had no significant effect on the sensitivity of the fast phase of the phenylephrine mediated response to diltiazem, but caused a small but significant increase in the sensitivity of the slow phase of the response to the calcium channel antagonist.

8.3.2.2. The influence of phenoxybenzamine and benextramine on EGTA resistant responses of the rat aorta to noradrenaline.

The next step in this section was to investigate the effect of pretreatment with the non-competitive antagonists phenoxybenzamine and benextramine on the component of the response of the rat aorta to $\alpha_1$-adrenoceptor mediated

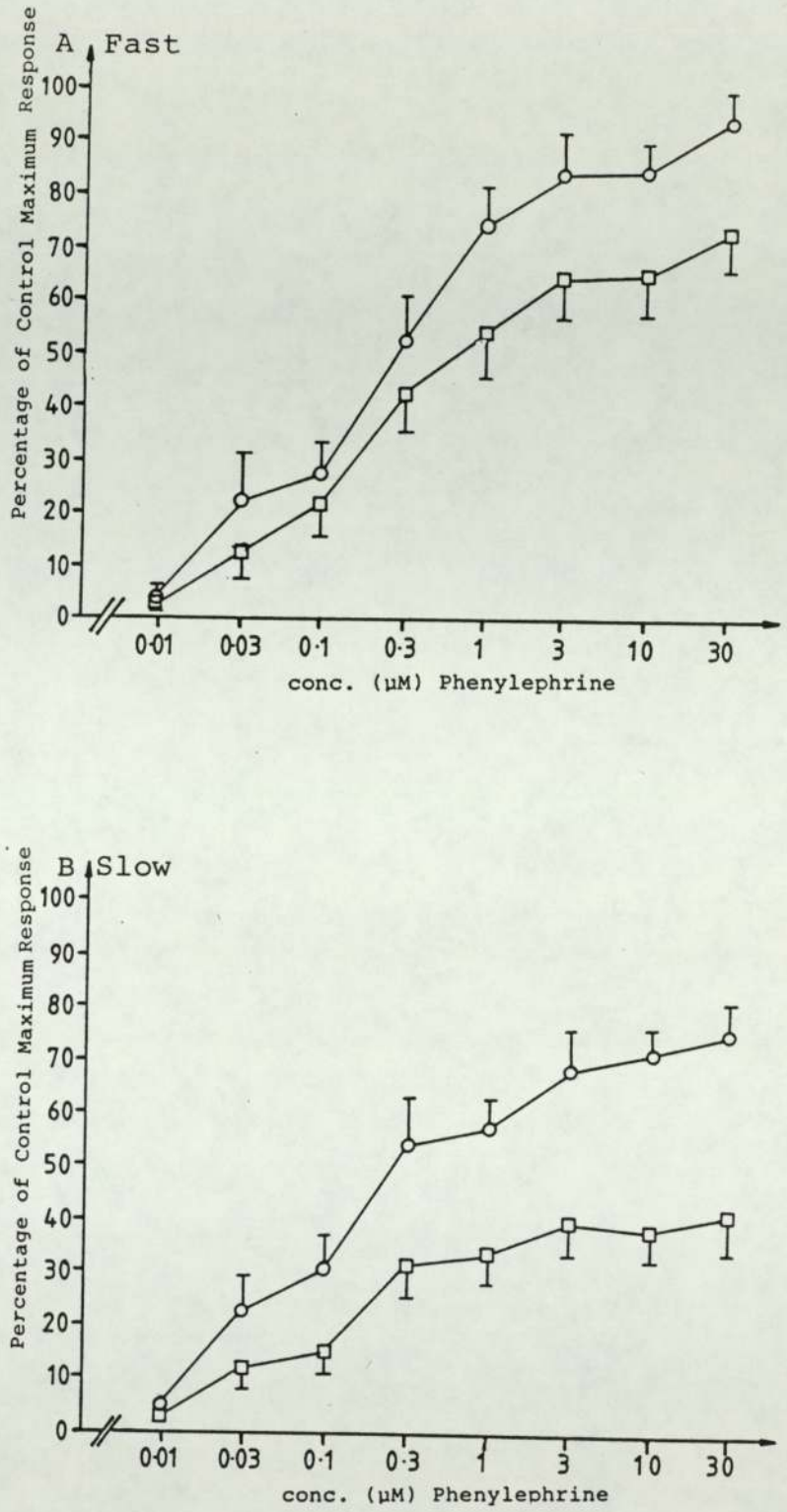



Figure 19: The effect of diltiazem on A. the fast and B. the slow phase of the response of the rat aorta to phenylephrine following treatment with 10nM phenoxybenzamine (PBZ) (n=9).
(O) phenylephrine following PBZ; (□) phenylephrine in the presence of 1μM diltiazem, following PBZ.

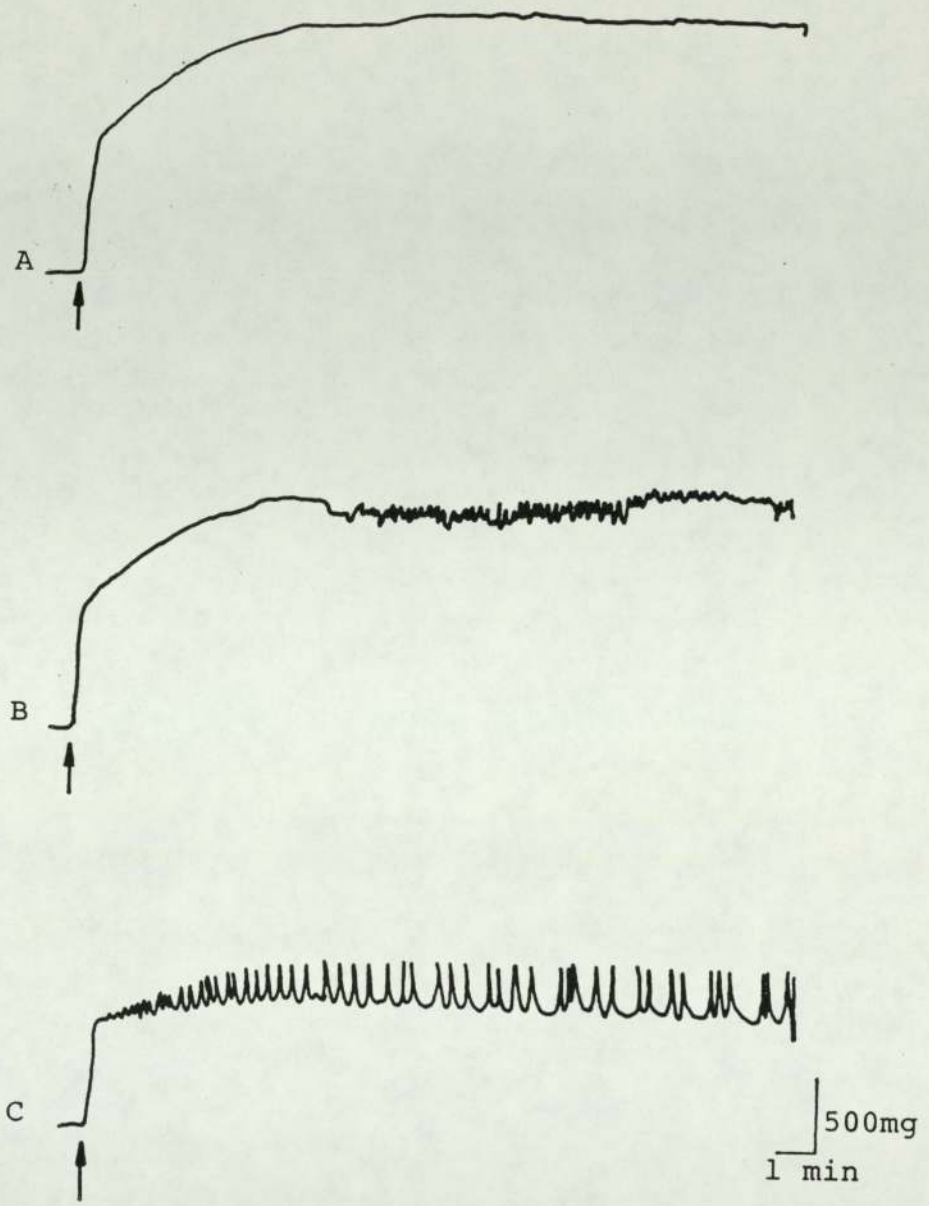



Figure 20: Representative traces of the response of the rat aorta to 3μM phenylephrine A. control, B. following treatment with 10nM phenoxybenzamine (PBZ) and C. in the presence of 1μM diltiazem, following PBZ.
Arrows indicate addition of phenylephrine.

vasoconstriction dependent on intracellular calcium release. The purpose was to see if any support could be found for the suggestion by Timmermans and co-workers (1985) that, in the pithed rat, phenoxybenzamine selectively inhibits that component of the $\alpha_1$-adrenoceptor mediated contraction which is independent of calcium influx.

Figure 21 illustrates the effect of phenoxybenzamine (10nM) and benextramine (1μM) on the peak and on the second phase of the EGTA resistant response of the rat aorta to noradrenaline (3nM - 3μM). Concentrations of antagonist were selected which would reduce the receptor reserve of noradrenaline in the aorta of the rat without significantly impairing the responsiveness of the tissue to agonist activation since if phenoxybenzamine had a selective effect on the component of the response not due to calcium influx, then this should be apparent without significant reduction of the component of the response that is due to calcium influx. The concentrations of antagonists chosen caused a small but not significant reduction of the maximum response of the aorta to noradrenaline in the presence of calcium.

Benextramine (1μM) caused a small but not-significant rightward shift of the curve for the peak EGTA resistant response of the rat aorta to noradrenaline. There was no change in the slope and no difference from control values at 3 and 30nM noradrenaline ($p>0.05$, $n=9$). The responses to the two higher concentrations of noradrenaline used were significantly different from control values (figure 18A, $p<0.05$). Benextramine did not significantly reduce the second phase of the EGTA resistant response except at the

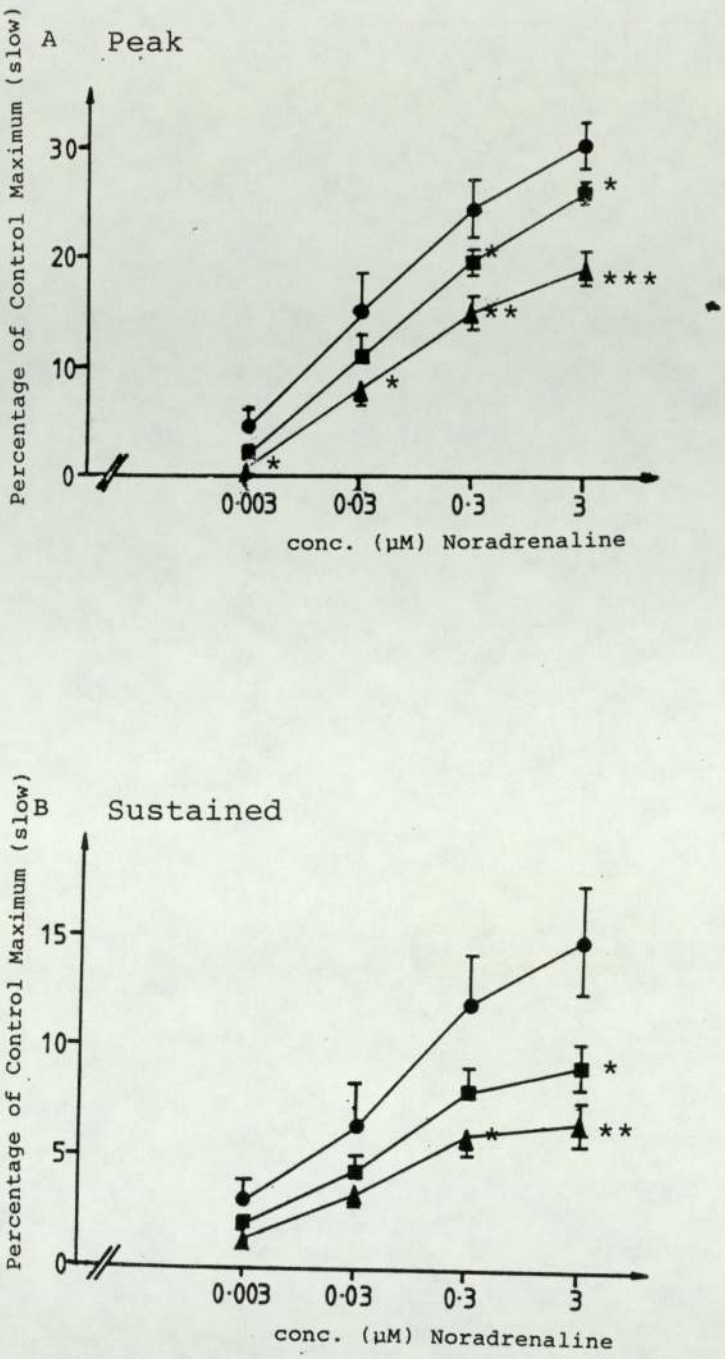



Figure 21: The effect of pretreatment with phenoxybenzamine or benextramine on A. the initial, transient peak and B. the second, sustained phase of the EGTA resistant response of the rat aorta to noradrenaline (n=8).
(●) control; (■) following 1μM benextramine; (▲) following 10nM phenoxybenzamine.
Asterisks indicate significant difference from control.
(* - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$).

highest concentration of noradrenaline tested (3μM). (figure 18B, $p<0.05$, $n=9$).

Phenoxybenzamine (10nM) caused a significant reduction of the peak EGTA resistant response at all concentrations of noradrenaline tested (figure 21A, $p<0.05$, $n=9$). However, only at the two highest concentrations of noradrenaline tested was there a significant reduction of the second phase of the EGTA resistant response. The slope of the concentration response curve for the peak EGTA resistant response was not affected by phenoxybenzamine, but the slope of the second phase was reduced (figure 21B, $p<0.05$, $n=9$).

Phenoxybenzamine and benextramine caused a similar reduction of the EGTA resistant response to all concentrations of noradrenaline studied. There was also no significant difference in the effect of these antagonists upon the peak EGTA resistant response to 3 and 30nM noradrenaline ($p>0.05$, $n=9$); but phenoxybenzamine caused a significantly greater reduction of the peak EGTA resistant response to 0.3 and 3μM noradrenaline, than did benextramine (figure 21, $p<0.05$). It has therefore been shown that both phenoxybenzamine and benextramine may reduce a component of the response of the rat aorta to noradrenaline that is not due to calcium influx.

8.3.2.3.EGTA resistant responses of the rat aorta to noradrenaline in the presence and absence of prazosin or corynanthine.

A similar experiment to 8.3.2.2. was performed in order to study the effects of the competitive antagonists prazosin

(1nM) and corynanthine (1μM) on the two phases of the EGTA resistant response of the rat aorta to noradrenaline. A fundamental difference between this experiment and the one described above is that tissues were exposed to the non-competitve antagonists for fifteen minutes, then the antagonists were washed away. Throughout this experiment, competitive antagonists remained in the bathing medium.

In the presence of calcium, corynanthine (1μM) or prazosin (1nM) caused small, but significant ($p<0.05$) reductions of the maximum sustained response of the rat aorta to 3μM noradrenaline by 4.1±2.4% and 5.9±2.9% (n=8) respectively. The fast component of the noradrenaline response, in the presence of calcium, which represented 38.8±1.3% of the control maximum, was reduced by 39.7±10.3% by the concentrations of corynanthine (1μM), and by 82.4±4.2% by prazosin (1nM - $p<0.01$, n=8). Figure 22 shows the effect of these antagonists on the shape of the responses to noradrenaline in the presence of calcium. Control responses of the rat aorta to 3μM noradrenaline showed an initial rapid increase, an inflection point, and a slow sustained phase. Following treatment with corynanthine, these features were still evident but the fast response appears to rise more slowly than for the control. Prazosin abolished the fast response whilst having little effect on the sustained contracture.

The EGTA resistant response of the rat aorta to noradrenaline is biphasic. There is an initial transient peak and a second sustained phase. This has been illustrated with representative traces in figure 23. The transient peak

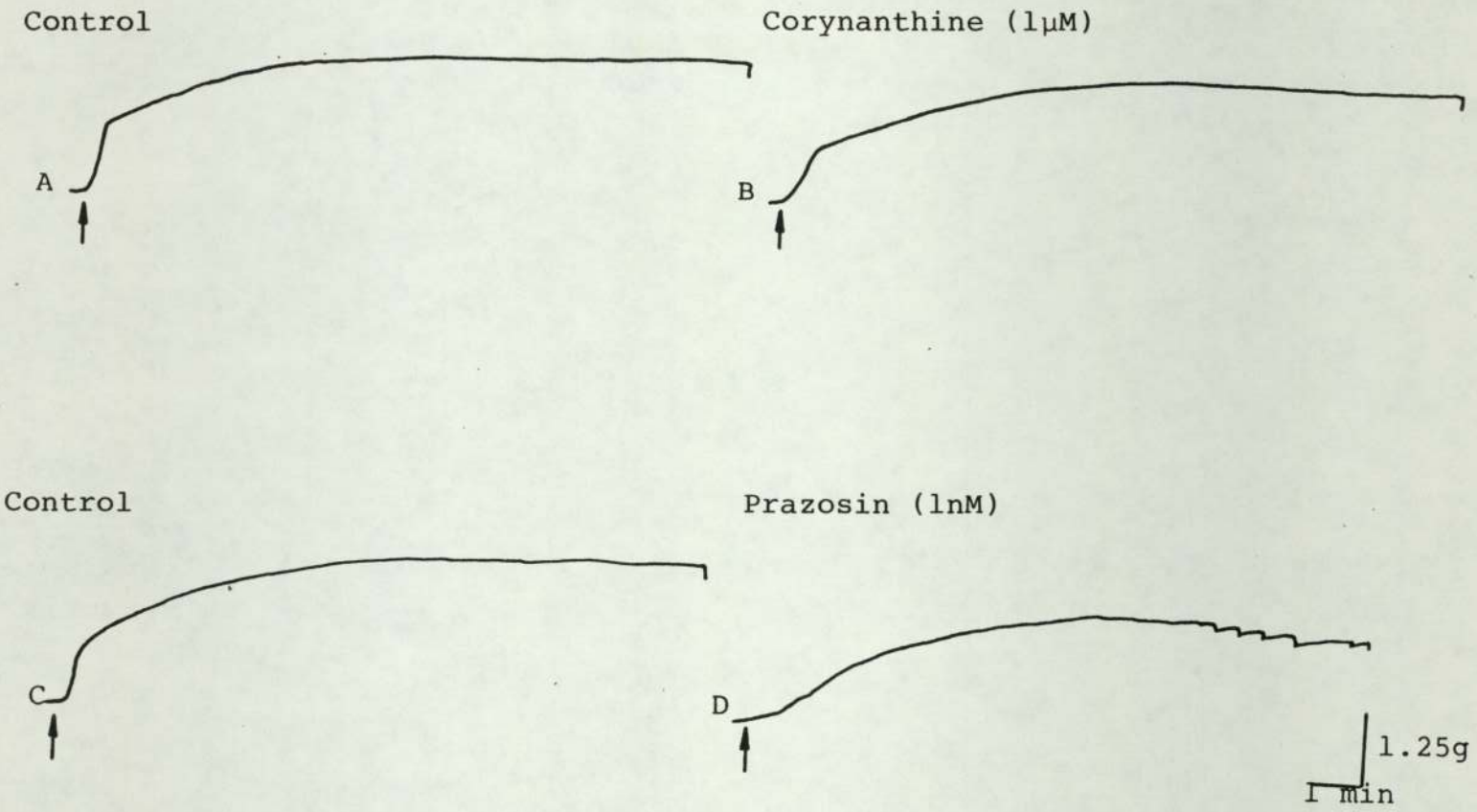



Figure 22: Representative traces to show the effect of corynanthine (1μM) or prazosin (1nM) upon responses of the rat aorta in calcium containing Krebs' solution, to 3μM noradrenaline Responses on the left are controls, before the addition of antagonist.
Arrows indicate addition of noradrenaline.

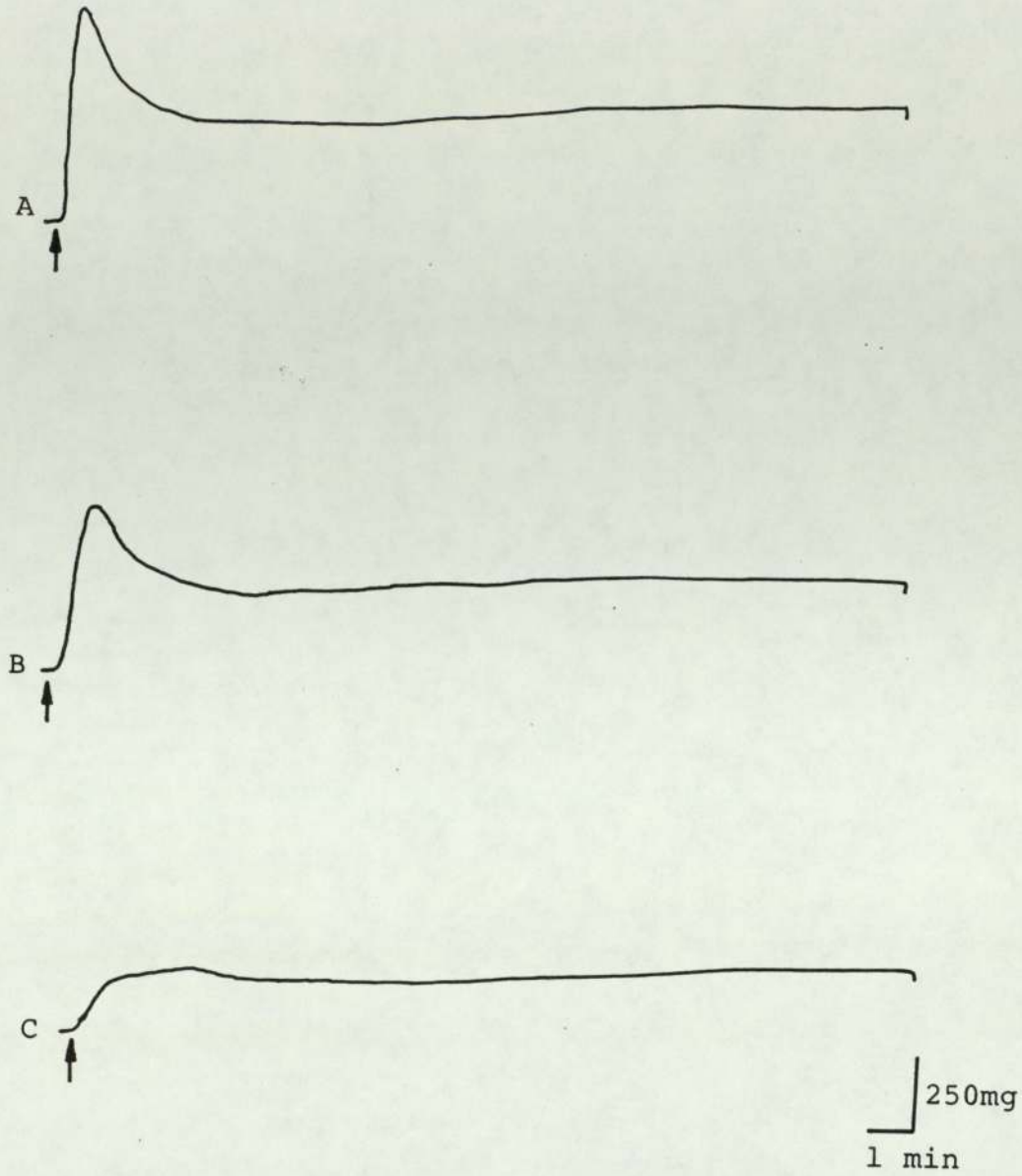



Figure 23: Representative traces to show the effect of corynanthine (1μM), or prazosin (1nM) upon the EGTA resistant response of the rat aorta to 3μM noradrenaline.
A. control; B. in the presence of corynanthine;
C. in the presence of prazosin.
Arrows indicate addition of noradrenaline.

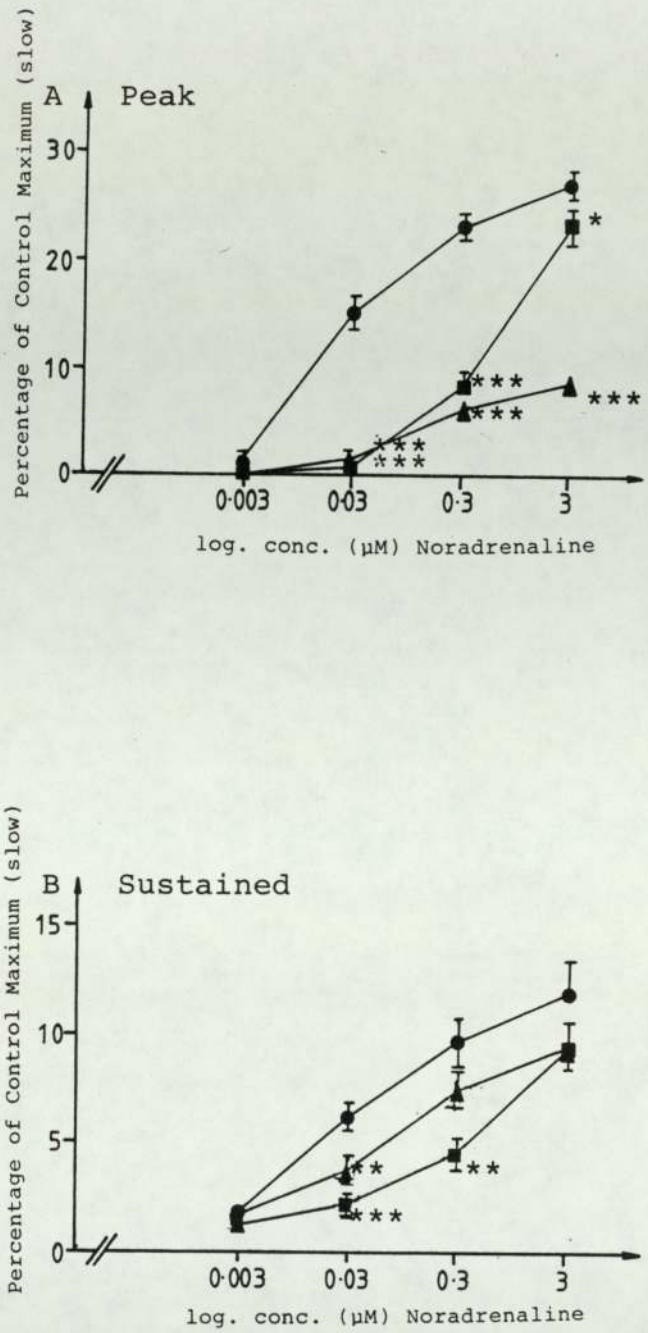



Figure 24: The effect of corynanthine (1μM) and of prazosin (1nM) on A. the initial, transient peak and B. the slow, sustained phase of the EGTA resistant response of the rat aorta to noradrenaline (n=8).
(●) control; (■) 1μM corynanthine; (▲) 1nM prazosin.
Asterisks indicate significant difference from control.
(* - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$).

of the EGTA resistant response was significantly reduced by prazosin and corynanthine for all concentrations of noradrenaline tested (figure 24A). However, the shape of the curves differ in the presence of prazosin, compared with corynanthine. There was no significant difference between the size of the peak EGTA resistant responses of the rat aorta to 3, 30 or 300nM noradrenaline; but in response to 3μM noradrenaline, the peak of the EGTA resistant response in the presence of corynanthine (1μM) was significantly greater than that in the presence of prazosin (1nM). The difference between the effect of these antagonists on the EGTA resistant response to 3μM noradrenaline has been illustrated in figure 23, where, in the presence of corynanthine, the initial peak rose more slowly than for the control. In the presence of prazosin, the initial transient peak appeared to have been abolished.

The second phase of the EGTA resistant response to 0.03μM and 0.3μM noradrenaline was significantly reduced by both antagonists (figure 24B). At these concentrations of noradrenaline, the reduction produced by corynanthine was significantly greater than that produced by prazosin ($p<0.05$, $n=8$). It has therefore been shown that the two phases of the EGTA resistant response have been differentially reduced by prazosin and corynanthine

8.3.2.4. The influence of α-adrenoceptor antagonists on the diltiazem sensitivity of the response of the rat aorta to noradrenaline.

Cauvin et al (1984) have suggested that reduction of the component of the α-adrenoceptor mediated vasoconstrictor response to noradrenaline due to intracellular calcium release increases the susceptibility of the response to calcium channel antagonists. If this hypothesis is correct then prazosin and corynanthine, which have been shown to reduce the EGTA resistant response of the rat aorta to noradrenaline (section 8.3.2.3.) should increase the susceptibility of the calcium present noradrenaline response to inhibition by diltiazem.

The slow sustained phase of the response of the rat aorta to 1μM noradrenaline was 2.3±0.23g (n=7). Diltiazem (1μM) added to the bathing medium during this phase, reduced the contraction by 33.8±3.6%. Corynanthine (1μM) or prazosin (1nM) caused a significant reduction of the sustained response to 1μM noradrenaline (by 12.7±4.8% and 14.2±2.7% respectively), but there was no significant difference between the effect of the two antagonists at these concentrations.

Following pretreatment with 1μM corynanthine or with 1nM prazosin, diltiazem (1μM) reduced the noradrenaline response by 45.7 ±2.1% and 44.3±3.6% respectively. These values are significantly greater than reductions by diltiazem in the absence of antagonist but do not differ from each other (figures 25 and 26, $p<0.01$, n=7). Thus the size of the contraction to 1μM noradrenaline following reduction by

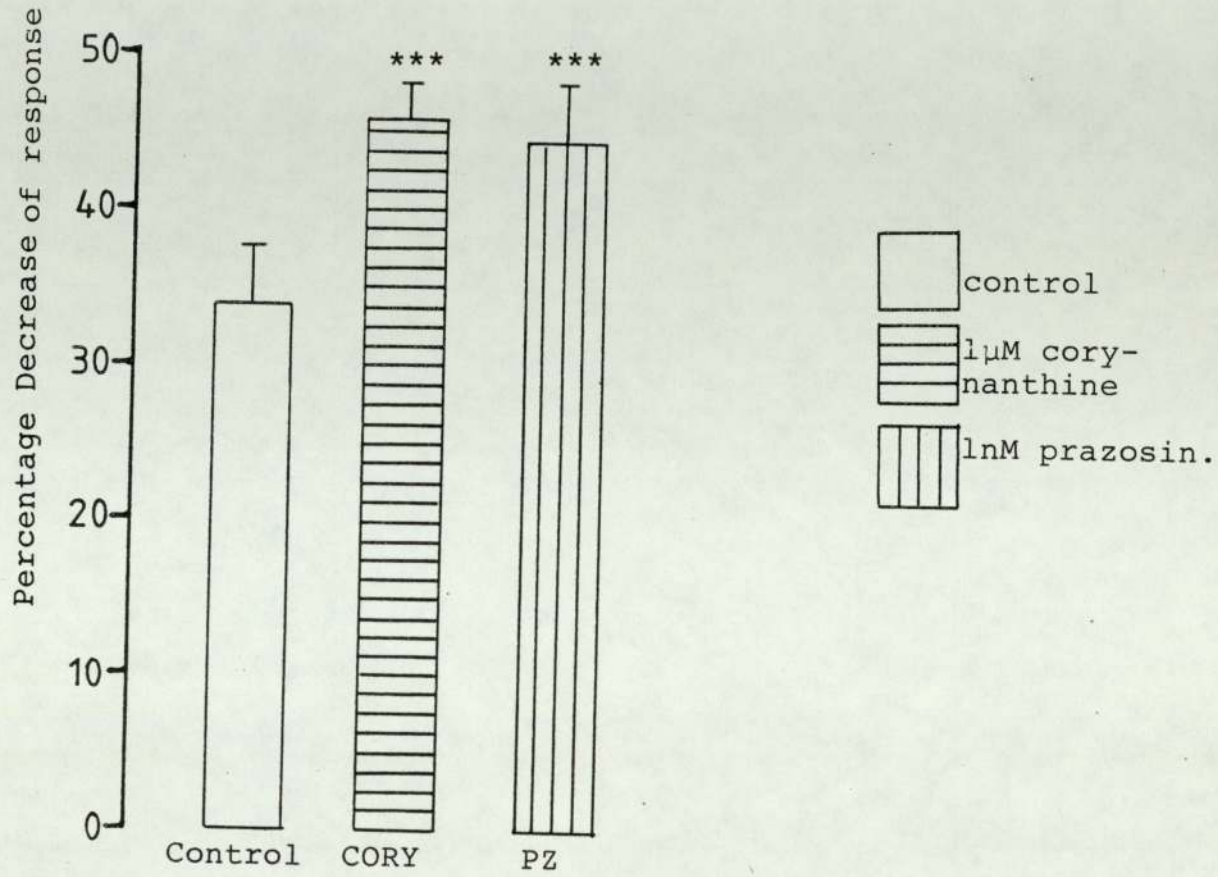



Figure 25: The effect of 1μM diltiazem on the response of the rat aorta to 1μM noradrenaline in the presence or absence of prazosin or corynanthine.
Open - control; Horizontal bars - 1μM corynanthine;
Vertical bars - 1nM prazosin.
Asterisks indicate significant difference from control ($p<0.001$).

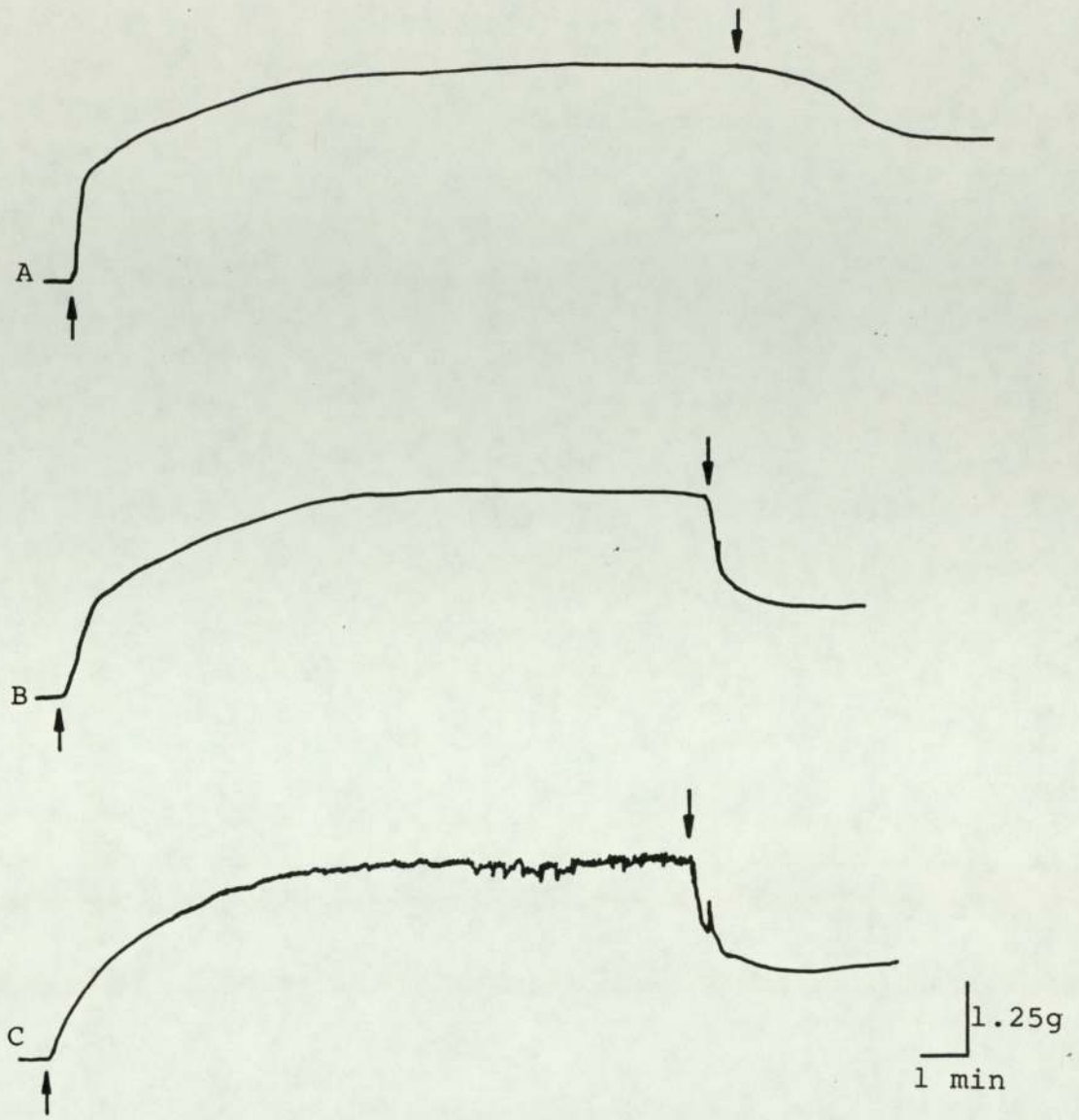



Figure 26: Representative traces of the effect of 1μM diltiazem on the response of the rat aorta to 1μM noradrenaline A. alone, B. following 1μM corynanthine and C. following 1nM prazosin.

Upward arrows indicate addition of noradrenaline, downward arrows indicate addition of diltiazem.

diltiazem was 2009±238mg for control values, which was significantly greater than the size of the contraction to 1μM noradrenaline remaining following diltiazem in the presence of corynanthine (1431±174mg) or prazosin (1398±151mg; $p<0.05$, $n=7$).

These results provide additional support for the hypothesis of Cauvin et al (1984) that reduction of the intracellular calcium component of the α-adrenoceptor mediated vasoconstriction, increases the susceptibility of the response to calcium channel antagonism. However, the assumption has been made that the reduction of the EGTA resistant response to noradrenaline by 1μM corynanthine and by 1nM prazosin, may be extrapolated to the reduction by these antagonists of the component of the response due to intracellular calcium release when the response has been elicited in the presence of extracellular calcium.

### 8.3.2.5. Diltiazem sensitivity, and EGTA resistant responses of the rat aorta following cumulative or bolus administration of agonist.

Heaslip and Rahwan (1982) have shown that there are two pools of intracellular calcium involved in the contraction of the rat aorta to noradrenaline. Therefore it was considered pertinent to investigate the relative contribution of these two pools to the total response.

The initial rapid rise of the response of the rat aorta to noradrenaline appears to be a threshold phenomenon in that it is not seen clearly when concentrations of

noradrenaline less than 10nM are administered (Downing et al 1983). Similarly, EGTA resistant responses of the rat aorta to noradrenaline do not have an initial, transient peak when the agonist has been added cumulatively (this section). Therefore, experiments were performed in which noradrenaline was added to the organ bath either by bolus administration or cumulatively. The objective was to determine whether the calcium released in the initial, rapid phase of either the EGTA resistant, or the calcium present response to α-adrenoceptor agonists contributed to the second, sustained phase.

In the presence of calcium the response of the rat aorta to phenylephrine showed an initial rapid rise, an inflection point and a second, sustained phase. When the agonist was given cumulatively, the initial rapid rise was not evident (figure 29). Similarly, the EGTA resistant response to noradrenaline caused an initial transient peak that was not seen during cumulative administration of agonist (figure 30).

The calcium present response of the rat aorta to 1μM phenylephrine following administration of agonist either as a bolus or cumulatively was found to be equally sensitive to 1μM diltiazem. Responses were reduced by 38.4±3.9% and 40.5±3.2% for cumulative or bolus administration respectively. These values are not significantly different from each other (figures 27 and 29, $p<0.05$, $n=8$).

The maximum sustained phase of the EGTA resistant response of the rat aorta to noradrenaline was the same whether the agonist was given cumulatively or as a bolus.

The percentage of the control maximum achieved by these methods was 14.0 ±1.2% and 14.0±0.4% respectively (figures 28 and 30, $p<0.05$, $n=8$).

Thus these results demonstrate that the maximum, sustained response to an $\alpha_1$-adrenoceptor agonist in the presence of calcium is the same whether the agonist is given as a bolus or cumulatively. Similarly, the second sustained phase of the EGTA resistant response to an agonist is unaffected by the rate of administration of that agonist.

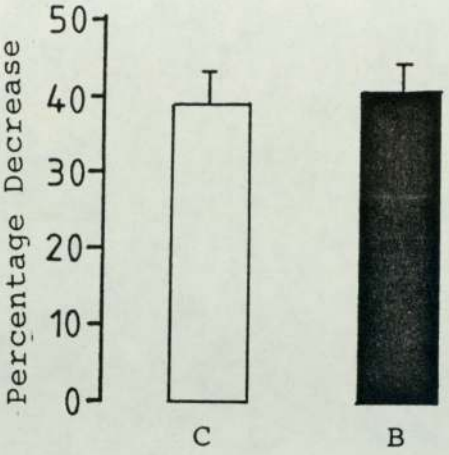



Figure 27: The reduction by 1μM diltiazem of the slow phase of the response of the rat aorta to cumulative (C) or bolus (B) administration of 1μM phenylephrine (n=8).

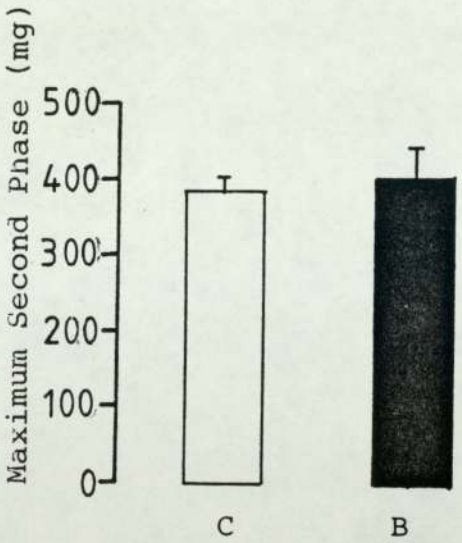



Figure 28: The magnitude of the second, sustained phase of the EGTA resistant response of the rat aorta to cumulative (C) or bolus (B) administration of 1μM noradrenaline (n=8).

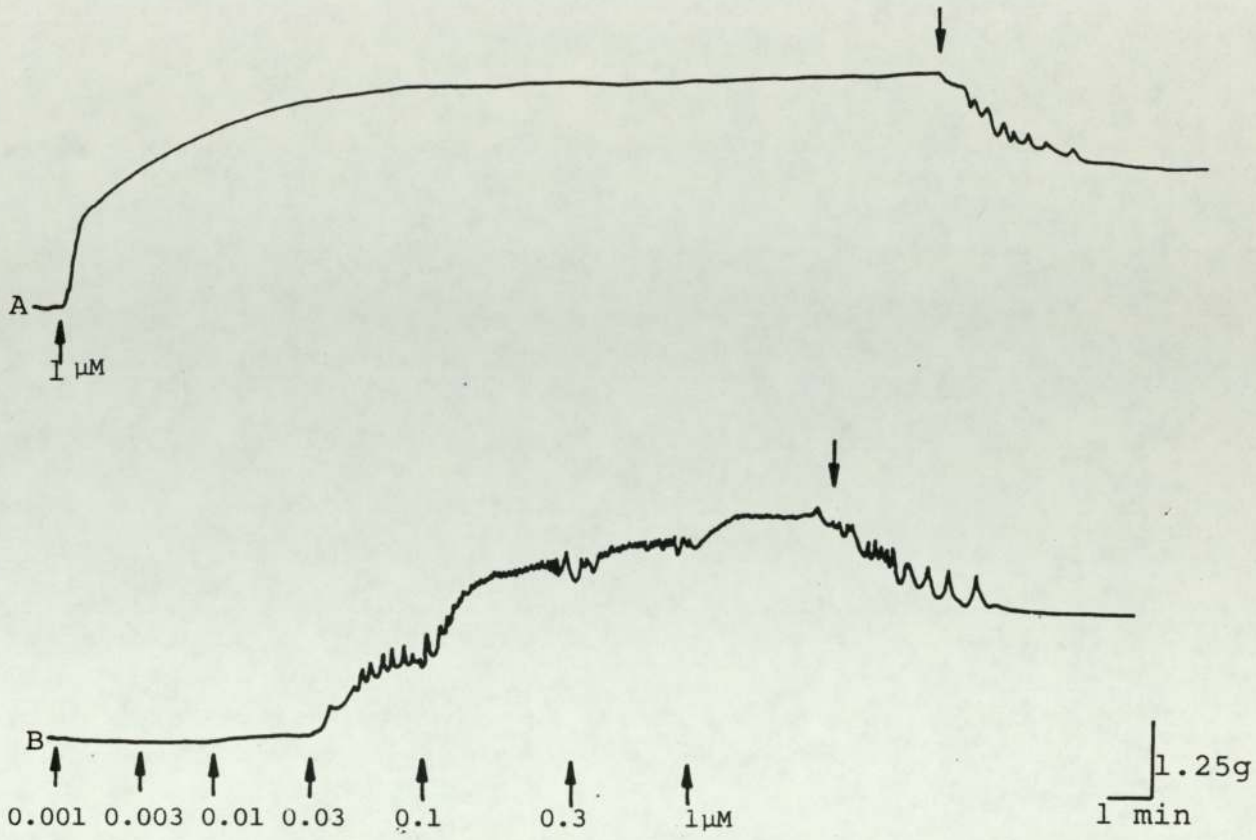



Figure 29: Representative traces showing the effect of 1µM diltiazem on the response of the rat aorta to A. bolus or B, cumulative administration of 1µM phenylephrine. Upward arrows indicate addition of agonist. Downward arrows indicate addition of diltiazem.

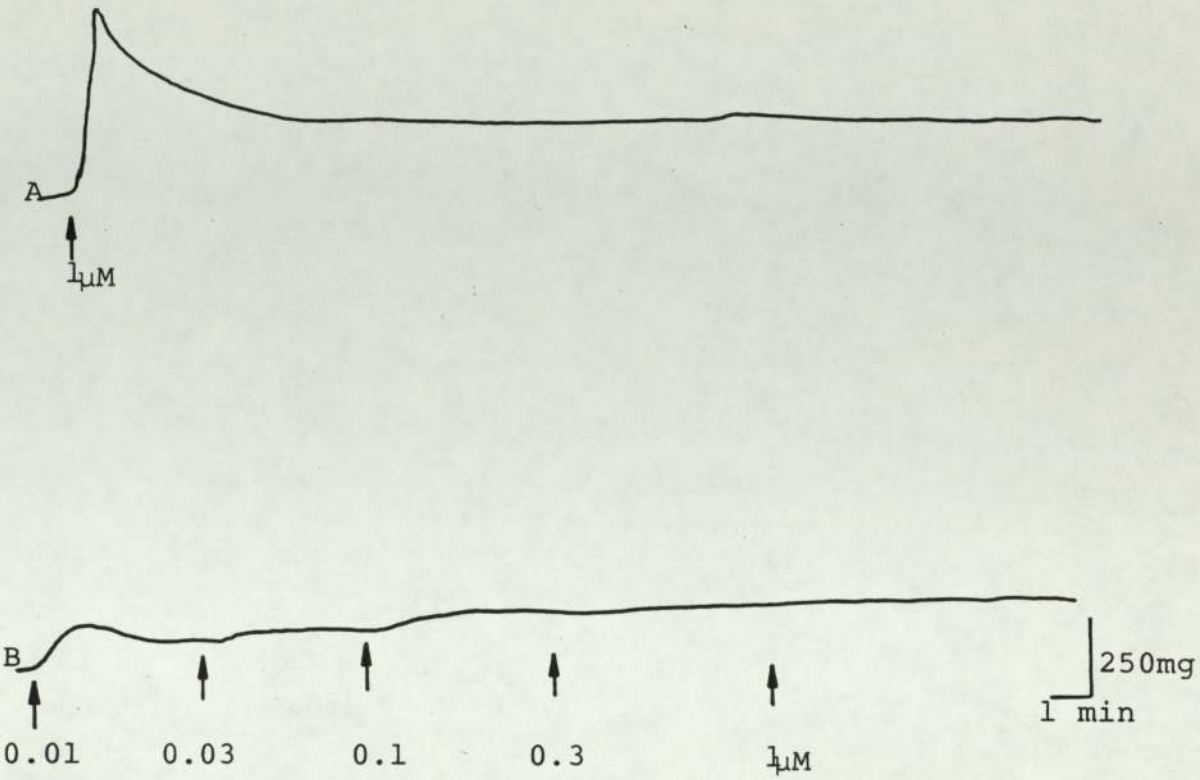



Figure 30: Representative traces showing EGTA resistant responses of the rat aorta to A. bolus or B. cumulative administration of 1μM noradrenaline.
Upward arrows indicate addition of agonist.

8.3.3. Discussion

The purpose of this section was to examine the reasons behind the observation that both competitive and non-competitive antagonists can alter the sensitivity of $\alpha_1$-adrenoceptor mediated pressor responses to diltiazem *in vivo* .

The *in vitro* situation is simpler than *in vivo* in that the pressor response of the whole animal is comprised of the responses of many vascular beds. *In vitro* the rat aorta gives rise to reproducible contractions in response to α-adrenoceptor agonists. In addition, all α-adrenoceptor agonists may be assumed to be acting at the $\alpha_1$-adrenoceptor subtype (Beckeringh *et al* 1984). Therefore, using this model, the influence of α-adrenoceptor antagonists on $\alpha_1$-adrenoceptor mediated calcium activation mechanisms was examined.

The first step in this investigation was to consider the effect of diltiazem on the fast and slow phases of the response of the rat aorta to phenylephrine, before and after treatment with phenoxybenzamine. It was found that the fast phase of the response was unaffected by diltiazem at all except the maximum two concentrations of phenylephrine used. Since diltiazem is a calcium channel antagonist which has no direct effect on either intracellular release or the contractile protein system (Cauvin *et al* 1984a), it would not be expected to have any effect on the fast phase; which may be attributed to the release of intracellular calcium (Godfraind and Kaba 1972). It is not immediately obvious why the inhibition of the responses to the maximum two

concentrations of phenylephrine used were inhibited by diltiazem, unless the fast responses at the two highest concentrations of phenylephrine are not entirely homogeneous; and involve some calcium influx. This may arise through an overlap of the two phases of the response. Bolton (1979) has suggested that the release of membrane bound calcium may be necessary in order to trigger calcium release from intracellular sites. Furthermore, it has been suggested that the fast phase may also be associated with the entry of a small extracellularly bound store which enters the cell through receptor linked channels (Leijten et al 1985). It is therefore possible that in the presence of calcium, a reduction of the slow phase of the response by diltiazem may be associated with a decrease in the fast response. Consequently the two phases of the response of the rat aorta to phenylephrine, may not be reduced by the same proportion.

The slow phase of the response to phenylephrine was inhibited by the calcium channel antagonist diltiazem. However, when receptor reserve was reduced with phenoxybenzamine, the effect of diltiazem on the slow response was greater than when the total receptor population was active. A possible explanation provided by Timmermans et al (1985) is that phenoxybenzamine may have inhibited that component of the response not due to calcium influx thus allowing the calcium channel antagonist to have more effect. But if this were true, a greater effect of phenoxybenzamine on the fast phase of the calcium present response than on the slow would have been expected. Therefore, the next step in the investigation was to consider the intracellular calcium

release component of the response of the rat aorta to $\alpha_1$-adrenoceptor agonists. However, initial consideration must be given to the method employed for the quantification of the components of the responses of the rat aorta which have been attributed to the release of intracellular calcium.

If calcium is simply not included in the Krebs' solution, trace quantities of the ion remain bound to the tissue and in solution from other sources. Consequently, a contraction to an agonist in such a medium cannot be attributed to intracellular release because of an unknown contribution of trace extracellular calcium to the total response.

Lanthanum has been used to block calcium influx and to displace extracellularly bound calcium, but this method requires the use of a Tris or HEPES buffer gassed with pure oxygen (Van Breemen, Farinas, Casteels, Gerba, Whytack and Deth 1973, Altura, Carella and Altura 1980). It has been shown by Altura et al (1980) that Tris and HEPES buffers may interfere with the binding, translocation and utilisation of calcium ions in the rat aorta. In addition, use of this method would necessitate repetition of all associated results in the same buffer for calcium present responses. This was not considered a satisfactory protocol.

Biochemical methods have been used to measure calcium fluxes (Deth and Lynch 1981) but these methods may only measure calcium that leaves the smooth muscle cells subsequent to contraction and may not fully express calcium ions used in contraction.

The method it was decided to use, was to add 0.5mMol EGTA to calcium free Krebs' solution. EGTA is a calcium chelating

agent and sequesters trace impurities of cations. With this method, direct comparisons between responses of the rat aorta to agonists in the presence and absence of calcium may be made. The main disadvantage of this method is that exposure of the tissue to EGTA for excessive periods of time may result in leaching of membrane bound calcium. Possibly for this reason the initial peak of the EGTA resistant response is smaller than the fast phase measured in the presence of calcium; although some of the fast response measured in the presence of calcium may include a contribution from the slow phase of the response (see above).

It is understood that force development by smooth muscle cells is directly regulated by the concentration of ionised calcium in the myoplasm (Rüegg 1971). It has therefore been assumed for the purpose of these experiments, that EGTA resistant responses of the rat aorta to α-adrenoceptor agonists represent the utilisation of calcium ions released from intracellular sources.

EGTA resistant responses of the rat aorta to noradrenaline following treatment with phenoxybenzamine (10nM) or benextramine (1μM) were studied. The results are presented in section 8.3.2.3. It has been shown that phenoxybenzamine reduced the peak of the EGTA resistant response for all concentrations of noradrenaline tested. Benextramine inhibited the peak for only the two highest concentrations of noradrenaline (0.3 and 3μM). Both of these antagonists reduced the second phase of the EGTA resistant response of the rat aorta to all the concentrations of

noradrenaline tested. These results do not suggest a selective reduction by phenoxybenzamine compared with benextramine.

Prazosin (1nM) has been shown in this study to be more effective than corynanthine (1μM) at inhibiting the initial, transient peak of the EGTA resistant response of the rat aorta to noradrenaline (figure 24A). However, the same is not true for the second, sustained phase of the response where for two of the concentrations of noradrenaline tested (0.03 and 0.3μM) corynanthine was more effective an antagonist than prazosin (figure 24B). Despite these differences, it has been shown that both prazosin (1nM) and corynanthine (1μM) cause a reduction in the α-adrenoceptor mediated vasoconstrictor response of the rat aorta that may be attributed to the release of intracellular calcium. It was therefore considered worthwhile to examine further the hypothesis of Cauvin et al (1984) to determine whether a reduction of the intracellular calcium release component of the α-adrenoceptor mediated vasoconstrictor response of the rat aorta to α-adrenoceptor agonists could increase the sensitivity of the response to diltiazem. It was decided to use only the competitive antagonists. The reasons were firstly, that the non-competitive antagonists were rather unpredictable in effectiveness and secondly because prazosin selectively reduces the fast phase of the response of the rat aorta to noradrenaline (Downing et al 1983) and was considered interesting for comparison with corynanthine.

The effect of diltiazem on the response of the rat aorta to noradrenaline in the presence or absence of $\alpha_1$-

adrenoceptor antagonists has been illustrated in figures 25 and 26. The results support the view that prazosin is no more effective than corynanthine at inhibiting that component of the response due to intracellular calcium release. If one antagonist were more effective than the other, then according to Cauvin et al (1984) there would have been a difference in the effectiveness of diltiazem depending on the antagonist and its degree of inhibition of intracellular calcium release. However, it is not certain whether any support has been provided for the postulate by Cauvin et al (1984) that the susceptibility to calcium channel blockade increases when the intracellular release component of the response is reduced. In this study, the absolute value of the reduction of the response to noradrenaline by diltiazem was approximately the same whether or not the maximum response in the presence of calcium had been reduced by the presence of $\alpha_1$-adrenoceptor antagonists. It is possible that the concentration of diltiazem used, caused a certain reduction of the maximum response regardless of the magnitude of that response. To test this question properly, the effect of a single concentration of diltiazem should be tested on the response of the tissue to a range of concentrations of noradrenaline, particularly over maximal and supramaximal concentrations where the size of the response in the presence of calcium changes little, but the release of intracellular calcium does change.

The experiments were taken further to consider the relative contribution of the two major pools of

intracellular calcium released in response to α-adrenoceptor activation, as described by Heaslip and Rahwan (1982). In figures 27 and 28 it has been shown that in the presence or absence of calcium, cumulative or bolus administration of agonist did not affect either the sensitivity of calcium present responses to diltiazem or the absolute size of the second phase of the EGTA resistant responses to an α-adrenoceptor agonist. It was reasoned that if the second phase had been larger as a result of bolus administration of agonist then it is possible that the initial peak was involved in some way in enhancing the second phase. If the second phase had been larger when the agonist was given cumulatively, then the calcium ions giving rise to the first phase may still have been released but over a longer time period. If however, as observed, the second phase was not significantly different regardless of the method of administration of agonist then it may be assumed that the first phase did not contribute significantly to the second. Support may then be given to the hypothesis by Heaslip and Rahwan (1982) that the two phases of the EGTA resistant response represent two independent pools of intracellular calcium and as such must be considered separately when investigating the release of calcium in vascular muscle.

There was no difference in the size of the second phase of the EGTA resistant response whether the agonist was given as a bolus, giving rise to an initial transient peak, or given cumulatively so that only a second, sustained phase was seen. This also leads to the conclusion that the first phase of the response does not contribute to the second.

Additional support is provided in figure 29. In the presence of calcium, a response to cumulative addition of agonist, which has no fast response, was shown to be as sensitive to calcium channel antagonism as responses to bolus administration of agonist, where a clear fast response exists.

It follows from the above that the second, sustained phase of the EGTA resistant response of the rat aorta to noradrenaline represents the source of intracellular calcium that is important in the influence of α-adrenoceptor antagonists on the sensitivity to calcium channel antagonists. There are two reasons for this conclusion. Firstly, in the presence of calcium, the presence or absence of the fast phase of the response did not influence the sensitivity of the second phase to diltiazem. Secondly prazosin, at a concentration which had more effect than corynanthine on the initial transient peak, but not the second sustained phase of the EGTA resistant response of the rat aorta to 1μM noradrenaline, was no more effective than corynanthine at increasing the sensitivity of the calcium present response to diltiazem.

In summary:

1. The aorta of the rat was examined with the objective of establishing the influence of α-adrenoceptor antagonists on α-adrenoceptor mediated calcium mobilisation.
2. Pretreatment of the isolated rat aorta with 10nM phenoxybenzamine had no significant effect on the sensitivity of the phenylephrine mediated fast phase of the response to

diltiazem, but had a small augmenting effect on the sensitivity of the slow, sustained phase in the presence of calcium.

3. The two phases of the EGTA resistant response to noradrenaline were susceptible to inhibition by competitive and non-competitive antagonists.

4. Prazosin (1nM) was no more effective than corynanthine (1μM) at increasing the sensitivity of the phenylephrine mediated slow, sustained phase of the calcium present response to 1μM diltiazem.

5. Cumulative or bolus administration of agonist affected neither the size of the second phase of the EGTA resistant response, nor the sensitivity of the calcium present slow response to 1μM diltiazem.

6. It is concluded that α-adrenoceptor antagonists cause a reduction in the second phase of the intracellular calcium release component of the response of the rat aorta to α-adrenoceptor agonists; thereby increasing the susceptibility of $\alpha_1$-adrenoceptor mediated responses to inhibition by calcium channel antagonists.

## 8.4. Rat Aorta - Rate of agonist binding to adrenoceptors.

### 8.4.1. Introduction

In the previous section, the α-adrenoceptor mediated vasoconstrictor response of the rat aorta was studied, before and after treatment with non-competitive antagonists. During the course of the study it was observed that the shape of the contractile response of the aorta following treatment with these agents differed from that before treatment. In particular, following treatment with phenoxybenzamine, bolus administration of phenylephrine produced a biphasic response with a more exaggerated inflection point compared with responses in untreated preparations. This was considered very interesting because phenoxybenzamine is an extreme example of an antagonist which dissociates slowly from adrenoceptors. It has been suggested that in the rat aorta, the ability of α-adrenoceptor antagonists to selectively block the component of contraction mediated by intracellular calcium release is related to the potency of the antagonist (Downing et al 1983), which in turn is related to the rate of dissociation of the antagonist from receptors (Van Rossum 1963). i.e. if an antagonist dissociates slowly from receptors, then the initial rate of binding of an agonist to those same receptors will be reduced (Downing et al 1985). If the hypothesis of Downing and co-workers (1985) is true, then the corollary would be expected to be true i.e. the rate at which agonists initially bind to the receptors will determine the degree to which they are able to activate the

receptors and mobilise the initial release of intracellular calcium. However, a tissue that has been exposed to a non-competitive antagonist (which dissociates very slowly from receptors) would have reduced receptor number, but the remaining active receptors would be unchanged. This is because the tissue would have been exposed to the non-competitive antagonist, then the antagonist would be washed away from the tissue. Consequently, subsequent addition of an agonist to the bathing medium would not affect the agonist's efficacy (i.e. the intrinsic ability of that agonist to initiate a response). Neither would pretreatment of a tissue with a non-competitive antagonist affect the affinity of the agonist for the remaining, active receptors. Therefore, the observation that pretreatment of the rat aorta with phenoxybenzamine may have had less effect on the fast phase of the response to phenylephrine, than the slow, may suggest that the fast phase is related to the affinity as well as the efficacy of the agonist. Therefore, it was considered worthwhile to examine the relationship between the parameters which define the agonist and the ability of the agonist to initiate the release of intracellular calcium.

This section studies two points. The first part of the section examines α-adrenoceptor mediated responses of the rat aorta before and after treatment with non-competitive antagonists, to determine if the observation that this treatment has less effect on the fast phase of the calcium present response than on the slow, is supported. The study was then extended to investigate whether there is any

evidence to link the ability of an agonist to release intracellular calcium with the rate at which the agonist associates with the receptors.

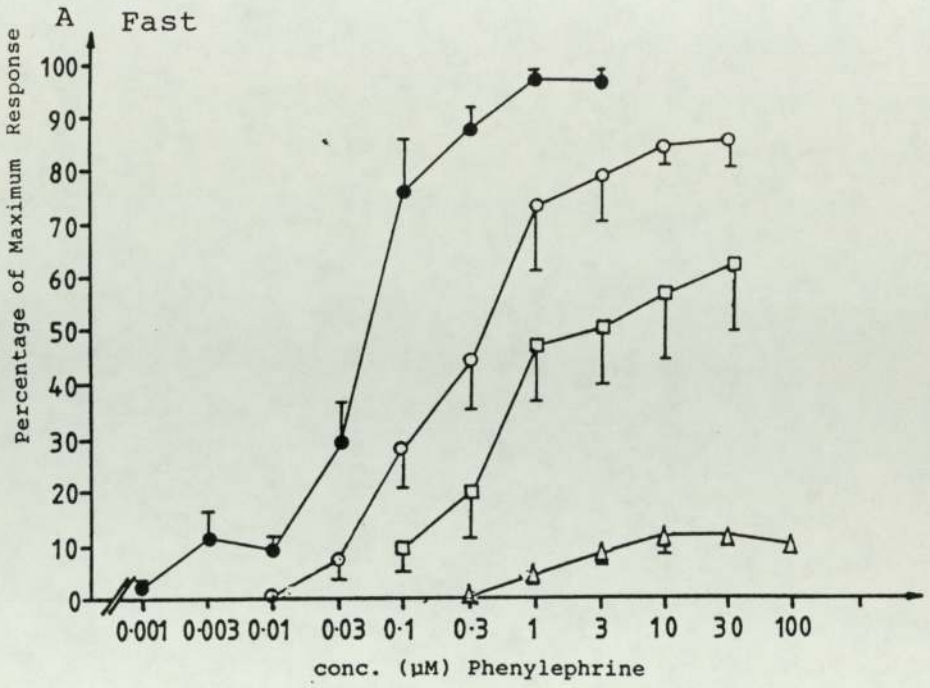
8.4.2. Results

8.4.2.1. The effect of phenoxybenzamine on the response of the rat aorta to phenylephrine.

The first step in this investigation was to study the effect of pretreatment of the rat aorta with a series of concentrations of phenoxybenzamine and to consider the effect of this treatment on the response to the potent α-adrenoceptor agonist phenylephrine.

The results are shown in figure 31. The fast and slow components of the responses have been plotted separately and both phases were antagonised non-competitively, i.e. the concentration response curve to phenylephrine was progressively shifted to the right and the maximum was reduced. However, it appeared that at all concentrations studied, phenoxybenzamine caused a greater percentage reduction of the slow phase of the response than of the fast. Thus 10nM phenoxybenzamine shifted the concentration response curve for the fast and slow phases of the response to phenylephrine by 1.99±0.58 and 2.89±0.67 log cycles respectively, measured at the $EC_{50}$. Using a paired t-test the mean difference between the shifts was 1.02±0.46 log cycles ($p=0.05$, $n=8$).

The maximum fast phase of the response to phenylephrine following irreversible receptor inactivation by 10 and 20nM phenoxybenzamine was 85.2±5.2% and 61.4±12.5% respectively, which is significantly greater than the maximum slow phase of the response (72.7±7.2% and 43.5±9.5% respectively) comparing each phase with its own control ($p<0.05$, $n=7-8$). It would therefore appear that the fast phase of the



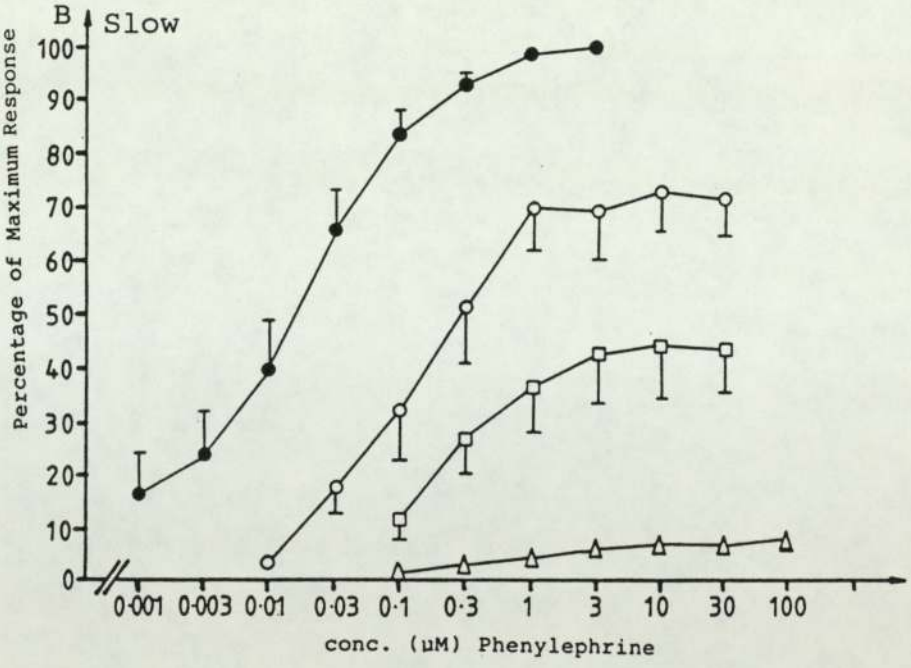




Figure 31: The effect of pretreatment with phenoxybenzamine on A. the fast and B. the slow phase of the response of the rat aorta to phenylephrine (n=7-16).
(●) control; (○) 10nM; (□) 20nM; (Δ) 50nM phenoxybenzamine.

vasoconstrictor response of the rat aorta to phenylephrine is affected less by irreversible receptor antagonism, than the slow phase of the response.

8.4.2.2. The effect of benextramine on the response of the rat aorta to phenylephrine.

To determine whether the above differential inhibition of the fast and slow components of the response of the rat aorta to phenylephrine was exclusive to phenoxybenzamine, the experiments were repeated using the non-competitive antagonist benextramine. The results are shown in figure 32.

Benextramine, at concentrations of 0.5 and 1μM, shifted the curve for the fast phase of the response of the rat aorta to phenylephrine to the right. At the lower concentration, benextramine caused a parallel rightward shift of the concentration response curve to phenylephrine with a reduction in the maximum response. At 1μM, benextramine caused a reduction in the slope of the concentration response curve. The curve for the slow response to phenylephrine was shifted to the right with a reduction of the slope and suppression of the maximum at both concentrations of benextramine tested. Measured at the $EC_{50}$, 0.5μM benextramine shifted the fast phase of the response significantly less than the slow. The mean difference was 0.49±0.15 log cycles ($p<0.05$, $n=8$).

Pretreatment with 1μM benextramine caused a significantly greater reduction of the maximum slow phase of the response than of the fast when each phase was compared with its own maximum (figure 32, $p<0.05$, $n=8$).

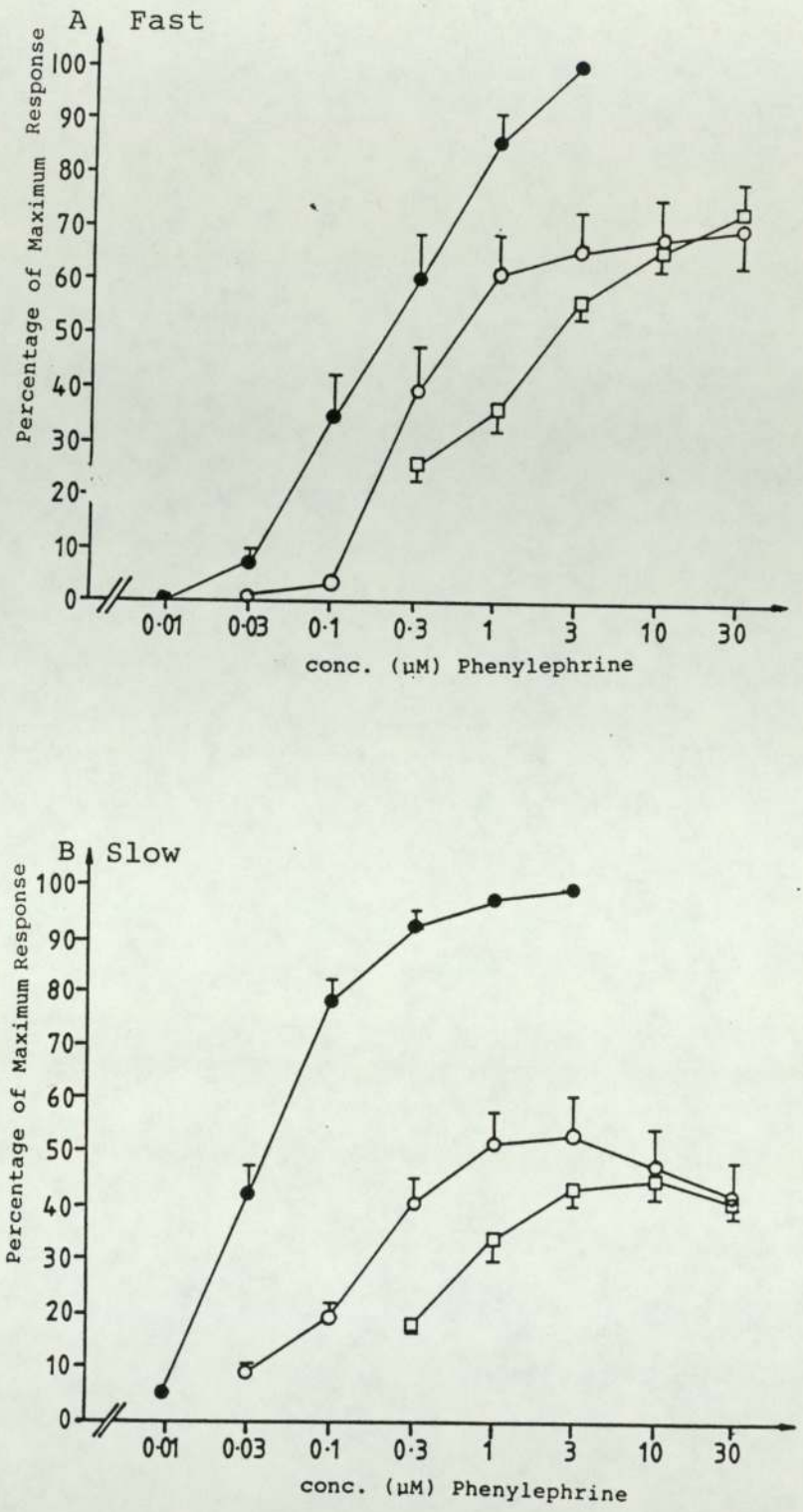



Figure 32: The effect of pretreatment with benextramine on A. the fast and B. the slow phase of the response of the rat aorta to phenylephrine (n=8-16).
(●) control; (○) 0.5μM; (□) 1μM benextramine.

It would therefore appear that pretreatment with benextramine, or with phenoxybenzamine, has less effect on the fast phase of the phenylephrine mediated response than on the slow.

8.4.2.3. Agonist Dissociation Constants.

In order to compare the rate of rise of receptor occupancy of an agonist with the magnitude of component of the response due to intracellular calcium release, it was first necessary to determine the dissociation constant for each agonist tested.

Table 1 summarises the values obtained to describe a series of six agonists acting at $\alpha_1$-adrenoceptors on the rat aorta:- noradrenaline (NA); phenylephrine (PE); guanfacine (GUAN); UK-14,304 (UK); amidephrine (AMID) and St 587 (St). The agonist dissociation constant ($K_A$ - Furchgott 1966) describes the concentration of drug required to occupy 50% of the receptor population and was found to be in the order St<NA<PE<UK<GUAN<AMID. The reciprocal of the $K_A$ is the affinity (Stephenson 1956) hence the order of affinities for the agonists is the reverse of the order of $K_A$.

The potency of an agonist may be represented by the $EC_{50}$ and is the concentration of agonist required to produce a response that is 50% of the maximum attained for that agonist. Thus the order of potency in this series was found to be NA>PE>GUAN>St>UK>AMID. The $EC_{50}$ of an agonist is less than the $K_A$ where receptor reserve exists, and the magnitude of the difference reflects the extent of the

Table 1: Receptor parameters for a number of $\alpha$-adrenoceptor agonists on the rat aorta calculated from the equilibrium responses in calcium containing Krebs' solution, and the EGTA resistant responses for these agonists measured at the peak and second, sustained phase.
(n)- denotes number of observations.

Table 1.

| Parameter | | Agonist (No. of observations) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Noradrenaline (5) | Phenylephrine (8) | UK-14,304 (4) | Guanfacine (8) | Amidephrine (8) | St 587 (8) |
| Dissociation Constant | | $2.87(\pm0.63) \times 10^{-7}$ | $4.27(\pm0.88) \times 10^{-7}$ | $1.39(\pm0.88) \times 10^{-6}$ | $3.13(\pm1.04) \times 10^{-6}$ | $3.42(\pm0.73) \times 10^{-6}$ | $4.31(\pm1.10) \times 10^{-8}$ |
| Intrinsic Activity | | 100% | 95.2±3.3 % | 50.9±6.6 % | 78.2±4.9 % | 22.0±3.6 % | 9.2±1.6 % |
| Potency ($EC_{50}$) | | $7.36(\pm1.27) \times 10^{-9}$ | $3.55(\pm0.55) \times 10^{-8}$ | $1.04(\pm0.22) \times 10^{-6}$ | $3.59(\pm0.83) \times 10^{-7}$ | $1.14(\pm0.22) \times 10^{-5}$ | $5.95(\pm1.47) \times 10^{-7}$ |
| Relative Efficacy (at 10%) | | 1.0 | 0.187 | 0.138 | 0.019 | 0.006 | 0.004 |
| Receptor Reserve (at 95%) | | 77.9±8.7% | 73.1±4.5% | 23.5+13.8% | 59.7+4.7% | 2.9±0.7% | 2.0±1.1% |
| EGTA resistant response as a proportion of the calcium present response | Peak | 0.25±0.01 | 0.20±0.02 | 0.07±0.01 | 0.10±0.01 | 0.44±0.16 | 0.25±0.07 |
| | 2nd Phase | 0.09±0.01 | 0.08±0.01 | 0.03±0.01 | 0.08±0.01 | 0.31±0.15 | 0.20±0.04 |

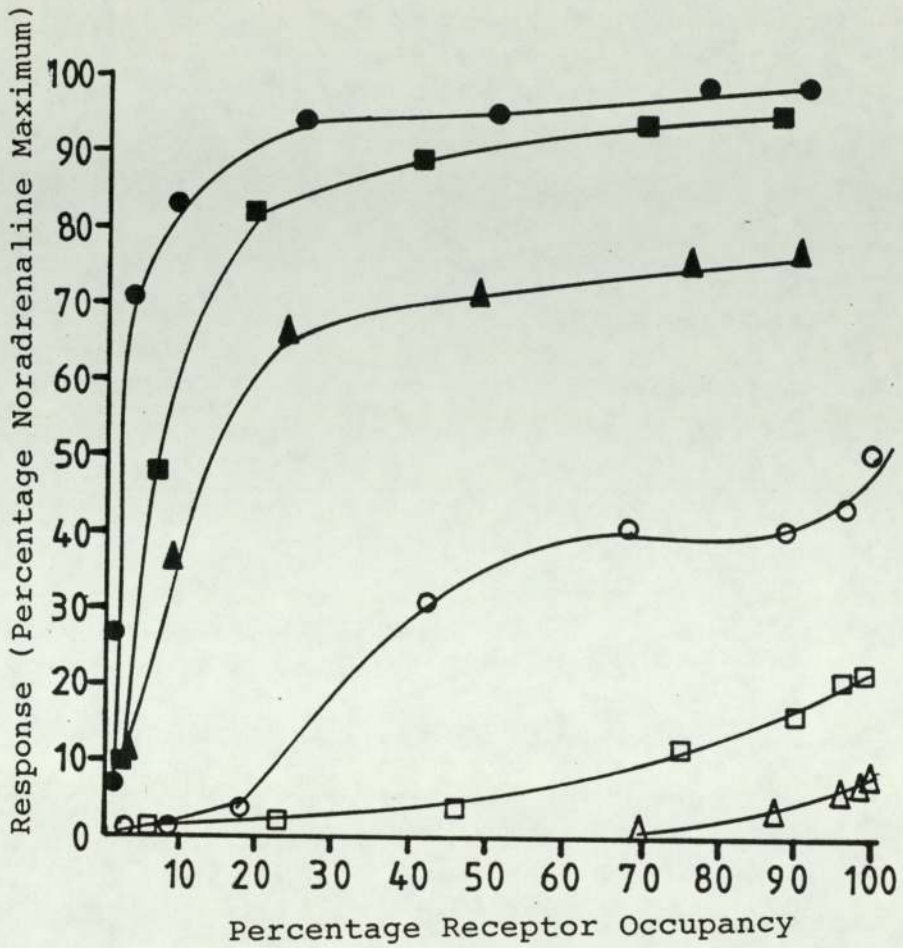



Figure 33: Response - occupancy curves for a range of α-adrenoceptor agonists on the rat aorta calculated from mean $K_A$ values in table 1.

(●) noradrenaline; (■) phenylephrine; (▲) guanfacine; (○) UK-14,304; (□) amidephrine; (△) St 587.

receptor reserve (Furchgott 1966, p41).

The intrinsic activity of an agonist is a measure of the maximum response attainable by that agonist, as a proportion of the maximum response to a potent, full agonist. This has been defined such that the intrinsic activity of NA is said to be 100% and the order of intrinsic activities for the agonists tested here were found to be NA>PE>GUAN>UK>AMID>St.

The relative efficacy of an agonist is also a comparative value and represents the relative position of agonists on the response vs occupancy curve at some defined value, usually 50% (Furchgott and Burstzyn 1967, p887). Figure 33 is a plot of the percentage receptor occupancy for the agonist series against the response, which in this case is the percentage of the noradrenaline maximum. These curves are related to the intrinsic activity. Therefore it is not possible to compare relative efficacies at 50% because only three of the agonists tested have an intrinsic activity greater than 50%. To assess the relative efficacies at a different level of the percentage of NA maximum changes the absolute values obtained but does not affect the order of the agonist series. At 10% of the NA maximum the order of relative efficacies for the agonists tested was found to be NA>PE>GUAN>UK>AMID>St; which is the same order as the intrinsic activities of the agonists.

The receptor reserve of an agonist represents the proportion of the receptor population occupied by an agonist in order to produce a maximum response (Furchgott 1966, p40). However, the occupancy-response curves for potent agonists gradually tend towards a maximum (see figure 33).

This may be interpreted as follows; 95% of the response to the potent agonist may be achieved by occupation of less than 30% of the receptor population, but the maximum may not be achieved until the total active receptor population has been occupied by the agonist. Therefore the percentage receptor occupancy was calculated for 95% of the maximum response as an assessment of the receptor reserve. The receptor reserve was found to be in the order NA>PE>GUAN>UK>AMID>St; which is the same order as the relative efficacies and intrinsic activities of the agonists.

The results of this section were then used for comparison with the ability of the agonists to produce EGTA resistant responses.

8.4.2.4. EGTA resistant responses of the rat aorta to α-adrenoceptor agonists.

The EGTA resistant responses of the rat aorta to a range of agonists acting at $\alpha_1$-adrenoceptors is illustrated in figure 34.

Originally, the intention was to study the EGTA resistant responses of a range of α-adrenoceptor agonists at concentrations that gave equivalent sized responses in the presence of calcium. However, the low intrinsic activities of St and AMID restricted the size of the responses that would have been measured by this method to some 10% of the noradrenaline maximum (see table 1). EGTA resistant responses of the rat aorta to noradrenaline represent up to only 30% of the response attainable in the presence of

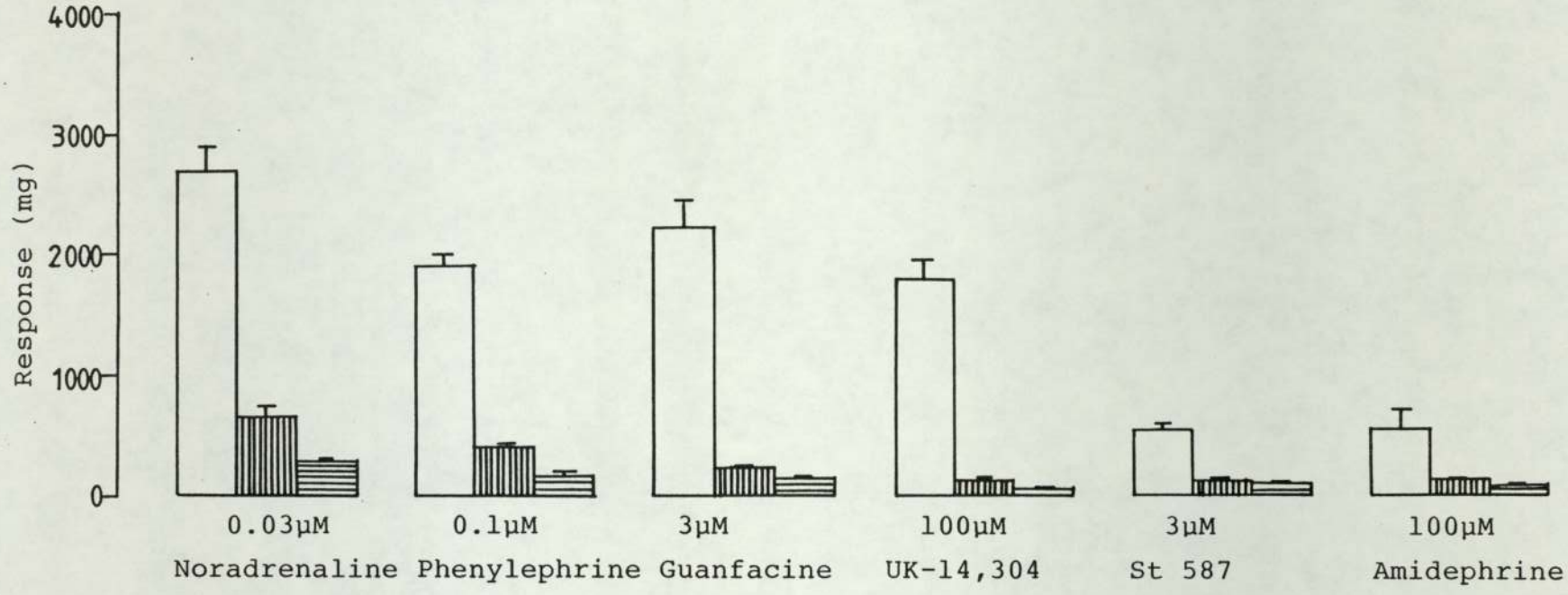



Figure 34: Responses (in mg) of the rat aorta to a range of α-adrenoceptor agonists.
responses in the presence of calcium; initial, transient peak of the EGTA resistant response;
second, sustained phase of the EGTA resistant response.

The concentrations used for each agonist are given under the appropriate figure (n=6-8).

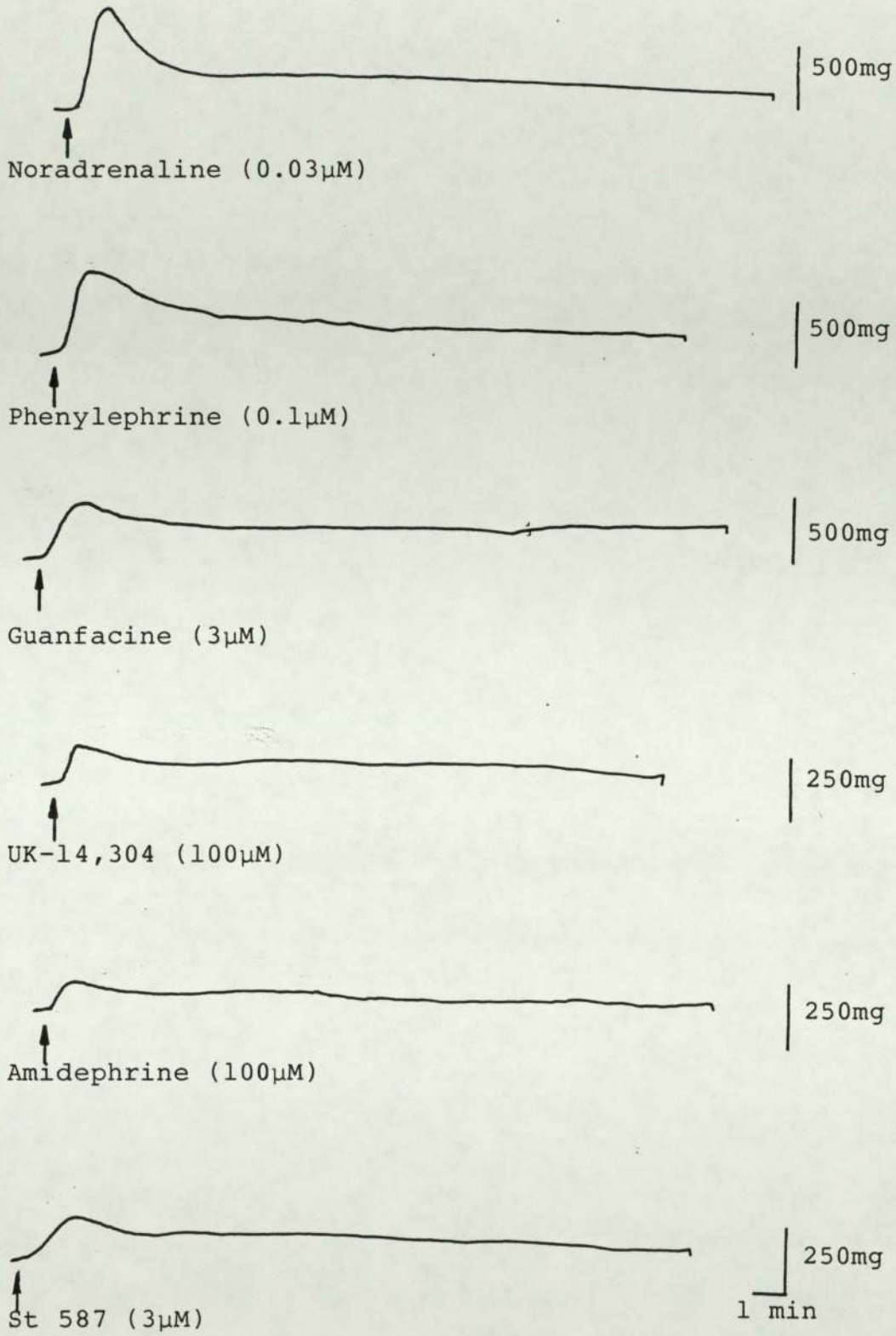



Figure 35: Representative traces showing the EGTA resistant responses of the rat aorta to a range of $\alpha$-adrenoceptor agonists.
Arrows indicate addition of agonist.

calcium (section 8.3.2.2.). Therefore to measure EGTA resistant responses to the range of agonists at 10% of the noradrenaline maximum would give such small responses that little could be determined in this way.

Since the purpose of the experiment was to determine which parameter described the ability of that agonist to produce an EGTA resistant response relative to the total response, it was considered that by expressing the EGTA resistant response as a proportion of the response in the presence of calcium, this would standardise the results to give a measure independent of the absolute size of the contractions.

In the presence of calcium, PE (0.1μM), GUAN (3μM) and UK (100μM), were equieffective in terms of the slow phase of the response. St (3μM) and AMID (100μM) also produced similar responses but the maximum responses attainable by St and by AMID were less than those for the other agonists tested. The calcium present response to NA (0.03μM) was significantly greater than responses to all the other agonists except GUAN ($p<0.05$, $n=6-8$).

EGTA resistant responses expressed as a proportion of the response in the presence of calcium were in the order AMID>NA≥St>PE>GUAN≥UK for the initial, transient peak and in the order St≥AMID>NA≥PE=GUAN>UK for the second, sustained phase.

When the order of magnitude of the EGTA resistant responses were compared with the order of agonist parameters (above) it could be seen that there were no conclusive association between the agonists and their ability to

produce either phase of an EGTA resistant response. It should be noted that the order of agonists differs for the peak and second phases. This suggests that the two phases of the EGTA resistant responses may be activated by independent mechanisms.

The agonists with the lowest intrinsic activities AMID and St, also have the lowest relative efficacies but do not have the smallest EGTA resistant responses of the agonists tested when expressed as a proportion of the total response. PE, GUAN and UK, show the same rank order (i.e. PE>GUAN>UK) for the EGTA resistant responses measured at both the peak and at the second phase for intrinsic activity, potency, relative efficacy and receptor reserve. In comparisons of the dissociation constants for the agonists the order becomes PE>UK>GUAN.

The absolute size of the EGTA resistant responses of the rat aorta to 0.03μM NA was greater than those of all the agonists tested but the second phase, as a proportion of the response in the presence of calcium was less than that for St and AMID.

It would seem then that the EGTA resistant proportion of a response to a given concentration of agonist cannot be attributed to one descriptive property of that agonist.

8.4.3. Discussion.

This section examined firstly, the effect of non-competitve antagonism on the fast and slow components of the α-adrenoceptor mediated calcium present response of the rat aorta to phenylephrine, and secondly, the property of an agonist that may be related to the ability to cause vaso-constriction due to the release of intracellular calcium.

The response of rat aorta to phenylephrine following irreversible receptor inactivation may be described as classical non-competitive antagonism, i.e. dose response curves were progressively shifted to the right and the maximum supressed (see figures 31 and 32). However, it was observed that the inhibition of the slow phase of the response was greater than that of the fast. The slow phase is indicative of the tissue response to agonists under equilibrium conditions. The fast response may arise from the initial binding of the drug with the receptor. If this is true then the fast response may be predominantly a function of the agonist rather than of the size of the active receptor population, i.e. the ability of an agonist to combine with and stimulate any given receptor would be unchanged. This may be because inactivation of part of the receptor population does not affect the remainder, although the lowered receptor number would reduce the number of possible agonist/receptor interactions when the drug first comes into contact with the tissue. The slow response is attained under equilibrium conditions at which the maximum sustained response could be limited by the size of the active receptor population.

Following this reasoning (see above) the role of the agonist in initiating the release of intracellular calcium was considered. However, the experiments described in sections 8.4.2.3. and 8.4.2.4. failed to show any conclusive association between agonist dissociation constants and the release of intracellular calcium. It should be noted that the $K_A$ for St 587 was particularly low. However, there is reason to believe that this value was inaccurate because it was lower than the $EC_{50}$, which contradicts Furchgott's (1966) theory, validated by Ruffolo et al 1979. The discrepancy is therefore difficult to explain.

It would appear that the principle problem was due to the difference in the intrinsic activities of the agonists. In order to establish whether the $K_A$ of an agonist may be used to predict its ability to cause the release of intracellular calcium, it would be necessary to use a range of agonists with the same definitive parameters - but with different $K_A$s. This issue is considered further in the general discussion.

Although there is some evidence to support the hypothesis that the dissociation constant of an antagonist is a measure of its ability to inhibit the initial, rapid release of intracellular calcium (Downing et al 1985); there is no conclusive evidence that this same measure predicts the ability of an agonist to activate this calcium pool. However, it is considered that the ability of an agonist to initiate and/or sustain a response, in the presence or absence of extracellular calcium cannot be described in terms of any single property of that agonist. This is due to

the interrelationship between affinity, efficacy, intrinsic activity, potency and also the stimulus-response coupling of the tissue. The composite nature of drug-receptor interactions forms the basis of the general discussion of this study.

In summary:

1. The α-adrenoceptor mediated vasoconstricor response of the rat aorta was studied firstly, to evaluate the effect of non-competitive antagonism on the fast and on the slow components of the response. Secondly, to establish that property of the agonist that describes its ability to cause the release of intracellular calcium.
2. Pretreatment with phenoxybenzamine (10nM) and with benextramine (1μM) had less effect on the fast phase of the response to phenylephrine, than on the slow.
3. Of the agonists tested, no property could be found with the same rank order as the proportion of the response due to intracellular calcium release.
4. It is concluded that, in the rat aorta the size of the active receptor population is less important for the fast phase of the α-adrenoceptor mediated response than of the slow. The ability of an agonist to induce the release of intracellular calcium in this tissue cannot be attributed to one descriptive property of that agonist.

## 8.5. Rat Aorta - Endothelium

### 8.5.1. Introduction.

The response of the rat aorta to α-adrenoceptor agonists is influenced by the presence or absence of a functional endothelium (Egleme, Godfraind and Miller 1984). The continuous release of an endothelium derived relaxant factor (EDRF) decreases the responsiveness of vascular smooth muscle, particularly arterial smooth muscle, to agonists (Miller et al 1984).

Egleme et al (1984) found that the contractile effect of noradrenaline and clonidine on the rat aorta was enhanced by the removal of endothelium. Clonidine was particularly affected by endothelium removal. Lues and Schumann (1984) extended this study by investigating the response of this tissue to a range of α-adrenoceptor agonists that varied widely in their structure, and selectivity for α-adrenoceptor subtypes. They found that all of the agonists tested showed a profound increase in potency and intrinsic activity when the endothelium was absent, as compared to the responses in its presence. Furthermore they found a direct correlation between the increase in the maximum response of the tissue to the agonist due to the removal of the endothelium, and the potency of the agonist.

However, all of the studies described above considered cumulative concentration response curves to agonists. Such a regime does not give rise to a fast response representing an initial, rapid release of intracellular calcium (Godfraind and Kaba 1972). Therefore, in this section, the effect of a range of α-adrenoceptor agonists, given by bolus

administration, were compared on normal and on endothelium denuded sections of aortae. The objective of these experiments was to determine whether there was a change in the fast response of the tissue, particularly to agonists such as UK-14,304, amidephrine and St 587. The question addressed was whether removal of the endothelium would reveal a component of the response that may be attributed to intracellular calcium release.

8.5.2. Results.

8.5.2.1. Removal of the endothelium by mechanical rubbing.

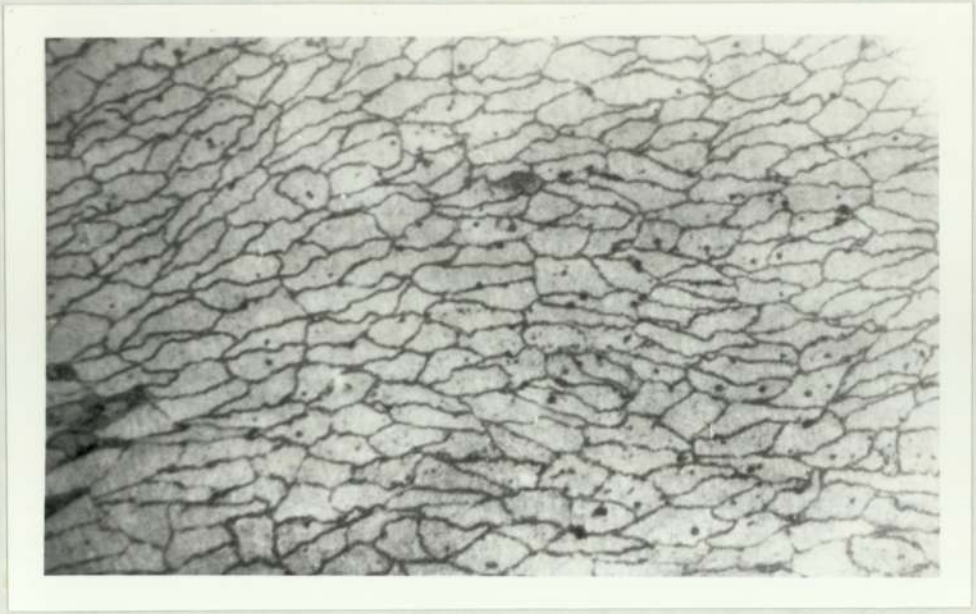
In this series of experiments, concentration response curves to the agonists under test were constructed on rings of rat aorta that had an intact endothelium. Following washout, tissues were taken out of the organ bath and the endothelium removed by mechanical rubbing before remounting. A full description of this method has been given in section 7.2.4.

In each experiment, the presence of a functional endothelium was assessed by the demonstration of a relaxation by 1μM acetylcholine of rings pretreated with 1μM noradrenaline. The method was further validated in a single experiment by histology, using a modified version of the method described by Poole, Sanders and Florey (1958). Figure 36 shows two photographs of the inner surface of isolated rings of rat aorta, viewed through a light microscope. In figure 36A (unrubbed) there is a layer of squamous cells while in 36B (rubbed), the view is of smooth muscle. Thus the method of mechanical rubbing of the inner surface of the aorta has been shown to remove the endothelial cell layer.

8.5.2.2. The response of the rat aorta to a range of α-adrenoceptor agonists in the preparations with or without endothelium.

The effect of removal of the endothelium on the response of the rat aorta to phenylephrine is illustrated in figure 37. Removal of the endothelium caused a significant shift to

A. With endothelium.



B. Without endothelium.

Figure 36: Photographs of the rat aorta viewed under the light microscope (x100) A. with endothelium and B. without endothelium.

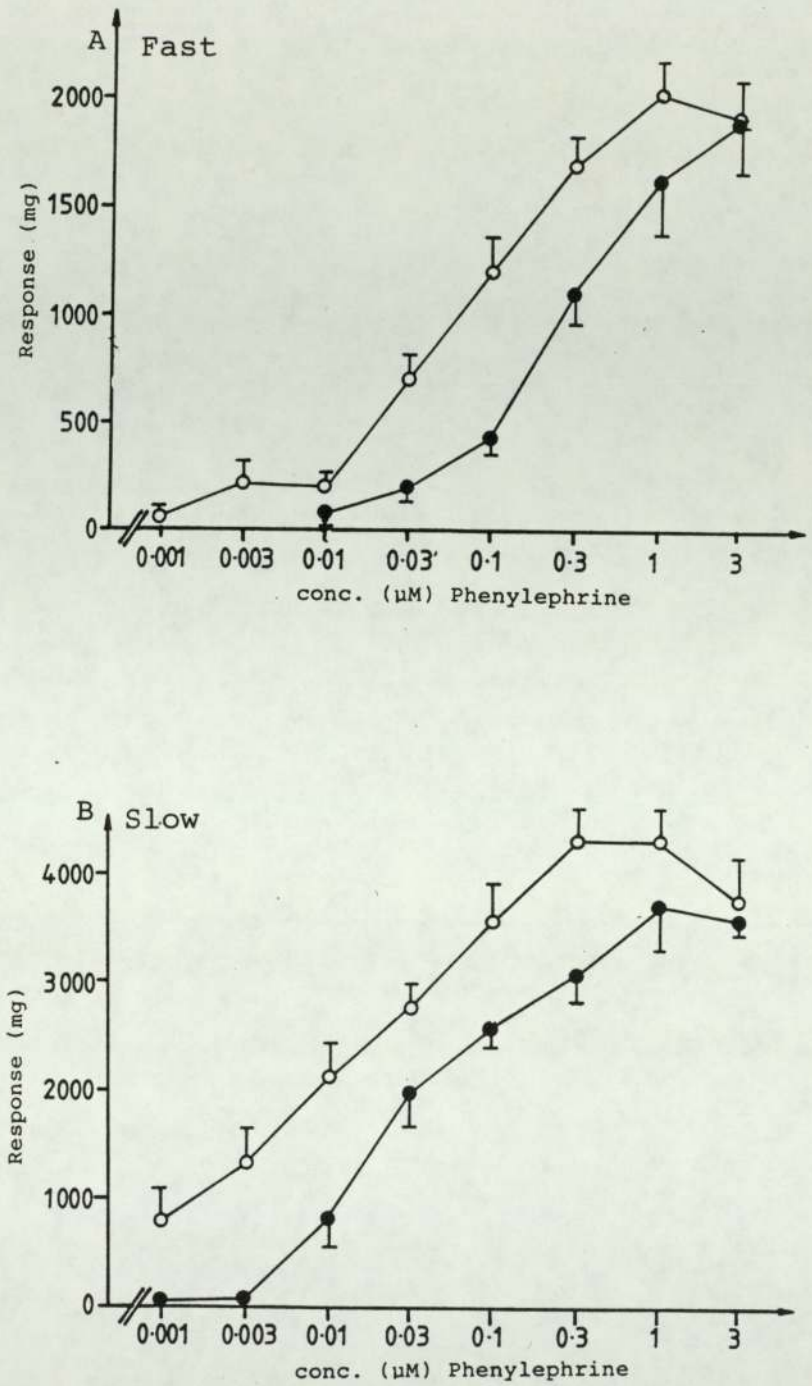



Figure 37: The effect of removal of the endothelium on A. the fast and B. the slow phase of the response of the rat aorta to phenylephrine (n=8).
(●) with endothelium; (O) without endothelium.

the left of the concentration response curve for the fast phase of the response (0.70±0.23 log cycles measured at the $EC_{50}$, $p<0.05$, $n=8$). However, the maximum was not significantly changed; 2200±229mg and 2280±124mg in the presence and absence of endothelium respectively.

The slow phase of the response was similarly affected. There was a significant shift to the left (0.96±0.25 log cycles) of paired responses measured at the $EC_{50}$, while the maximum response was not significantly changed (4514±262mg and 4943±328mg in the presence and absence of endothelium respectively, $p<0.05$, $n=8$).

The greatest effect of endothelium removal in this series of experiments was observed with the responses of the rat aorta to UK-14,304 (figure 38). The maximum fast response was increased from 142±59mg in the presence of endothelium, to 1267±238mg in its absence ($p<0.05$, $n=6$). Similarly the maximum slow response was increased from 404±161mg in the presence of endothelium to 3100±255mg in its absence ($p<0.05$). The concentration response curve for the slow phase of the response to UK-14,304 was significantly shifted to the left following endothelium removal. Thus the $EC_{50}$ was (1.62±2.45) $x10^{-5}M$ in the presence of endothelium and (1.05±1.2) $x10^{-6}M$ ($n=8$) after endothelium removal ($p<0.05$). Although the concentration response curve for the fast phase of the response was shifted to the left, there was no significant difference between the $EC_{50}$ in the presence of endothelium, (1.02±2.45) $x10^{-5}M$, and in its absence, (2.40±1.58) $x10^{-6}M$ ($p>0.05$, $n=8$).

Responses to guanfacine were not affected by the removal

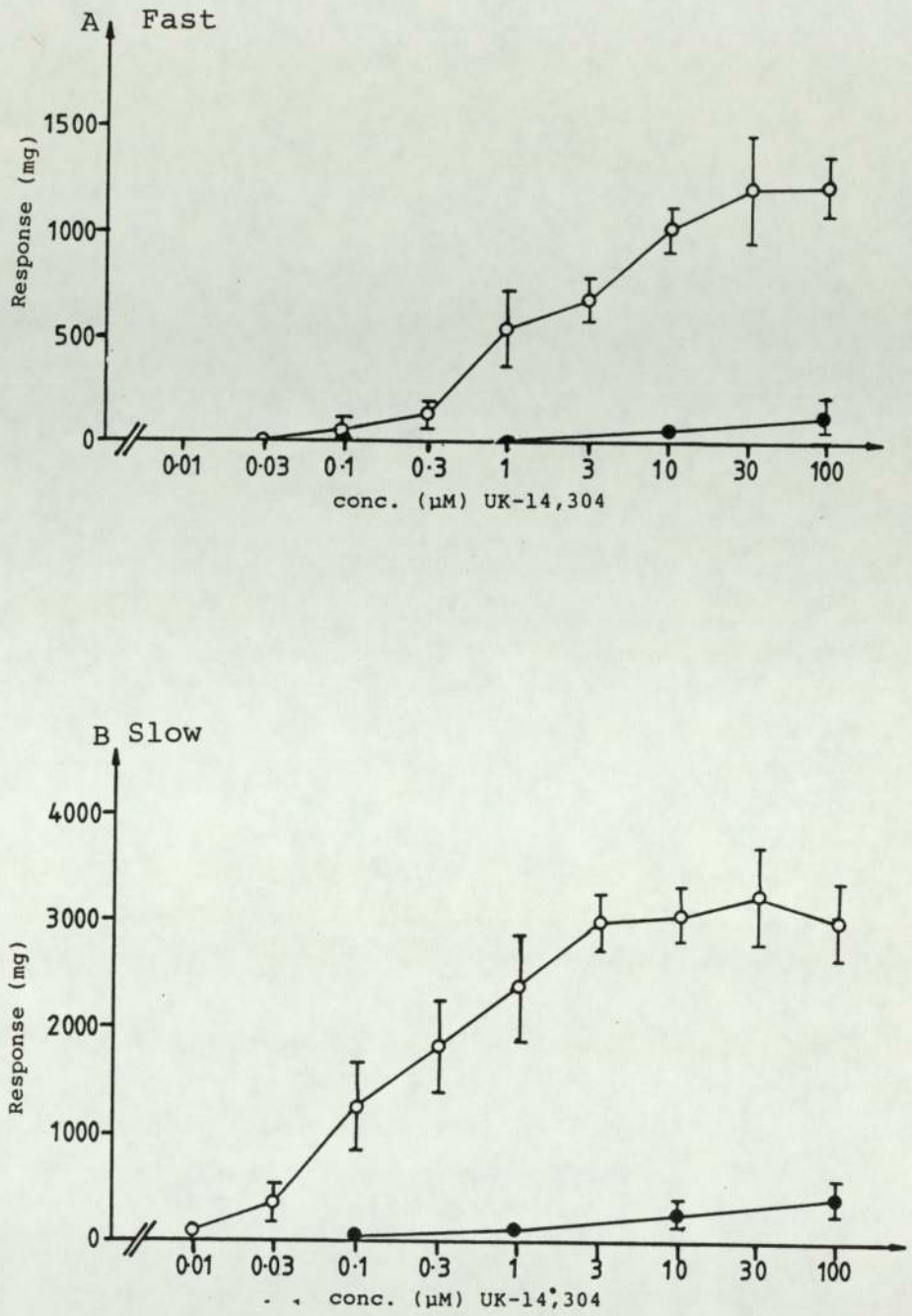



Figure 38: The effect of removal of the endothelium on A. the fast and B. the slow phase of the response of the rat aorta to UK-14,304 (n=6).
(●) with endothelium; (O) without endothelium.

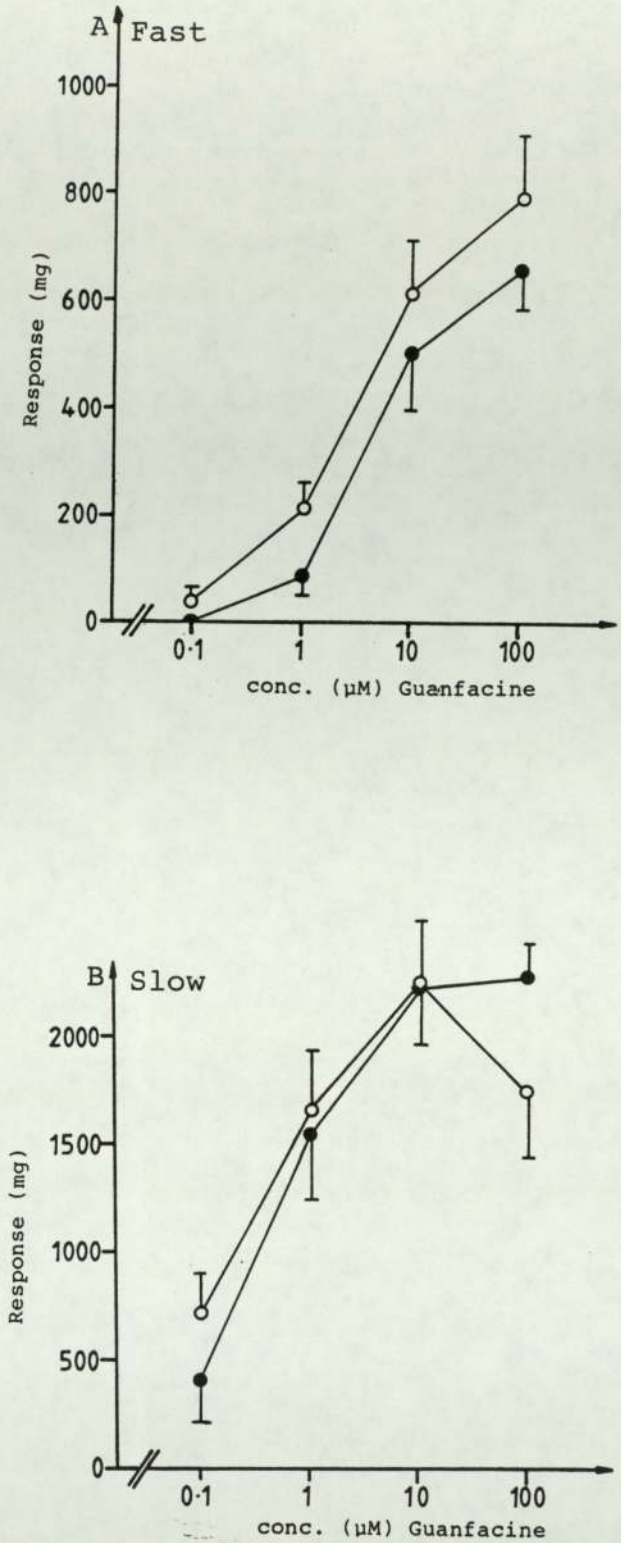



Figure 39: The effect of removal of the endothelium on A. the fast and B. the slow phase of the response of the rat aorta to guanfacine (n=8).
(●) with endothelium; (O) without endothelium.

of the endothelium (figure 39). Although there appears to be a separation in the curves for the fast response in the presence and absence of endothelium, the $EC_{50}$ for the response in the presence of endothelium is not significantly different from the $EC_{50}$ in its absence. The maximum value for the fast and slow phases of the response was unaffected by endothelium removal (see table 2).

Amidephrine produced little response in the rat aorta in the presence of endothelium. The mean maximum fast response measured was only 80±16mg, and the slow response was found to be 105±20mg (n=8). However, after removal of the endothelium, the fast response increased to 331±76mg and the slow response to 359±48mg. Both of these values are significantly greater than the maximum values obtained in the presence of endothelium ($p<0.05$, n=8). There was no significant difference between the $EC_{50}$s for either the fast or slow responses in the presence or absence of endothelium (figure 40, $p>0.05$, n=8).

There was little response of the rat aorta to St 587 in the presence of endothelium (figure 41). The mean maximum fast response was found to be 43±14mg and the slow response 134±16mg. After removal of the endothelium the mean maximum fast response became 136±56mg, which was not significantly different from the value obtained in the presence of endothelium ($p>0.05$, n=8). However, the mean maximum slow response was significantly increased by endothelium removal to 483±114mg. The mean $EC_{50}$ values for both the fast and slow responses were not significantly changed by removal of the endothelium.

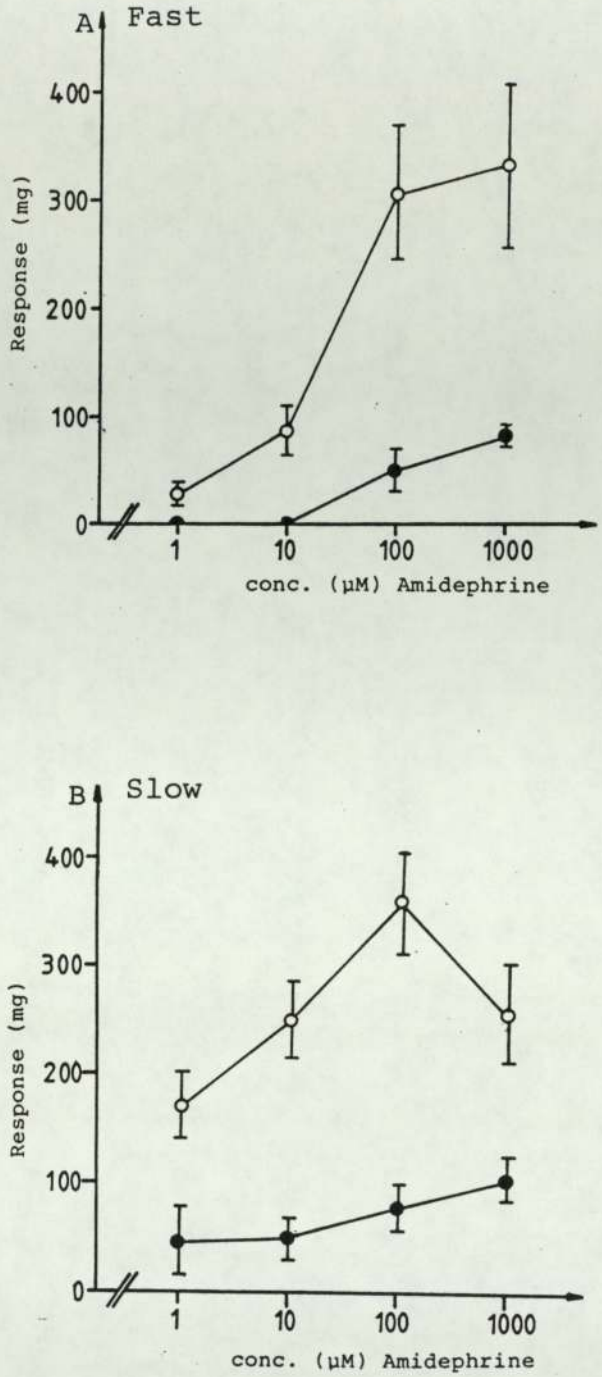



Figure 40: The effect of removal of the endothelium on A. the fast and B. the slow phase of the response of the rat aorta to amidephrine (n=8).
(●) with endothelium; (O) without endothelium.

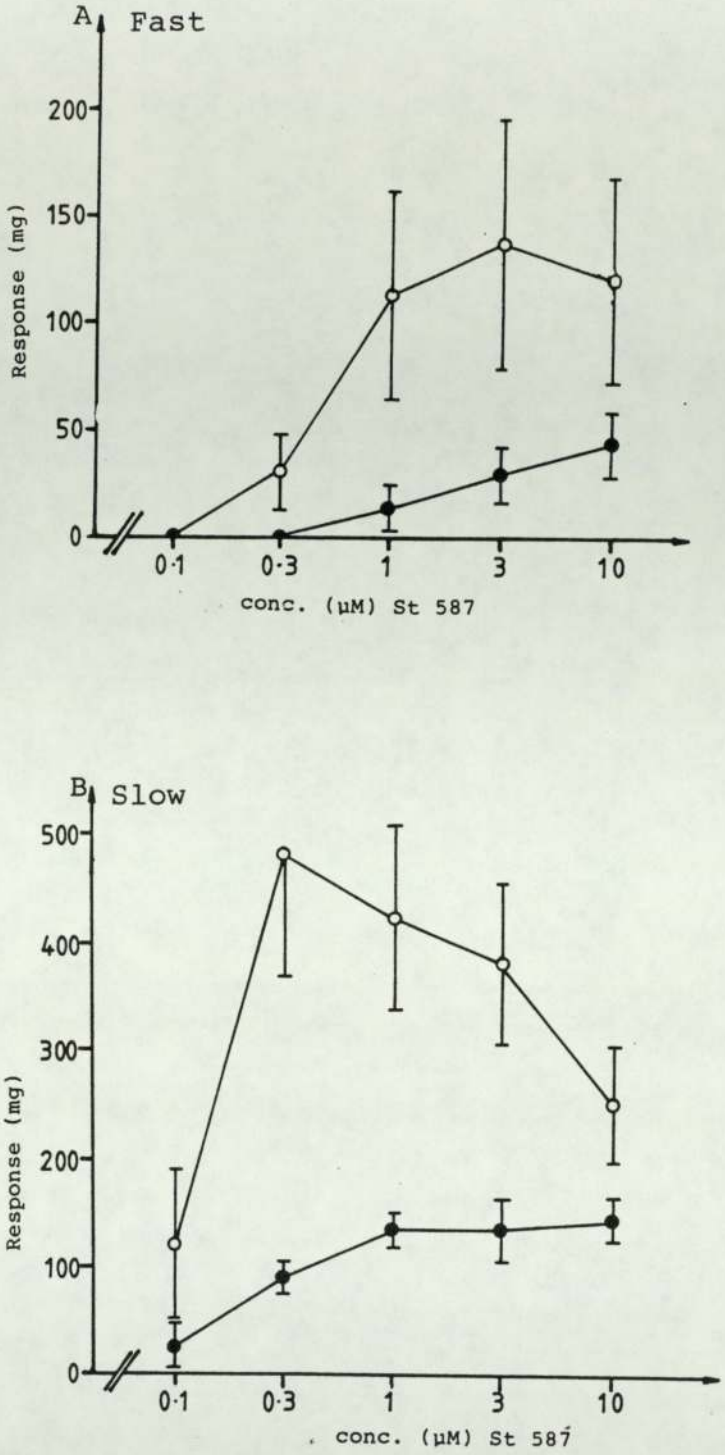



Figure 41: The effect of removal of the endothelium on A. the fast and B. the slow phase of the response of the rat aorta to St 587 (n=8).
(●) with endothelium; (○) without endothelium.

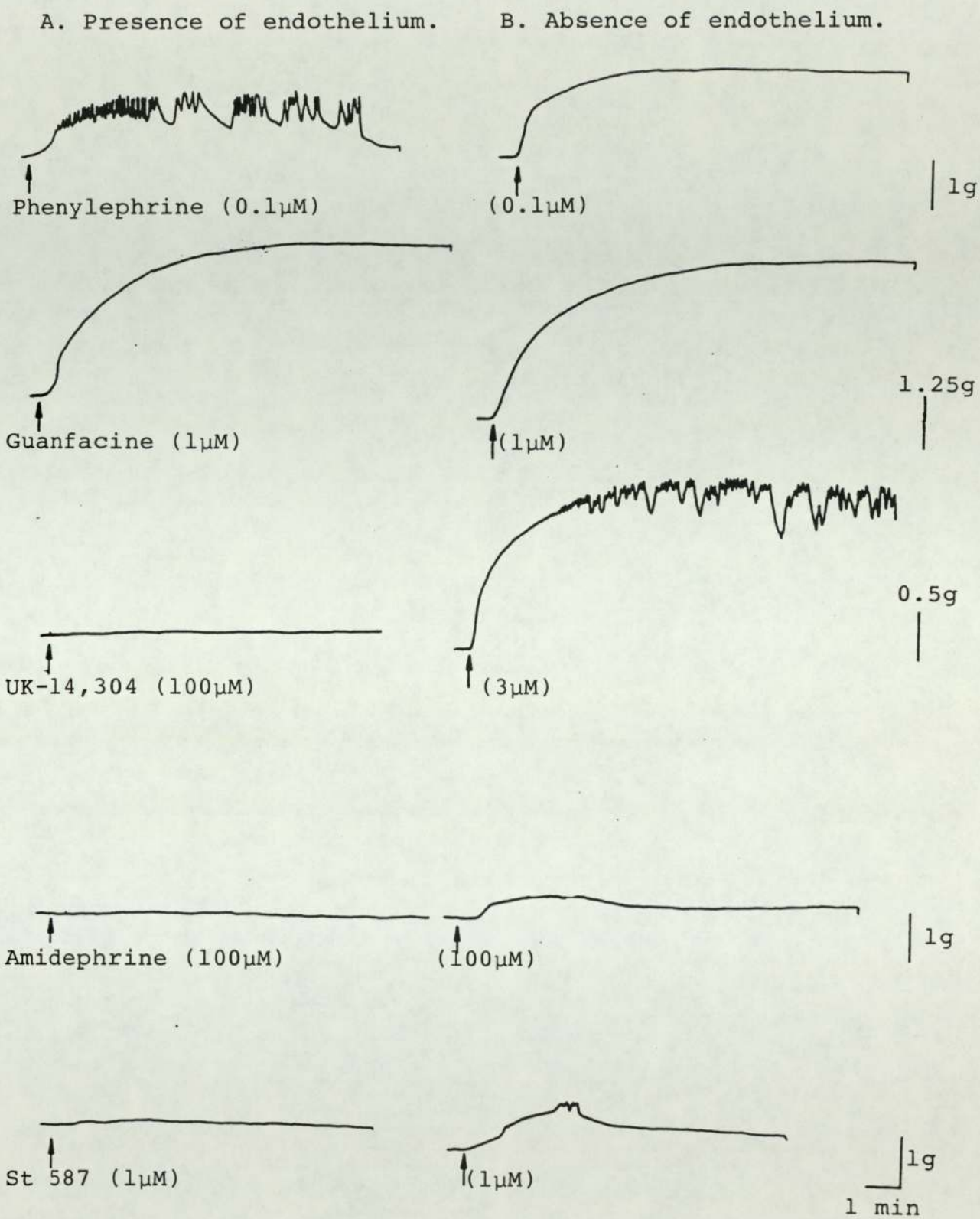



Figure 42: Representative traces showing the effect of removal of the endothelium on the response of the rat aorta to a range of α-adrenoceptor agonists.
A. with endothelium; B. without endothelium.
Arrows indicate addition of agonist.

Table 2: The $EC_{50}$ and $E_{max}$ values for the fast component of the response of the rat aorta to a range of $\alpha$-adrenoceptor agonists in preparations with and without endothelium.
The difference in $EC_{50}$ values is shown as a log shift measured at the $EC_{50}$.
* indicates significant difference between values ($p<0.05$).

Table 2. Fast phase

| Agonist | Number of Observations | $EC_{50}$ | | Log shift | Emax (mg) | |
|---|---|---|---|---|---|---|
| | | with endothelium | without endothelium | | with endothelium | without endothelium |
| Phenylephrine | 8 | $3.24 (\pm 1.23) \times 10^{-7}$ * | $6.46 (\pm 1.74) \times 10^{-8}$ | 0.70±0.23 | 2200±229 | 2287±124 |
| UK-14,304 | 6 | $1.02 (\pm 2.45) \times 10^{-5}$ | $2.40 (\pm 1.58) \times 10^{-6}$ | 0.63±0.34 | 142±59 * | 1267±238 |
| Guanfacine | 8 | $1.20 (\pm 1.48) \times 10^{-5}$ | $5.89 (\pm 6.02) \times 10^{-5}$ | 0.30±0.35 | 653±75 | 725±125 |
| Amidephrine | 8 | $1.26 (\pm 1.95) \times 10^{-4}$ | $3.63 (\pm 1.55) \times 10^{-5}$ | 0.54±0.35 | 80±16 * | 331±76 |
| St 587 | 8 | $2.09 (\pm 14.45) \times 10^{-4}$ | $1.78 (\pm 13.18) \times 10^{-5}$ | 1.07±1.58 | 43±14 | 136±58 |

Table 3: The $EC_{50}$ and $E_{max}$ values for the slow component of the response of the rat aorta to a range of $\alpha$-adrenoceptor agonists in preparations with and without endothelium.
The difference in $EC_{50}$ values is shown as a log shift measured at the $EC_{50}$.
* indicates significant difference between values ($p<0.05$).

Table 3. Slow phase

| Agonist | Number of Observations | $EC_{50}$ | | Log shift | Emax (mg) | |
|---|---|---|---|---|---|---|
| | | with endothelium | without endothelium | | with endothelium | without endothelium |
| Phenylephrine | 8 | 6.92 (±1.44) $x10^{-8}$ * | 7.59 (±1.44) $x10^{-9}$ | 0.96±0.25 | 4514±262 | 4943±328 |
| UK-14,304 | 6 | 1.62 (±2.45) $x10^{-5}$ * | 1.05 (±1.20) $x10^{-6}$ | 1.20±0.36 | 404±161 * | 3100±255 |
| Guanfacine | 8 | 2.34 (±1.90) $x10^{-6}$ | 1.58 (±1.90) $x10^{-6}$ | 0.17±0.29 | 2281±147 | 2276±390 |
| Amidephrine | 8 | 1.62 (±2.57) $x10^{-5}$ | 4.79 (±2.75) $x10^{-6}$ | 0.16±0.88 | 105±20 * | 359±48 |
| St 587 | 8 | 2.51 (±1.29) $x10^{-7}$ | 2.82 (±1.48) $x10^{-7}$ | 0.04±0.21 | 134±16 * | 483±114 |

Thus it has been shown that removal of the endothelium may allow increases in the fast phase as well as the slow phase of the response of the rat aorta to α-adrenoceptor agonists. The results described above are summarised in tables 2 and 3, and representative traces have been shown in figure 42.

8.5.2.3. The effect of endothelium removal on the response of the rat aorta to caffeine.

It was considered of interest to evaluate the effect of removal of the endothelium on the response of the rat aorta to an agent that caused the release of intracellular calcium other than by action at adrenoceptors, for example caffeine (Deth and Lynch 1981b).

It was found that the response of the rat aorta to caffeine was unaffected by the removal of endothelium. In the presence of endothelium, the magnitude of the response elicited by caffeine (35mMol) was 1424±177mg. Following removal of the endothelium the response was 1391 ±156mg (n=8). These values are not significantly different ($p>0.05$).

Therefore the evidence does not suggest a direct effect of the endothelium on intracellular calcium mobilisation.

8.5.2.4. The effect of removal of the endothelium on the efficacy of agonists.

If EDRF acts directly on smooth muscle, then it would not be expected to affect the rate at which agonists bind to and dissociate from adrenoceptors. It was therefore considered

of interest to make a brief examination of the dissociation constant for an agonist in the presence and absence of endothelium. In these experiments, separate segments of aorta were used, with and without endothelium, because the method for determination of agonist dissociation constants (Furchgott 1966) requires the use of irreversible receptor inactivation.

The $K_A$ for noradrenaline in the presence of endothelium was found to be $(2.01\pm0.48)\times10^{-7}$M (n=8), and in its absence $(2.92\pm0.50)\times10^{-7}$M (n=6). These values are not significantly different from each other. Similarly, the $K_A$ for phenylephrine in the presence of endothelium was $(9.43\pm3.40)\times10^{-7}$M (n=8) and in its absence $(1.12\pm0.43)\times10^{-6}$M (n=11). Again these values are not significantly different from each other ($p>0.05$).

However, removal of a functional endothelium caused a significant increase in the potency of the agonists with consequent changes in the $EC_{50}$ values (section 8.5.2.2.). Consequently, there was an increase in the relative efficacy of the agonists. In the presence of endothelium, the percentage receptor occupancy required to cause a 50% of maximum response by noradrenaline was approximately 7%. For tissues without endothelium, this value was 2% which is roughly a three and a half fold increase in the efficacy of noradrenaline in the rat aorta following removal of endothelium. In the same way, the efficacy of phenylephrine was approximately doubled.

Unfortunately it was not possible to determine the $K_A$ for weak agonists in the presence of endothelium because the

intrinsic activities of these agonists were too low to allow determination by the method of Waud (1969). However, it has been shown that in the presence of endothelium the ability of the potent agonists noradrenaline and phenylephrine to induce a response, is reduced.

8.5.3. Discussion.

The α-adrenoceptor mediated responses of the rat aorta were examined to determine the influence of the endothelium upon the fast and slow phases of the response to α-adrenoceptor agonists. There is evidence that the response of the rat aorta to cumulative addition of α-adrenoceptor agonists is modified by the presence of the endothelium. Lues and Schümann (1984) investigated the response of this preparation to a number of α-adrenoceptor agonists and for each, found that removal of the endothelium caused a leftward displacement of the concentration response curve and an increase in the maximum response. In contrast, Carrier and White (1985) failed to observe an increase in the maximum contractile force elicited by noradrenaline.

In this series of experiments, the concentration response curve for the potent $\alpha_1$-adrenoceptor agonist phenylephrine was shifted to the left in the absence of endothelium. But as observed by Carrier and White (1985) and Godfraind et al (1985), there was no increase in the maximum response. A number of experiments were performed in order to investigate the changes in the maximum response to potent agonists described by Carrier and White (1985), but not found in this study. However it was found repeatedly that the maximum response to the potent agonists tested in this study, was no different in the presence or absence of endothelium (results not shown). Nevertheless, an increase in potency caused by endothelium removal was shown, and was associated with an increase in the fast response to the agonist. Furthermore, agonists with very low intrinsic activities in the presence

of endothelium (i.e. UK-14,304, St 587 and amidephrine) showed not only a marked increase in the maximum response attained but also a measurable fast response.

Unfortunately, attempts to confirm the influence of EDRF on intracellular calcium have met with limited success, principally because the release of EDRF is dependent on the presence of extracellular calcium (Singer and Peach 1982). The present study has consistently used calcium free Krebs' solution containing 0.5mMol EGTA to estimate the component of responses due to intracellular calcium release. Therefore the same method could not be used to determine the magnitude of the component of the response due to intracellular calcium release in the presence or absence of endothelium, because EDRF would not be expected to be released in the absence of extracellular calcium. In confirmation of the above, a small number of experiments with UK-14,304 revealed that EGTA resistant responses in the presence and absence of endothelium were not significantly different (n=8, results not shown). However, the response of the rat aorta to caffeine was unchanged by the removal of endothelium. Caffeine has been suggested to release intracellular calcium from the same sites as noradrenaline (Deth and Lynch 1981) which suggests that EDRF affects receptor response coupling rather than producing a direct effect on the vascular muscle contractility.

The potency of full agonists is affected by the removal of endothelium. Potency is typically assessed at the $EC_{50}$ and this same value is used to determine the relative efficacy whereby:

$$\text{percentage receptor occupancy} = \frac{[A]}{K_A + [A]}$$

where [A] is the concentration of agonist in the region of the receptors, and $K_A$ is the agonist dissociation constant (Furchgott and Burstzyn 1967).

Then, if the $K_A$ of the agonist is unchanged by the presence or absence of endothelium, and the concentration of agonist required to elicit 50% of maximum response differs; then the percentage receptor occupancy will change and hence the relative efficacy will be changed. This has been shown here to be true for both noradrenaline and phenylephrine, but the same reasoning cannot be applied to the weak agonists where the $EC_{50}$ is not significantly changed. For these agonists there is an increase in the intrinsic activity following removal of endothelium. The intrinsic activity is related to efficacy in that receptor occupancy is plotted as a percentage of the maximum response to a potent agonist in order to determine relative efficacies. Where there is no receptor reserve, there is litle or no change in the $EC_{50}$ of curves in which the slopes and maxima are declining, as described by (Furchgott 1966 p41). Therefore, for weak agonists, a change in efficacy cannot be measured according to the method of Furchgott and Burstzyn (1967). It is possible that for the weak agonists an increase in intrinsic activity is associated with an increased fast response and hence a probable increase in the release of intracellular calcium.

In summary:

1. The effect of the endothelium on the response of the rat aorta to α-adrenoceptor agonists was examined. The objective was to determine whether EDRF affects the fast phase of $\alpha_1$-adrenoceptor mediated responses in this tissue.

2. The concentration response curve for the fast phase of the response to phenylephrine was significantly shifted to the left at the $EC_{50}$. There was a significant increase in the maximum fast phase of the response to UK-14,304 and to amidephrine.

3. The response of the rat aorta to 35mMol caffeine was unaffected by the presence or absence of endothelium.

4. It is concluded that the endothelium depresses the receptor response coupling of α-adrenoceptor agonists on the rat aorta, causing a decrease in relative efficacy of potent agonists and a decrease in intrinsic activity of weak agonists. It is further concluded that the ability of an α-adrenoceptor agonist to cause the release of intracellular calcium is a tissue related property. Since the agonist is unchanged by endothelium removal, changes in tissue responsiveness must be due to receptor response coupling.

## 9. GENERAL DISCUSSION.

This thesis is a study of the calcium dependence of α-adrenoceptor induced contraction of vascular muscle. Based on experiments in the pithed rat, it was suggested by Van Meel and co-workers (1981 a and b) that postjunctional α-adrenoceptors may be differentiated according to the calcium pools utilised by the receptor subtypes. These workers demonstrated that $\alpha_2$-adrenoceptor mediated pressor responses of the pithed rat were susceptible to blockade by calcium channel antagonists, while $\alpha_1$-adrenoceptor mediated responses were not. (Van Meel, De Jonge, Kalkman, Wilffert, Timmermans and Van Zwieten 1981 a and b). Support for this work has been given *in vivo* in pithed rats (Cavero *et al* 1983) and in the canine autoperfused hindlimb (Llenas and Massingham 1983) and *in vitro* in strips of canine saphenous vein (Cavero *et al* 1983).

However, while most studies support the view that $\alpha_2$-adrenoceptor mediated responses may be attenuated by the presence of calcium channel antagonists, it has not been universally demonstrated that $\alpha_1$-adrenoceptor mediated responses are resistant to these antagonists. For example, Morita *et al* (1984) showed that in anaesthetised, spinal dogs, the peripheral arterial bed gave pressor responses to $\alpha_1$-adrenoceptor agonists that involved calcium influx as much as, or more than, those mediated by $\alpha_2$- adrenoceptors (Morita, Maniwa, Satoh and Taira 1985).

Pedrinelli and Tarazi (1985) considered the sensitivity of $\alpha_1$- and $\alpha_2$-adrenoceptor mediated pressor responses of the pithed rat to nitrendipine and compared the results with

responses in the pithed rat autoperfused hindlimb. These workers found that in the whole animal, $\alpha_1$-adrenoceptor mediated pressor responses were resistant to inhibition by nitrendipine, but in the autoperfused hindlimb both $\alpha_1$- and $\alpha_2$-adrenoceptor mediated vasoconstrictor responses were equally sensitive to calcium channel antagonism.

Janssens and Verhaeghe (1984) studied the effect of verapamil on the response of the canine saphenous vein to α-adrenoceptor agonists. They concluded that activation of either the $\alpha_1$- or the $\alpha_2$- subtype of adrenoceptor caused both influx of extracellular calcium ions and the release of calcium from intracellular sites. Lues and Schümann (1984), demonstrated that in the rabbit aorta, the selective $\alpha_2$-adrenoceptor agonist B-HT 920 acted exclusively through $\alpha_1$-adrenoceptors. Further, that the response to this agonist was dependent on the presence of extracellular calcium. They went on to suggest that calcium dependency of a response is determined by the agonist, and that one receptor subtype could be involved in both calcium entry and calcium release (Lues and Schümann 1984a). The observations made in this thesis (section 8.3.) support this conclusion, for the $\alpha_1$-adrenoceptor on the rat aorta.

Therefore, in a variety of both <u>in vivo</u> and <u>in vitro</u> systems, it has been shown that $\alpha_1$- and $\alpha_2$-adrenoceptor mediated responses cannot be simply differentiated by their sensitivity to calcium channel antagonists. However, it is then necessary to consider why $\alpha_1$-adrenoceptor mediated pressor responses of the pithed rat appear to be resistant to calcium channel antagonists.

It has been suggested by Korstanje et al (1984) that $\alpha_1$-adrenoceptors may have two separate recognition sites representing either release or influx of calcium ions. However, these workers later dispute this themselves (Timmermans et al 1985). They observed that -log $ID_{50}$ values for nifedipine against three $\alpha_1$-adrenoceptor agonists eventually increase, following treatment with phenoxybenzamine, to values identical to those found for nifedipine against vasoconstriction induced by B-HT 920. They suggested that calcium channels activated by either $\alpha_1$- or $\alpha_2$-adrenoceptor agonists may be similar. If this is true, then the sensitivity of a response to calcium channel antagonism would not be strictly related to the adrenoceptor subtype being activated, but to some other property of the total response.

The possibility that $\alpha_1$- and $\alpha_2$-adrenoceptor mediated responses may have mobilised different pools of calcium as a result of differences in the time course of the responses was examined in section 8.2.. Phenylephrine and UK-14,304 were administered to pithed rats by the method of continuous infusion, with or without diltiazem, in order that pressor responses to the agonists could be measured at equivalent time points. It was shown that concentrations of diltiazem which attenuated the pressor response of the pithed rat to UK-14,304 failed to affect the response to phenylephrine. Hence it was concluded that differences in the susceptibility of $\alpha_1$- and $\alpha_2$-adrenoceptor mediated responses in the pithed rat were not due to differences in the time course of the pressor responses.

However, in contrast to the results obtained in the present study, recent work by McGrath and O'Brien (1987) demonstrated that nifedipine reduced pressor responses of the pithed rat to infusions of $\alpha_1$- or $\alpha_2$-adrenoceptor agonists with no preference for either subtype. However, apart from the use of a different calcium channel antagonist, there are two major differences between the methods employed by McGrath and O'Brien, and the present study. The first is the ventilation of the preparation. McGrath and O'Brien used pure oxygen, while in the present study, room air was used (section 7.1.3.). It had previously been suggested (Grant, McGrath and O'Brien 1984) that respiratory acidosis enhances the responsiveness of the pithed rat to $\alpha_2$-adrenoceptor agonists. Therefore, the protocol of ventilation of the pithed rat with pure oxygen might be expected to exhibit different $\alpha_1$- and $\alpha_2$-adrenoceptor mediated vascular responsiveness compared with a preparation ventilated with room air. Possibly as a consequence of this, differences in the responsiveness of the pithed rat to phenylephrine could have occurred. In support of this, McGrath and O'Brien (1987) emphasised that the pressor response of the pithed rat to continuous infusion of phenylephrine were not stable, whereas in the present study these responses were stable (see figure 9).

The second difference concerns the measurement of responses. McGrath and O'Brien (1987) reported that bolus injection of certain $\alpha_1$-adrenoceptor agonists produced a rapid peak followed by a variable decline. They chose to measure the area under the curve for the responses because

they found that measurement of the peak failed to show an effect of nifedipine, which could be shown using the area as the measure of responsiveness. Preliminary experiments for the present study also revealed a biphasic response to phenylephrine when the agonist was administered via the jugular vein (results not shown) which was the method employed by McGrath and O'Brien (1987). However, a transient peak only was observed when the agonist was administered through the femoral vein; which was the method used throughout the present study for bolus administration of agonist.

To explain the above two differences between the studies, would require an investigation specific for this purpose. However, at the time of the present study, the results of McGrath and O'Brien were not available. Consequently, other explanations for the insensitivity of $\alpha_1$-adrenoceptor mediated pressor responses of the pithed rat to calcium channel antagonists were investigated.

In 1984, Ruffolo, Morgan and Messick showed that a diltiazem sensitive component of the pressor response of the pithed rat to cirazoline could be exposed by reducing the receptor reserve of the preparation for this agonist. They concluded that differences in the effect of calcium channel antagonists on vascular $\alpha_1$- and $\alpha_2$-adrenoceptors could result from differences in their receptor reserves and may not reflect differences in the utilisation of calcium by the receptor subtypes. Support for this observation by Ruffolo et al (1984) has been given in vivo by Korstanje, Wilffert, De Jonge, Thoolen, Timmermans and Van Zwieten

(1984); Jim, Macia and Matthews (1986); Pedrinelli and Tarazi (1985) and in this study (section 8.2.2.4.). However, *in vitro* support has not been found, for example in the isolated rat aorta (Beckeringh, Thoolen, De Jonge, Wilffert, Timmermans and Van Zwieten 1984; this study, section 8.3.2.1.), or in the rabbit aorta or canine saphenous vein (Bou and Massingham 1986).

It therefore appeared worthwhile to investigate the role of receptor reserve in the sensitivity of α-adrenoceptor mediated responses to calcium channel antagonism in greater detail. Ruffolo *et al* (1984) studied the effect of diltiazem on the response of the pithed rat to a range of $\alpha_1$-adrenoceptor agonists of varying intrinsic activity; and to cirazoline after progressively increasing concentrations of phenoxybenzamine. It was found that a direct relationship existed between the intrinsic activity of the agonist, or the degree of inhibition by phenoxybenzamine, and the sensitivity of the response to diltiazem. However, their conclusions were based on measurement of the area under the dose pressor response curve for agonist in the presence of diltiazem, and expressing the response as a percentage of that in its absence. If the diagrams in this paper are studied (Ruffolo *et al* 1984 pp 590-591), it would appear that the reduction in diastolic blood pressure, in mmHg, by calcium channel antagonist is approximately the same for all agonists, and for cirazoline, after different degrees of receptor inactivation by phenoxybenzamine. An alternative explanation for the results could then be offered, that the calcium channel antagonist is capable of effecting a fixed

inhibition of the response, regardless of the absolute value of that response, provided there is no receptor reserve to "buffer" the effect.

A feature of examining the role of receptor reserve in the pithed rat is that responses in the whole animal are not measured under equilibrium conditions which are necessary for the determination of agonist parameters (Furchgott 1966). Furthermore, at the time of measuring the effector response, the irreversible antagonist is probably still present. In particular, phenoxybenzamine, in addition to α-adrenoceptor blockade, also blocks neuronal uptake and facilitates noradrenaline release (Starke 1977). These effects of phenoxybenzamine may additionally modify the actions of noradrenaline in the whole animal where the pressor response represents a combination of several contributing factors. The question of the role of receptor reserve in α-adrenoceptor mediated responses would be better examined in vitro where, in contrast, unbound phenoxybenzamine is washed away before responses to agonists are measured. It is therefore reasonable to assume that any influence on the responsiveness of a tissue to phenoxybenzamine in vitro, would be due only to inactivation of receptors and not to a direct action of the drug. In section 8.3.2.1. of the present study it was shown that 10nM phenoxybenzamine had a minor augmenting effect on the diltiazem sensitivity of the response of the isolated rat aorta to phenylephrine. These in vitro results are in marked contrast to the significant increase by phenoxybenzamine of the sensitivity of the cirazoline

mediated pressor response of the pithed rat to diltiazem.

An alternative action of phenoxybenzamine was considered by Timmermans et al (1985). In a comparison of the influence of either phenoxybenzamine or benextramine on the nifedipine sensitivity of the pressor response of the pithed rat to cirazoline, these workers concluded that phenoxybenzamine selectively inhibited that component of the $\alpha_1$- response that was independent of an influx of extracellular calcium; an effect not observed with benextramine (Timmermans, Thoolen, Mathy, Wilffert, De Jonge and Van Zwieten 1985). However, Nwako et al (1980) have shown that phenoxybenzamine blocks calcium channels in the rat vas deferens (Nwako, Lin and Swamy 1980). It therefore seems unlikely that phenoxybenzamine selectively inhibits that component of the response not due to calcium influx.

However, if it were true that phenoxybenzamine reduces the intracellular calcium release component of the $\alpha_1$-mediated pressor response of the pithed rat, it is possible that the same is true for prazosin and corynanthine which were shown in this study (sections 8.2.2.5. and .6.) to expose a diltiazem sensitive component of the pithed rat to cirazoline. Thus it was shown that a diltiazem sensitive component of the pressor response of the pithed rat to cirazoline could be uncovered, other than by reduction of receptor reserve. It was therefore considered worthwhile to investigate the effect of competitive and non-competitive antagonists on the component of the $\alpha_1$-adrenoceptor mediated response that has been attributed to intracellular calcium release in vitro .

In sections 8.3.2.2. and .3. of this study, it was shown that phenoxybenzamine (10nM), benextramine (1μM), prazosin (1nM) and corynanthine (1μM) reduced both phases of the EGTA resistant response of the rat aorta to noradrenaline. The concentrations of antagonist used had little effect on the response to noradrenaline in the presence of calcium. Therefore, the results mitigate against a difference between the effect of phenoxybenzamine and benextramine on the component of the $\alpha_1$-adrenoceptor mediated response that is not due to calcium influx. However, it is necessary to consider that two different systems have been studied ,i.e. the pithed rat preparation and the isolated aorta of the rat. Perhaps the difference in effectiveness of phenoxybenzamine and benextramine in influencing the sensitivity of the $\alpha_1$-adrenoceptor mediated pressor response of the pithed rat to nifedipine, as described by Timmermans et al (1985), may be explained in terms of a difference in the local, non receptor, effects (i.e. blockade of noradrenaline uptake; facilitation of noradrenaline release) rather than a change in receptor response coupling.

Nevertheless, for all of the antagonists tested, a diltiazem sensitive component of the pithed rat to cirazoline was exposed. It was considered that the next step in the investigation was to examine factors which could influence the component of the α-adrenoceptor mediated vasoconstrictor response that is due to the release of intracellular calcium.

It has been shown by Cauvin, Saida and Van Breemen (1984),in rabbit aorta and mesenteric resistance vessels, that the greater the noradrenaline induced release of intracellular calcium the less the susceptibility of the total contractile response to inhibition by diltiazem. If this is generally true, then it follows that an assessment of the contribution of intracellular calcium release to the total response of any given vascular muscle to an agonist, should be inversely related to the sensitivity of that muscle to calcium channel antagonists.

However, the likelihood that there is more than one pool of intracellular calcium (Godfraind and Kaba 1972, Heaslip and Rahwan 1982 and this study section 8.3.) must be accounted for. Godfraind and Kaba (1972) identified three sources of $^{45}Ca^{++}$ loss from the rat aorta following stimulation by noradrenaline. They divided these sources into slow, fast and very fast. They attributed the very fast calcium loss to ions loosely bound in the extracellular space. The slow and fast sources were considered to be mainly of cellular origin. Heaslip and Rahwan (1982) identified two sources of intracellular calcium that may be utilised for the contraction of the aorta following stimulation by noradrenaline.

In section 8.3.2.5. of the present study, it was shown that the magnitude of the second phase of either the EGTA resistant response or the calcium present response of the rat aorta to α-adrenoceptor agonists did not differ in magnitude whether the agonist was given as a bolus or cumulatively. If the first phase of either response

contributed to the second, then a difference in the magnitude of the second phase would have been expected (section 8.3.3.). Since no such difference was observed, it was concluded that the peak and the second, sustained phase of the EGTA-resistant response, and the fast and slow components of the calcium present response of the rat aorta to α-adrenoceptor agonists were largely independent of one another. This is in agreement with the conclusions of Heaslip and Rahwan (1982). The question then arises as to which is the pool of calcium responsible for modulation of the sensitivity of α-adrenoceptor mediated responses to calcium channel antagonists in the rat aorta?

In the experiments described in section 8.3.2.4. of the present study, the inhibition by diltiazem (1μM) of the α-adrenoceptor mediated response of the rat aorta was increased to the same extent by both prazosin (1nM) and by corynanthine (1μM). It was shown in section 8.3.2.3. that at these concentrations, prazosin had a greater effect than corynanthine at inhibiting the initial transient peak of the EGTA resistant response of the rat aorta to 3μM noradrenaline. The same was not true for the second, sustained phase. Following the reasoning of Cauvin et al (1984) that decreasing the intracellular calcium component of the response increases the sensitivity to calcium channel antagonists; if the initial transient release of intracellular calcium was important in determining the sensitivity of the α-adrenoceptor mediated contraction of the rat aorta to diltiazem, then it would be expected that prazosin would have been more effective than

corynanthine at increasing the sensitivity of the response to diltiazem. This is because prazosin was more effective than corynanthine at inhibiting the first phase of the EGTA resistant response to 3μM noradrenaline. However, different vessels rely on different pools of calcium to varying extents (Cauvin et al (1984). Therefore these suggestions can only be applied to the aorta of the rat and not to the whole animal.

Following this examination of the means by which α-adrenoceptor mediated intracellular calcium release in vitro may be inhibited, and the consequent effect on diltiazem sensitivity, the next step in this investigation was to consider the properties of the agonists which describe their ability to cause α-adrenoceptor mediated intracellular calcium release. Consider first, the hypothesis of Downing et al (1985) which relates the antagonist dissociation constant to the ability of that antagonist to inhibit the initial rate of binding of an $\alpha_1$-adrenoceptor agonist to the receptors (Downing, Wilson and Wilson 1985). If this is true, i.e. if an antagonist may inhibit the release of α-adrenoceptor mediated intracellular calcium release by reducing the rate of rise of receptor occupancy by the agonist, then the corollary would be expected to be true. That is, the rate at which an $\alpha_1$-adrenoceptor agonist binds to receptors may determine the ability of that agonist to cause the release of intracellular calcium.

However, a significant problem encountered when attempting to measure the initial intracellular calcium

release component of the response of the rat aorta to $\alpha_1$-adrenoceptor agonists may be found by considering work by Furchgott (1972). When measuring an initial, transient response, such as the fast response of the rat aorta to α-adrenoceptor agonists, it is unlikely that the concentration of agonist in the bathing medium is the concentration in equilibrium with the receptors. It can only be hoped that when a measurement of the response is made, the ratio of the concentration of agonist in the region of the receptors to that in the external solution is the same for all agonists and all concentrations of agonist tested. Experiments in which the dissociation constant of a drug has been determined and used for comparison of responses of the rat aorta during the fast phase, make the fundamental assumption that the properties of the drug at equilibrium may be extrapolated to all components of that response.

In addition to agonist dissociation constants, the efficacy of an agonist is also important when considering the ability of an agonist to cause the release of intracellular calcium. A series of experiments by McGrath in 1985 examined the relationship between α-adrenoceptor agonists and the sources of calcium utilised for smooth muscle contraction in the rat anococcygeus muscle. It was concluded by this worker that the mobilisation of calcium pools represented a continuous spectrum of activity across the range of agonists tested; which was likely to be correlated with efficacy. Put another way, in a series of agonists, the ability of any one agonist to cause the release of intracellular calcium is likely to be related to

its efficacy. This idea has been supported by Timmermans et al (1985). These workers suggested that the efficacy of an $\alpha_1$-adrenoceptor agonist to cause vasoconstriction in pithed rats is related to the ability of the agonist to trigger the vasoconstrictor mechanism not utilising calcium influx. In other words, the efficacy of an agonist determines its ability to cause the release of intracellular calcium for contraction.

However, the studies described above did not systematically attempt to relate calculated relative efficacy with the release of intracellular calcium. It was therefore considered of interest to determine these parameters for a range of α-adrenoceptor agonists and compare them with the EGTA resistant responses in the rat aorta. The reasons for using EGTA resistant responses as a measure of α-adrenoceptor mediated intracellular calcium release have been discussed in section 8.3.3.. It was found that the agonists available varied in their intrinsic activities in the rat aorta. To express the EGTA resistant response of an agonist as a proportion of the response to the same concentration of agonist in the presence of calcium was used as a means of standardising the results obtained; but then the intrinsic activity must be considered as a third variable when comparing dissociation constants or relative efficacies with intracellular calcium release. In order to logically compare either the efficacy or the dissociation constant with calcium mobilisation, all other parameters for that agonist series must be the same, leaving the property to be studied as the only variable.

Furthermore, since the dissociation constant and relative efficacy are mathematically related (Furchgott and Burstzyn 1967), a series of agonists with, for example, similar dissociation constants but different relative efficacies must have different potencies, which again is a third variable.

Jim, Macia and Matthews (1985) examined the ability of a series of full $\alpha_1$-adrenoceptor agonists to release intracellular calcium in the canine saphenous vein. They obtained a series of five $\alpha_1$-adrenoceptor agonists with intrinsic activities above 90% and compared these with phenylephrine. They reasoned that if a correlation exists between the efficacy of an agonist and its ability to release intracellular calcium, then the contractile responses of these agonists to equieffective concentrations should be the same in both zero external calcium medium and in normal calcium medium containing 5mM lanthanum. They found that no correlation could be shown between efficacy and the ability to release intracellular calcium for a series of full $\alpha_1$-adrenoceptor agonists in the canine saphenous vein. However, the results presented by Jim *et al* (1985) demonstrated a relationship between intracellular calcium release and agonist dissociation constants, although these workers were not looking for such a relationship. However, it must be emphasised that the agonists tested (phenylephrine, cirazoline, SK&F 29029, SK&F 102652 and SK&F *1* -89748) differred in efficacy, affinity and potency (Jim *et al* 1985). Thus there were three concurrent variables for comparison with the ability of the agonists to activate

intracellular calcium release.

It is clear that, with the available drugs, no simple method is currently available for the study of the role of efficacy or affinity in the mobilisation of calcium pools following α-adrenoceptor activation. It should be considered though, that a simple answer is unlikely to be obtained even if such a study were possible.

It was considered that the next step in the present investigation was to look at a factor which modulated the response of the tissue to agonist activation. Since no clear path forward could be seen with respect to agonist mediated calcium mobilisation in vascular muscle, the effect of the endothelium on the responsiveness of the rat aorta to α-adrenoceptor agonists was studied. The results have been presented in section 8.5.. It was found that the presence of endothelium depressed both the fast and slow phases of the calcium present response of the rat aorta to a variety of α-adrenoceptor agonists (section 8.5.2.2.). Preliminary experiments (section 8.5.2.4.) suggested that removal of the endothelium changed the efficacy of the agonists tested; but it was not possible, within the scope of these experiments, to determine whether removal of the endothelium increased α-adrenoceptor mediated intracellular calcium mobilisation. However, recent work by Malta and co-workers (1986) examined how the change in efficacy in the rat aorta might be manifest in the smooth muscle cell (Malta, Schini and Miller 1986). These workers studied the phenylephrine stimulated $^{45}Ca^{++}$ efflux from rings of rat aorta with and without endothelium. Experiments were performed in the

presence of flunarizine, on the grounds that this agent inhibited agonist induced stimulated influx of $Ca^{++}$ but did not antagonise either the spontaneous liberation of EDRF or that stimulated by acetylcholine. It was found that stimulated $^{45}Ca^{++}$ efflux was significantly greater in the absence of endothelium compared with in its presence. They concluded that the endothelium can affect intracellular calcium metabolism.

The question remains, as to whether the endothelium has affected the agonist itself, or the agonist-receptor combination, or whether it has affected the ability of the tissue to translate the stimulus into a response. Any of these mechanisms would bring about a change in the calculated efficacy of an agonist.

To summarise so far:-

1. The sensitivity of α-adrenoceptor mediated pressor responses to calcium channel antagonists <u>in vivo</u> is not determined by the receptor subtype. Neither is it dependent on the time course of the response.
2. Receptor reserve masks the component of the $\alpha_1$-adrenoceptor mediated pressor response <u>in vivo</u> that is sensitive to calcium channel antagonism, but elimination of receptor reserve is not necessary in order to expose a calcium channel antagonist sensitive component.
3. Results of <u>in vitro</u> investigations suggest that α-adrenoceptor antagonists reduce that component of the $\alpha_1$- mediated response that is due to intracellular calcium release, thus increasing the sensitivity of the total response to calcium

channel antagonism.

4. Although it is likely that the efficacy of an agonist may determine its ability to cause the release of intracellular calcium, and that the rate at which the agonist combines with the receptors may determine the magnitude of the initial, transient release of calcium in the rat aorta, the values are interrelated and may not be considered in isolation of each other.

5. The endothelium on the rat aorta modulates the intra- as well as the extracellular component of the response to α-adrenoceptor agonists.

Looking to the future in the investigation of α-adrenoceptor mediated calcium mobilisation, it is worth considering the work of Kenakin (1984) who studied the responses of the rat anococcygeous and vas deferens to noradrenaline and oxymetazoline. This worker was interested by the different potencies of the agonists for the two tissues. Oxymetazoline acted as a potent full agonist in the anococcygeous but as a partial agonist in the vas deferens, despite classical pharmacology classifying the α-adrenoceptors in these two tissues as being of the same type.

Kenakin reasoned that for an agonist to produce a response in a tissue it must have both affinity and efficacy. Noradrenaline and oxymetazoline both have high affinity for the α-adrenoceptor, but oxymetazoline has low efficacy and is therefore more susceptible to alterations in receptor response coupling than the agonist with high efficacy, noradrenaline (Kenakin 1984). He went on to

suggest that many classifications of receptors into subgroups may well have arisen from differences in the efficacies of the agonists used in the experiments. Kenakin also suggested that receptor reserve is not a tissue specific property but a relationship between the receptor density, the efficacy of the agonist and the efficiency of stimulus response coupling (Kenakin 1986).

Variation in tissue responsiveness may explain the apparent insensitivity to calcium channel antagonists of the response of the whole animal to certain $\alpha_1$-adrenoceptor agonists, as reported by Van Meel et al 1981, Ruffolo et al 1984 and this study section 8.2.2.. An explanation for this observation may be found in the distribution and relative contribution of vascular beds to the response of the whole animal. i.e. the pressor response of the pithed rat to $\alpha_1$-adrenoceptor agonists may be composed mostly of tissues and vascular beds that utilise intracellular calcium for contraction. Put another way, it is possible that $\alpha_1$-adrenoceptor agonists may be located largely in tissues which are efficient at receptor response coupling, while postjunctional $\alpha_2$-adrenoceptors may be located in those vessels which do not have the contractile mechanisms necessary for the release of intracellular calcium. Therefore the differential sensitivity of $\alpha$-adrenoceptor mediated responses to calcium channel antagonists may not be explained fully in terms of either receptor subtype or receptor reserve.

The concept that the ability of an agonist to induce intracellular calcium release is a tissue related property

was considered by Flavahan and co-workers (1985). They looked at the effect of cooling on the response of the canine saphenous vein to α-adrenoceptor agonists. It was found that cooling augmented the response to $\alpha_2$- but not to $\alpha_1$-adrenoceptor mediated responses. Cooling was suggested to act as a functional antagonist, probably by depressing calcium influx such that, in a cold environment, blood would be transferred to the deep veins thereby reducing heat loss (Flavahan, Lindblad, Verbeuren, Shepherd and Vanhoutte 1985).

An alternative suggestion for regional differences in the responsiveness of smooth muscle to α-adrenoceptor agonists was given by Laher and Bevan (1985). Studying the response of the basilar artery, the aorta and the small intrapulmonary arteries of the rabbit to noradrenaline, they suggested that the number of α-adrenoceptors is an important factor in the magnitude of the response to noradrenaline. It must therefore be considered that different regions of the body may utilise different mechanisms for local circulatory regulation.

Ruffolo and Zeid (1985) reported that the canine saphenous vein had a significant receptor reserve for both the $\alpha_1$-agonist cirazoline and for the $\alpha_2$-agonist B-HT 933. They went on to show that both were full agonists in this tissue and had high efficacies at their respective α-adrenoceptor subtypes. They hypothesised that there are fundamental differences in the contractile machinery utilised by $\alpha_1$- and $\alpha_2$-adrenoceptors; such that $\alpha_2$-adrenoceptor mediated vasoconstriction is not capable of producing

the degree of vasoconstriction observed with $\alpha_1$-adrenoceptor agonists, regardless of efficacy, number of receptors or efficiency of coupling.

Following the theme that $\alpha_1$- and $\alpha_2$-adrenoceptor subtypes utilise different machinery for contractile processes, there has been much speculation recently over the role of membrane phospholipids in the mobilisation of calcium in vascular muscle. In 1987, Chiu and co-workers examined the relationship between $^{45}Ca^{++}$ efflux, as an index of intracellular calcium release, and the turnover of phosphatidylinositol (PI) in the rat aorta. They demonstrated a positive correlation between intracellular calcium release and agonist induced PI turnover (Chiu, Bozarth and Timmermans 1987). It has been suggested (Berridge 1984) that following receptor activation in vascular muscle, PI is hydrolysed to give the end products inositol trisphosphate ($IP_3$) and diacylglycerol (DAG). These were said to act as second messengers with $IP_3$ acting by mobilising intracellular calcium, and DAG stimulating protein phosphorylation (Berridge 1984).

It has been shown that the biphasic contraction of the rabbit aorta in response to $\alpha_1$-adrenoceptor stimulation may activate distinct intracellular second messenger pathways, $IP_3$ and DAG linked, which may lead to calcium release and influx respectively (Campbell, Deth, Payne and Honeyman 1985). The question now arises, is PI turnover a tissue related property? i.e. do the tissues which do not respond to α-adrenoceptor activation by a release of intracellular calcium, lack the biochemical pathway necessary for this

effect? This question remains open, but some interesting work by Wick et al (1987) has examined the role of efficacy in PI turnover. These workers studied a series of partial agonists for their ability to affect phospholipids, intracellular calcium release, influx of extracellular calcium and contraction in the rabbit aorta. They described a series of seven steps from formation of the receptor-agonist complex to the formation of active phospholipase C which hydrolyses phosphatidylinositol diphosphate ($PIP_2$) to $IP_3$ and DAG. It was hypothesised that the release of intracellular calcium was dependent on the initial production of $IP_3$ (Wick, Keung, Bowler and Deth 1987). Presumably in the absence of spare receptors, the initial rate of formation of $IP_3$ is insufficient to activate the release of intracellular calcium. However, it should be noted that the $\alpha$-adrenoceptor mediated contraction of the rat aorta gives rise to the release of two independent pools of intracellular calcium (Heaslip and Rahwan 1982, this study section 8.3.). Thus an explanation for the slow, sustained release of intracellular calcium is called for.

There has been no link demonstrated between $\alpha_2$-adrenoceptor mediated vasoconstriction and PI turnover (for review see Jakobs and Schultz 1982). Thus it would appear that activation of postjunctional $\alpha_2$-adrenoceptors could not mobilise calcium by the same biochemical pathway as that taken by $\alpha_1$-adrenoceptors. In the light of this, the mechanism by which a calcium channel antagonist sensitive component of an $\alpha_1$-adrenoceptor mediated response cannot arise in the same way as an $\alpha_2$- response.

In conclusion, the experimental findings currently available strongly support the hypothesis that post-junctional $\alpha_2$-adrenoceptor mediated vasoconstriction in vascular muscle occurs primarily, if not exclusively, by an influx of extracellular calcium. $\alpha_1$-adrenoceptor mediated vasoconstriction may occur by both influx of extracellular calcium and release of intracellular calcium, but the proportion of the response due to intracellular calcium release varies according to total effect of efficacy, affinity and potency of the agonist. The component of the $\alpha_1$-adrenoceptor mediated response resulting in the release of intracellular calcium (both the initial, transient peak and the second, sustained phase) may be reduced by competitive and by non-competitive antagonists. The greater the component of the response due to influx of extracellular calcium, the more susceptible the response to inhibition by calcium channel antagonists. Thus α-adrenoceptor antagonists may reduce the component of the $\alpha_1$- response due to calcium release thereby increasing the sensitivity of the total response to calcium channel antagonism. Vascular muscle contraction mediated by postjunctional $\alpha_2$-adrenoceptor agonists, full $\alpha_1$- agonists in the presence of selective antagonists and weak or partial $\alpha_1$- agonists may all be attenuated by calcium channel antagonists, but the mechanism by which $\alpha_1$- and $\alpha_2$-adrenoceptor activation stimulates the influx of extracellular calcium, is not the same.

It is considered that the future of this work lies in an examination of tissue related properties in receptor-

response coupling. Consider the following by Kenakin and Beek (1980). These workers studied the agonist activity of prenalterol, a partial β-adrenoceptor agonist, compared with isoprenaline, in six isolated tissues: guinea-pig trachaea, left atria, extensor digitorum longus, rat left atria, papillary muscle and aorta. They found that the equilibrium dissociation constants of prenalterol on the isoprenaline receptors were similar in all six tissues, but that the stimulus response coupling differed markedly. Thus they demonstrated that a partial agonist can demonstrate organ selectivity, not from differences in receptors but from differences in the stimulus response relationships of each tissue (Kenakin and Beek 1980).

A separate series of experiments conducted by Flavahan and Vanhoutte (1986) obtained different equilibrium dissociation constants for phenylephrine in the canine saphenous and femoral veins. However, their conclusion was similar to that of Kenakin and Beek (1980). That is, differences in the affinity of $\alpha_1$-adrenoceptor agonists between these blood vessels may result from differences in the way the receptors are coupled in the membrane.

The question then arises as to whether the peripheral vessels lack the necessary contractile machinery, or biochemical pathways for the release of intracellular calcium; or if receptor number limits the receptor-response coupling of the tissues below a critical stimulus which may be necessary to trigger intracellular calcium release. To investigate this point a variety of tissues from a range of species of animal should be used. At least one tissue in

which the presence of postjunctional $\alpha_2$-adrenoceptors have been demonstrated should be included, and one in which they have not. Then, using the approach of Kenakin and Beek (1980), the parameters describing two agonists selective for $\alpha_1$- and $\alpha_2$-adrenoceptors (eg cirazoline and UK-14,304 respectively) should be estimated in each tissue. It is important that the agonists used should have high affinity and efficacy for their respective receptor subtypes, because both agonists should be capable of stimulating the release of intracellular calcium. The calcium sources utilised for the contraction should then be differentiated into intra- and extracellular components, either using the approach of the present study i.e. EGTA resistant responses and sensitivity to calcium channel antagonists, or a biochemical approach, e.g. $^{45}Ca^{++}$ uptake and efflux. The study should examine receptor number (eg by radioligand binding). The objective would be to ascertain whether tissues in which $\alpha_2$-adrenoceptor mediated vasoconstriction have insufficient receptor number to allow the critical stimulus necessary for the release of intracellular calcium or whether the biochemical pathways utilised by the receptor subtypes differ.

It is possible that $\alpha_2$-adrenoceptor activation may mobilise intracellular calcium in some vessels. If this is the case then it would be interesting to determine whether such activation may initiate PI turnover. If it does not, then it would be true that the receptor subtypes utilise different biochemical pathways for calcium mobilisation. If it is found that postjunctional $\alpha_2$-adrenoceptors are present

in some vessels, in the same number as $\alpha_1$- are found in others, yet $\alpha_2$-adrenoceptor mediated vasoconstriction does not mobilise intracellular calcium for contraction, then it may be concluded that receptor number is the limiting factor in the mobilisaton of intracellular stores of calcium.

## 10. REFERENCES


AHLQUIST, R.P. (1948).
A study of the adrenotropic receptors.
Am. J. Physiol. 153 : 586-600.

ALTURA, B.M., CARELLA, A. and ALTURA, B.T. (1980).
Adverse effects of TRIS, HEPES and MOPS buffers on contractile responses of arterial and venous smooth muscle induced by prostaglandins.
Prostaglandins and Med. 5 : 123-130.

ARIËNS, E.J. (1954).
Affinity and intrinsic activity in the theory of competitive inhibition.
Arch. Int. Pharmacodyn. 99 : 32-49.

ARIËNS, E.J. and SIMONIS, A.M. (1983).
Physiological and pharmacological aspects of adrenergic receptor classification.
Biochem. Pharmacol. 32 : 1539-1545.

ARIËNS, E.J. and Van ROSSUM, J.M. (1957).
pDx, pAx and pD'x values in the analysis of pharmacodynamics.
Arch. Int. Pharmacodyn. 110 : 275-299.

ARUNLAKSHANA, O. and SCHILD, H.O. (1959).
Some quantitative uses of drug antagonists.
Br. J. Pharmac. 14 : 48-58.

AXELSSON, J., WAHLSTROM, B., JOHANSSON, B. and JONSSON, O. (1967).
Influence of the ionic environment on spontaneous electrical and mechanical activity of the rat portal vein.
Circ. Res. 21 : 609-618.

BARGER, G. and DALE, H.H. (1910).
Chemical structure and sympathomimetic action of amines.
J. Physiol. 41 : 19-59.

BARLOW, R.B., SCOTT, N.C. and STEPHENSON, R.P. (1967).
The affinity and efficacy of onium salts on the frog rectus abdominis.
Br. J. Pharmac. Chemother. 31 : 188-196.

BAUM, T. (1984).
In: Cardiovascular Pharmacology. Antonaccio M. (ed.).
New York: Raven press.

BECKERINGH, J.J., THOOLEN, M.J.M.C., De JONGE, A., WILFFERT, B., TIMMERMANS, P.B.M.W.M. and Van ZWIETEN, P.A. (1984)a.
The contractions induced in rat and guinea-pig aortic strips by the $\alpha_2$-adrenoceptor selective agonists B-HT 920 and UK-14,304 are mediated by $\alpha_1$-adrenoceptors.
Eur. J. Pharmac. 104 : 197-203.

BECKERINGH, J.J., THOOLEN, M.J.M.C., De JONGE, A., WILFFERT, B., TIMMERMANS, P.B.M.W.M. and Van ZWIETEN, P.A. (1984)b.
Differential effects of the calcium entry blocker D600 on contractions of rat and guinea-pig aortas, elicited by various alpha-1 adrenoceptor agonists.
J. Pharmac. Exp. Ther. 229 : 515-521.

BELLEAU, B. (1964).
A molecular theory of drug action based on induced conformational perturbations of receptors.
J. Med. Chem. 7 : 776-784.

BERRIDGE, M.J. (1984).
Inositol trisphosphate and diacylglycerol as second messengers.
Biochem. J. 220 : 345-360.

BIGAUD, M., SCHOEFFTER, P., STOCLET, J.C. and MILLER, R.C. (1984).
Dissociation between endothelium-mediated increases in tissue cGMP levels and modulation of aortic contractile responses.
Naunyn-Schmied. Arch. Pharmacol. 328 : 221-223.

BOHR, D.F. (1963).
Vascular smooth muscle: Dual effect of calcium.
Science. 139 : 597-599.

BOLTON, T.B. (1979).
Mechanisms of action of transmitters and other substances on smooth muscle.
Physiol. Rev. 59 : 606-718.

BOU, J. and MASSINGHAM, R. (1986).
Effect of diltiazem upon contractile responses to phenylephrine, cirazoline, Sgd 101/75, St 587 and B-HT 920 in rabbit aorta and dog saphenous vein preparations.
Eur. J. Pharmac. 121 : 319-325.

CAMBRIDGE, D. (1981).
UK-14,304. A potent and selective $\alpha_2$-agonist for the characterisation of α-adrenoceptor subtypes.
Eur. J. Pharmac. 72 : 413-415.

CAMBRIDGE, D., DAVEY, M.J. and MASSINGHAM, R. (1977).
Prazosin, a selective antagonist of postsynaptic α-adrenoceptors.
Br. J. Pharmac. 59 : 514P-515P.

CAMPBELL, M.D., DETH, R.C., PAYNE, R.A. and HONEYMAN, T.W. (1985).
Phosphoinositide hydrolysis is correlated with agonist-induced calcium flux and contraction in the rabbit aorta.
Eur. J. Pharmac. 116 : 129-136.

CARRIER, G.O. and WHITE, R.E. (1985).
Enhancement of alpha-1 and alpha-2 adrenergic agonist-induced vasoconstriction by removal of endothelium in rat aorta.
J. Pharmac. Exp. Ther. 232 : 682-687.

CAUVIN, C., LOUTZENHISER, R., HWANG, O. and Van BREEMEN, C. (1982).
$\alpha_1$-adrenoceptors induce Ca influx and intracellular Ca release in isolated rabbit aorta.
Eur. J. Pharmac. 84 : 233-235.

CAUVIN, C., LUKEMAN, S., CAMERON, J., HWANG, O., MEISHERI, K., YAMAMOTO, H. and Van BREEMEN, C. (1984)a.
Theoretical bases for vascular selectivity of $Ca^{++}$ antagonists.
J. Cardiovasc. Pharmacol. 6 : s630-s638.

CAUVIN, C., SAIDA, K. and Van BREEMEN, C. (1984).
Extracellular $Ca^{++}$ dependence and diltiazem inhibition of contraction in rabbit conduit arteries and mesenteric resistance vessels.
Blood Vessels. 21 : 23-31.

CAVERO, I., SHEPPERSON, N., LEFEVRE-BORG, F. and LANGER, S. Z. (1983).
Differential inhibition of vascular smooth muscle responses to $\alpha_1$- and $\alpha_2$-adrenoceptor agonists by diltiazem and verapamil.
Circ. Res. 52 (suppl. 1): 69-76.

CHIU, A.T. BOZARTH, J.M. and TIMMERMANS, P.B.M.W.M. (1987).
Relationship between phosphatidylinositol turnover and $Ca^{++}$ mobilization induced by alpha-1 adrenoceptor stimulation in the rat aorta.
J. Pharmac. Exp. Ther. 240 : 123-127.

CHIU, A.T., McCALL, D.E., THOOLEN, M.J.M.C. and TIMMERMANS, P. B. M. W. M. (1986).
$Ca^{++}$ utilization in the constriction of rat aorta to full and partial alpha-1 adrenoceptor agonists.
J. Pharmac. Exp. Ther. 238 : 224-231.

CLARKE, A.J. (1926).
The antagonism of acetyl choline by atropine.
J. Physiol. (Lond.) 61 : 547-556.

CONSTANTINE, J.W. and LEBEL, W. (1980).
Complete blockade by phenoxybenzamine of $\alpha_1$- but not $\alpha_2$-vascular receptors in dogs and the effects of propranolol.
Naunyn-Schmied. Arch. Pharmacol. 314 : 149-156.

COOKE, J.P., RIMELE, T.J., FLAVAHAN, N.A. and VANHOUTTE, P. M. (1985).
Nimodipine and inhibition of alpha adrenergic activation of the isolated canine saphenous vein.
J. Pharmac. Exp. Ther. 234 : 598-602.

DALE, H.H. (1906).
On some physiological actions of ergot.
J. Physiol. 34 : 163-206.

DALRYMPLE, H.W., HAMILTON, C.A., HANNAH, J.A.M. and REID, J. L. (1983).
Cardiovascular effects of RX781094 in the rabbit: Possible partial agonist effect in addition to $\alpha_2$-adrenoceptor antagonism.
Br. J. Pharmac. 78 : 128P.

De JONGE, A., KNAPE, J.Th.A., Van MEEL, J.C.A., KALKMAN, H. O., WILFFERT, B., THOOLEN, M.J.M.C., TIMMERMANS, P.B.M.W.M. and Van ZWIETEN, P.A. (1982).
Effect of converting enzyme inhibition and angiotensin receptor blockade on the vasoconstriction mediated by $\alpha_1$- and $\alpha_2$-adrenoceptor stimulation in pithed normotensive rats.
Naunyn-Schmied. Arch. Pharmacol. 321 : 309-313.

De MEY, J.G. and VANHOUTTE, P.M. (1980).
Differences in pharmacological properties of postjunctional alpha-adrenergic receptors among arteries and veins.
Arch. Int. Pharmacodyn. 244 : 328-329.

De MEY, J.G. and VANHOUTTE, P.M. (1981).
Uneven distribution of postjunctional $alpha_1$- and $alpha_2$-like adrenoceptors in canine arterial and venous smooth muscle.
Circ. Res. 48 : 875-884.

Den HERTOG, A. (1981).
Calcium and the α-action of catecholamines on guinea-pig taenia caeci.
J. Physiol. 316 : 109-125.

DETH, R.C. and LYNCH, C.J. (1981)a.
Inhibition of α-receptor-induced $Ca^{++}$ release and $Ca^{++}$ influx by $Mn^{++}$ and $La^{+++}$.
Eur. J. Pharmac. 71 : 1-11.

DETH, R.C. and LYNCH, C.J. (1981)b.
Mobilization of a common source of smooth muscle $Ca^{++}$ by norepinephrine and methylxanthines.
Am. J. Physiol. 240 : C239-C247.

DETH, R.C. and Van BREEMEN, C. (1977).
Agonist induced release of intracellular $Ca^{++}$ in the rabbit aorta.
J. Membrane Biol. 30 : 363-380.

DIGGES, K.G. and SUMMERS, R.J. (1983).
Characterization of postsynaptic α-adrenoceptors in rat aortic strips and portal veins.
Br. J. Pharmac. 79 : 655-665.

DOCHERTY, J.R. and McGRATH, J.C. (1980)a.
A comparison of pre and post-junctional potencies of several alpha-adrenoceptor agonists in the cardiovascular system and anococcygeus muscle of the rat.
Evidence for two types of post-junctional alpha-adrenoceptor.
Naunyn-Schmied. Arch. Pharmacol. 312 : 107-116.

DOCHERTY, J.R. and McGRATH, J.C. (1980)b.
An examination of factors influencing adrenergic transmission in the pithed rat, with special reference to noradrenaline uptake mechanisms and post-junctional α-adrenoceptors.
Naunyn-Schmied. Arch. Pharmacol. 313 : 101-111.

DOCHERTY, J.R. and McGRATH, J.C. (1980)c.
The factors influencing the time course of drug action at α-adrenoceptors: An investigation of the effects of clonidine in the pithed rat.
Br. J. Pharmac. 68 : 225-234.

DOWNING, O.A., WILSON, K.A. and WILSON, V.G. (1983).
Non-competitive antagonism of the α-adrenoceptor-mediated fast component of contraction of rat aorta, by doxazosin and prazosin.
Br. J. Pharmac. 80 : 315-322.

DOWNING, O.A., WILSON, K.A. and WILSON, V.G. (1985).
Perfusion with calcium free solution potentiates prazosin blockade of noradrenaline induced pressor responses in the mesenteric bed of the rat.
J. Auton. Pharmacol. 5 : 295-299.

DOXEY, J.C., ROACH, A.G. and SMITH, C.F.C. (1983).
Studies on RX781094: A selective, potent and specific antagonist of $\alpha_2$-adrenoceptors.
Br. J. Pharmac. 78 : 489-505.

DREW, G.M. and WHITING, S.B. (1979).
Evidence for two distinct types of postsynaptic α-adrenoceptor in vascular smooth muscle *in vivo* .
Br. J. Pharmac. 67 : 207-215.

EDMAN, K.A.P. and SCHILD, H.O. (1962).
The need for calcium in the contractile responses induced by acetylcholine and potassium in the rat uterus.
J. Physiol. 161 : 424-441.

EGLÈME, C., GODFRAIND, T. and MILLER, R.C. (1984).
Enhanced responsiveness of rat isolated aorta to clonidine after removal of the endothelial cells.
Br. J. Pharmac. 81 : 16-18.

FLAVAHAN, N.A., LINDBLAD, L.E., VERBEUREN, T.J., SHEPHERD, J.T. and VANHOUTTE, P.M. (1985).
Cooling and $\alpha_1$- and $\alpha_2$-adrenergic responses in cutaneous veins: Role of receptor reserve.
Am. J. Physiol. 249 : H950-H955.

FLAVAHAN, N.A. and McGRATH, J.C. (1980).
Blockade by yohimbine of prazosin-resistant pressor effects of adrenaline in the pithed rat.
Br. J. Pharmac. 69 : 355-357.

FLAVAHAN, N.A. and VANHOUTTE, P.M. (1986).
Alpha-1 and alpha-2 adrenoceptor: Response coupling in canine saphenous and femoral veins.
J. Pharmac. Exp. Ther. 238 : 131-138.

FLECKENSTEIN, A. (1977).
Specific pharmacology of calcium in myocardium, cardiac pacemakers, and vascular smooth muscle.
Ann. Rev. Pharmacol. Toxicol. 17 : 149-166.

FLECKENSTEIN, A. (1983).
History of calcium antagonists.
Circ. Res. 52 (Suppl. 1): 3-16.

FÖRSTERMANN, U., TROGISCH, G. and BUSSE, R. (1985).
Species-dependent differences in the nature of endothelium-derived vascular relaxing factor.
Eur. J. Pharmac. 106 : 639-643.

FURCHGOTT, R.F. (1955).
The pharmacology of vascular smooth muscle.
Pharmacol. Rev. 7 : 182-265.

FURCHGOTT, R.F. (1964).
Receptor mechanisms.
Ann. Rev. Pharmacol. 4 : 21-50.

FURCHGOTT, R.F. (1966).
The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes.
Advan. Drug Res. 3 : 21-55.

FURCHGOTT, R.F. (1972).
The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In Handbook of experimental pharmacology. Vol. 33. Catecholamines. Blaschko H. and Muscholl E. (eds.). Berlin: Springer-Verlag.

FURCHGOTT, R.F. (1984).
The role of endothelium in the responses of vascular smooth muscle to drugs.
Ann. Rev. Pharmacol. Toxicol. 24 : 175-197.

FURCHGOTT, R.F. and BURSZTYN, P. (1967).
Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors.
Ann. N. Y. Acad. Sci. 144 : 882-898.

FURCHGOTT, R.F. and ZAWADZKI, J.V. (1980).
The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine.
Nature. 288 : 373-376.

GEROLD, M. and HAEUSLER, G. (1983).
$\alpha_2$-adrenoceptors in rat resistance vessels.
Naunyn-Schmied. Arch. Pharmacol. 322 : 29-33.

GODFRAIND, T. (1976).
Calcium exchange in vascular smooth muscle. Action of noradrenaline and lanthanum.
J. Physiol. 260 : 21-35.

GODFRAIND, T. (1982).
Calcium entry blockers and receptor-response coupling.
J. Cardiovasc. Pharmacol. 4 (Suppl. 3): s269-s272.

GODFRAIND, T., EGLEME, C. and OSACHIE, I.A. (1985).
Role of endothelium in the contractile response of rat aorta to α-adrenoceptor agonists.
Clin. Sci. 68 (Suppl. 10): 65s-71s.

GODFRAIND, T. and KABA, A. (1969).
Blockade or reversal of the contraction induced by calcium and adrenaline in depolarized arterial smooth muscle.
Br. J. Pharmac. 36 : 549-560.

GODFRAIND, T. and KABA, A. (1972).
The role of calcium in the action of drugs on vascular smooth muscle.
Arch. Int. Pharmacodyn. 196 (Suppl.): 35-49.

GOLENHOFEN, K. (1972).
In: Vascular smooth muscle. Betz, E. (ed.). Berlin: Springer-Verlag.

GOLENHOFEN, K. (1976).
In: Physiology of smooth muscle. Bulbring, E. and Shuba, M.F. (eds.). New York: Raven Press.

GRAHAM, J.D.P. (1957).
The ethyleneiminium ion as the active species in 2-halo-alkylamine compounds.
Br. J. Pharmac. 12 : 489-497.

GRANT, T.L., McGRATH, J.C. and O'BRIEN, J.W. (1984).
The influence of blood gases on $\alpha_1$- and $\alpha_2$- adrenoceptor mediated pressor responses in the pithed rat.
Proceedings Brit. Pharmacol. Soc. (April) C.22.

GRIFFITH, T.M., EDWARDS, D.H., LEWIS, M.J., NEWBY, A.C. and HENDERSON, A.H. (1984).
The nature of endothelium-derived vascular relaxant factor.
Nature. 308 : 645-647.

HAMILTON, C.A., REID, J.L. and SUMNER, D.J. (1983).
Acute effects of phenoxybenzamine on α-adrenoceptor responses in vivo and in vitro : Relation of in vivo pressor responses to the number of specific adrenoceptor binding sites.
J. Cardiovasc. Pharmacol. 5 : 868-873.

HEASLIP, R.J. and RAHWAN, R.G. (1982).
Evidence for the existence of two distinct pools of intracellular calcium in the rat aorta accessible to mobilization by norepinephrine.
J. Pharmac. Exp. Ther. 221 : 7-13.

HEASLIP, R.J. and RAHWAN, R.G. (1983)a.
Norepinephrine-induced contractions of the rat aorta in the absence of extracellular calcium-I. Effects of α-adrenoceptor blockers.
Gen. Pharmac. 14 : 497-503.

HEASLIP, R.J. and RAHWAN, R.G. (1983)b.
Norepinephrine-induced contractions of the rat aorta in the absence of extracellular calcium-II. Effects of calcium antagonists.
Gen. Pharmac. 14 : 505-512.

HICKS, P.E. (1982).
Preferential antagonism of $\alpha_2$-receptor and voltage operated $Ca^{++}$ channels by diltiazem in rat portal vein.
Br. J. Pharmac. 77 : 469P.

HICKS, P.E. (1983).
α-adrenoceptor-mediated phasic and tonic activity in rat portal vein in vitro .
J. Auton. Pharmacol. 3 : 97-106.

HICKS, P.E., MEDGETT, I.C. and LANGER, S.Z. (1984).
Postsynaptic alpha$_2$-adrenergic receptor-mediated vasoconstriction in SHR tail arteries in vitro .
Hypertension. 6 (Suppl. 1): I-12-I-18.

HICKS, P.E., TIERNEY, C. and LANGER, S.Z. (1985).
Preferential antagonism by diltiazem of $\alpha_2$-adrenoceptor mediated vasoconstrictor responses in perfused tail arteries of spontaneous hypertensive rats.
Naunyn-Schmied. Arch. Pharmacol. 328 : 388-395.

HIRST, G.D.S., NEILD, T.O. and SILVERBERG, G.D. (1982).
Noradrenaline receptors on the rat basilar artery.
J. Physiol. 328 : 351-360.

JAKOBS, K.H. and SCHULTZ, G. (1982).
Signal transformation involving α-adrenoceptors.
J. Cardiovasc. Pharmacol. 4 (Suppl.): s63-s67.

JANSSENS, W. and VERHAEGHE, R. (1984).
Sources of calcium used during $\alpha_1$- and $\alpha_2$-adrenergic contractions in canine saphenous veins.
J. Physiol. 342 : 525-532.

JIE, K., Van BRUMMELEN, P., VERMEY, P., TIMMERMANS, P.B.M.W. M. and Van ZWIETEN, P.A. (1984).
Identification of vascular postsynaptic $\alpha_1$- and $\alpha_2$-adrenoceptors in man.
Circ. Res. 54 : 447-452.

JIM, K.F., De MARINIS, R.M. and MATTHEWS, W.D. (1985).
Measurements of $^{45}Ca^{++}$ uptake and contractile responses after activation of postsynaptic $\alpha_1$-adrenoceptors in the isolated canine saphenous vein: Effects of calcium entry blockade.
Eur. J. Pharmac. 107 : 199-208.

JIM, K.F., MACIA, R.A. and MATTHEWS, W.D. (1985).
An evaluation of the ability of a series of full alpha-1 adrenoceptor agonists to release internal calcium in venous smooth muscle.
J. Pharmac. Exp. Ther. 235 : 377-381.

JIM, K.F., MACIA, R.A. and MATTHEWS W.D. (1986).
Role of receptor reserve in the inhibition of alpha-1 adrenoceptor-mediated pressor responses by calcium antagonists in the pithed rat.
J. Pharmac. Exp. Ther. 238 : 89-94.

JONAS, L. and ZELCK, U. (1974).
The subcellular calcium distribution in the smooth muscle cells of the pig coronary artery.
Exp. Cell. Res. 89 : 352-358.

KALSNER, S. and QUILLAN, M. (1984).
A hypothesis to explain the presynaptic effects of adrenoceptor antagonists.
Br. J. Pharmac. 82 : 515-522.

KENAKIN, T.P. (1984)a.
The classification of drugs and drug receptors in isolated tissues.
Pharmacol. Rev. 36 : 165-222.

KENAKIN, T.P. (1984)b.
The relative contribution of affinity and efficacy to agonist activity: Organ selectivity of noradrenaline and oxymetazoline with reference to the classification of drug receptors.
Br. J. Pharmac. 81 : 131-141.

KENAKIN, T.P. (1985).
The quantification of relative efficacy of agonists.
J. Pharmacol. Meth. 13 : 281-308.

KENAKIN, T.P. (1986).
False premiss. Receptor reserve as a tissue misnomer.
T.I.P.S. 7 : 93-95.

KENAKIN, T.P. and BEEK, D. (1980).
Is prenalterol (H133/80) really a selective beta 1 adrenoceptor agonist? Tissue selectivity resulting from differences in stimulus-response relationships.
J. Pharmac. Exp. Ther. 213 : 406-413.

KOBINGER, W. and PICHLER, L. (1980).
Investigation into different types of post- and presynaptic α-adrenoceptors at cardiovascular sites in rats.
Eur. J. Pharmac. 65 : 393-402.

KOBINGER, W. and PICHLER, L. (1981).
$\alpha_2$-adrenoceptor agonistic effect of B-HT 920 in isolated perfused hindquarters of rats.
Eur. J. Pharmac. 76 : 101-105.

KORSTANJE, C., WILFFERT, B., De JONGE, A., THOOLEN, M.J.M.C. TIMMERMANS, P.B.M.W.M. and Van ZWIETEN, P.A. (1984).
Calcium influx-dependent and -independent $\alpha_1$-adrenoceptor-mediated processes of vasoconstriction in vivo do not operate via different $\alpha_1$-adrenoceptor subtypes.
J. Cardiovasc. Pharmacol. 6 : 1102-1108.

KOU, K., IBENGWE, J. and SUZUKI, H. (1984).
Effects of alpha-adrenoceptor antagonists on electrical and mechanical responses of the isolated dog mesenteric vein to perivascular nerve stimulation and exogenous noradrenaline.
Naunyn-Schmied. Arch. Pharmacol. 326 : 7-13.

LAHER, I. and BEVAN, J.A. (1985).
Alpha adrenoceptor number limits response of some rabbit arteries to norepinephrine.
J. Pharmac. Exp. Ther. 233 : 290-297.

LANDS, A.M., ARNOLD, A., McAULIFF, J.P., LUDUENA, F.P. and BROWN, T.G. (1967).
Differentiation of receptor systems activated by sympathomimetic amines.
Nature. 214 : 597-598.

LANGER, S.Z. (1974).
Presynaptic regulation of catecholamine release.
Biochem. Pharmacol. 23 : 1793-1800.

LANGER, S.Z. (1977).
Presynaptic receptors and their role in the regulation of transmitter release.
Br. J. Pharmac. 60 : 481-497.

LANGER, S.Z. (1981).
Presynaptic regulation of the release of catecholamines.
Pharmacol. Rev. 32 : 337-362.

LANGER, S.Z., MASSINGHAM, R. and SHEPPERSON, N.B. (1980).
Presence of postsynaptic $\alpha_2$-adrenoceptors of predominantly extrasynaptic location in the vascular smooth muscle of the dog hind limb.
Clin. Sci. 59 (Suppl.): 225s-228s.

LANGER, S.Z., MASSINGHAM, R. and SHEPPERSON, N.B. (1981).
Differential sensitivity to prazosin blockade of endogenously released and exogenously administered noradrenaline: Possible relationship to the synaptic location of $\alpha_1$- and the extrasynaptic location of $\alpha_2$-adrenoceptors in dog vascular smooth muscle.
Br. J. Pharmac. 72 : 123P.

LANGER, S.Z. and SHEPPERSON, N.B. (1981).
Antagonism of alpha-adrenoceptor mediated contractions of the isolated saphenous vein of the dog by diltiazem and verapamil.
Br. J. Pharmac. 74 : 942P-943P.

LANGLEY, J.N. (1909).
On the contraction of muscle chiefly in relation to the presence of "receptive" substances. Part IV. The effect of curari and of some other substances on the nicotine response of the sartorius and gastrocnemius muscles of the frog.
J. Physiol. 39 : 235-295.

LEFÈVRE, F., FENARD, S. and CAVERO, I. (1977).
Vascular β-adrenoceptor stimulating properties of phenylephrine.
Eur. J. Pharmac. 43 : 85-88.

LEGAN, E., CHERNOW, B., PARRILLO, J. and ROTH, B.L. (1985).
Activation of phosphatidylinositol turnover in rat aorta by $\alpha_1$-adrenergic receptor stimulation.
Eur. J. Pharmac. 110 : 389-390.

LEIJTEN, P., CAUVIN, C., LODGE, N., SAIDA, K. and Van BREEMEN, C. (1985).
$Ca^{++}$ sources mobilized by $\alpha_1$-receptor activation in vascular smooth muscle.
Clin. Sci. 68 (Suppl. 10): 47s-50s.

LLENAS, J. and MASSINGHAM, R. (1983).
A comparison of the effects of verapamil and cinnarizine upon responses elicited by selective $\alpha_1$- and $\alpha_2$-adrenoceptor agonists in the autoperfused canine hindlimb.
Eur. J. Pharmac. 87 : 53-59.

LONG, C.J. and STONE, T.W. (1985)a.
The release of endothelium derived relaxant factor is calcium dependent.
Br. J. Pharmac. 85 (Suppl.): 152P.

LONG, C.J. and STONE, T.W. (1985)b.
The release of endothelium derived relaxant factor is calcium dependent.
Blood Vessels. 22 : 205-208.

LOUTZENHISER, R. and Van BREEMEN, C. (1983).
The influence of receptor occupation on $Ca^{++}$ influx-mediated vascular smooth muscle contraction.
Circ. Res. 52 (Suppl. 1): 97-103.

LUES, I. and SCHÜMANN, H.J. (1984)a.
B-HT 920 acts as an $\alpha_1$-adrenoceptor agonist in the rabbit aorta under certain *in vitro* conditions.
Naunyn-Schmied. Arch. Pharmacol. 325 : 42-46.

LUES, I. and SCHÜMANN, H.J. (1984)b.
Effect of removing the endothelial cells on the reactivity of rat aortic segments to different α-adrenoceptor agonists.
Naunyn-Schmied. Arch. Pharmacol. 328 : 160-163.

MACKAY, D. (1966).
A general analysis of the receptor-drug interaction.
Br. J. Pharmac. 26 : 9-16.

MALTA, E., SCHINI, V. and MILLER, R.C. (1986).
Effect of endothelium on basal and α-adrenoceptor stimulated calcium fluxes in rat aorta.
Naunyn-Schmied. Arch. Pharmacol. 334 : 63-70.

MARTIN, W., FURCHGOTT, R.F., VILLANI, G.M. and JOTHIANANDAN, D. (1986).
Depression of contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor.
J. Pharmac. Exp. Ther. 237 : 529-538.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. and FURCHGOTT, R.F. (1985).
Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta.
J. Pharmac. Exp. Ther. 232 : 708-716.

McGRATH, J.C. (1982).
Evidence for more than one type of post-junctional α-adrenoceptor.
Biochem. Pharmacol. 31 : 467-484.

McGRATH, J.C. (1983).
The variety of vascular α-adrenoceptors (viewpoint).
T.I.P.S. 4 : 14-18.

McGRATH, J.C. (1983).
The variety of vascular α-adrenoceptors (reply).
T.I.P.S. 4 : 331-333.

McGRATH, J.C. (1985).
α-adrenoceptor agonists and the $Ca^{++}$-dependence of smooth muscle contraction: Evidence for subtypes of receptors or for agonist dependent differences in the agonist-receptor interaction?
Clin. Sci. 68 (Suppl. 10): 55s-63s.

McGRATH, J.C., FLAVAHAN, N.A. and McKEAN, C.E. (1982).
$\alpha_1$- and $\alpha_2$-adrenoceptor-mediated pressor and chronotropic effects in the rat and rabbit.
J. Cardiovasc. Pharmacol. 4 (Suppl.): s101-s107.

McGRATH, J.C. and O'BRIEN, J.W. (1987).
Blockade by nifedipine of responses to intravenous bolus injection or infusion of $\alpha_1$- and $\alpha_2$-adrenoceptor agonists in the pithed rat.
Br. J. Pharmac. 91 : 355-365.

MEDGETT, C., HICKS, P.E. and LANGER, S.Z. (1984).
Smooth muscle alpha-2 adrenoceptors mediate vasoconstrictor responses to exogenous norepinephrine and to sympathetic stimulation to a greater extent in spontaneously hypertensive than in Wistar Kyoto rat tail arteries.
J. Pharmac. Exp. Ther. 231 : 159-165.

MILLER, R.C., MONY, M., SCHINI, V., SCHOEFFTER, P. and STOCLET, J.C. (1984).
Endothelial mediated inhibition of contraction and increase in cyclic GMP levels evoked by the α-adrenoceptor agonist B-HT 920 in rat isolated aorta.
Br. J. Pharmac. 83 : 903-908.

MORITA, T., MANIWA, T., SATOH, K. and TAIRA, N. (1985).
Undifferentiated effects of calcium antagonists on pressor responses to selective alpha-1 and alpha-2 adrenoceptor agonists in anaesthetised, spinal dogs.
J. Pharmac. Exp. Ther. 234 : 728-734.

NICKERSON, M. (1956).
Receptor occupancy and tissue response.
Nature. 178 : 697-698.

NWAKO, N., LIN, C.S. and SWAMY, V.C. (1980).
Interaction of D600 with phenoxybenzamine-induced adrenergic blockade.
Eur. J. Pharmac. 62 : 205-207.

PALLUK, R., HOEFKE, W. and GAIDA, W. (1985).
Postjunctional α-adrenoceptors and influence of angiotensin II in different isolated blood vessels.
Arzneimittel Forsch. 35 : 395-401.

PALMER, R.M.J., FERRIGE, A.G. and MONCADA, S. (1987).
Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor.
Nature 327 : 524-526.

PATON, W.D.M. (1961).
A theory of drug action based on the rate of drug-receptor combination.
Proc. Roy. Soc. (Lond.) B 154 : 21-69.

PEACH, M.J., SINGER, H.A. and LOEB, A.L. (1985).
Mechanisms of endothelium-dependent vascular smooth muscle relaxation.
Biochem. Pharmacol. 34 : 1867-1874.

PEDRINELLI, R. and TARAZI, R.C. (1985).
Calcium entry blockade by nitrendipine and alpha adrenergic responsiveness *in vivo* : Comparison of systemic vs local effects.
J. Pharmac. Exp. Ther. 233 : 643-649.

PLOTEK, Y. and ATLAS, D. (1983).
Characterization of benextramine as an irreversible α-adrenergic blocker and as a blocker of potassium-activated calcium channels.
Eur. J. Biochem. 133 :539-544.

POOLE, J.C.F., SANDERS, A.G. and FLOREY, H.W. (1958).
The regeneration of aortic endothelium.
J. Path. and Bact. 75 :133-143.

POPESCU, L.M., PANOIU, C., HINESCU, M. and NUTU, O. (1985).
The mechanism of cGMP-induced relaxation in vascular smooth muscle.
Eur. J. Pharmac. 107 : 393-394.

RAPOPORT, R.M., DRAZNIN, M.B. and MURAD, F. (1983).
Endothelium-dependent relaxation in rat aorta may be mediated through cyclic GMP-dependent protein phosphorylation.
Nature. 306 : 174-176.

RAPOPORT,R.M., SCHWARTZ, K. and MURAD, F. (1985).
Effects of $Na^+$,$K^+$-pump inhibitors and membrane depolarizing agents on acetylcholine-induced endothelium-dependent relaxation and cyclic GMP accumulation in rat aorta.
Eur. J. Pharmac. 110 : 203-209.

RINGER, S. (1883).
A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart.
J. Physiol. 4 : 29-42.

RÜEGG, J.C. (1971).
Smooth muscle tone.
Physiol. Rev. 51 : 201-248.

RÜEGG, J.C. (1982).
Vascular smooth muscle: Intracellular aspects of adrenergic receptor contraction coupling.
Experientia. 38 : 1400-1404.

RUFFOLO, R.R. (1982).
Review. Important concepts of receptor theory.
J. Auton. Pharmacol. 2 : 277-295.

RUFFOLO, R.R.Jr., MORGAN, E.L. and MESSICK, K. (1984).
Possible relationship between receptor reserve and the differential antagonism of alpha-1 and alpha-2 adrenoceptor-mediated pressor responses by calcium channel antagonists in the pithed rat.
J. Pharmac. Exp. Ther. 230 : 587-594.

RUFFOLO, R.R.Jr., ROSING, E.L. and WADDELL, J.E. (1979).
Receptor interactions of imidazolines. 1. Affinity and efficacy for alpha adrenergic receptors in rat aorta.
J. Pharmac. Exp. Ther. 209 : 429-436.

RUFFOLO, R.R. and WADDELL, J.E. (1983).
Aromatic and benzylic hydroxyl substitution of imidazolines and phenethylamines: Differences in activity at alpha-1 and alpha-2 adrenergic receptors.
J. Pharmac. Exp. Ther. 224 : 559-566.

RUFFOLO, R.R.Jr., WADDELL, J.E. and YADEN, E.L. (1981).
Postsynaptic alpha adrenergic receptor subtypes differentiated by yohimbine in tissues from the rat. Existence of alpha-2 adrenergic receptors in rat aorta.
J. Pharmac. Exp. Ther. 217 : 235-240.

RUFFOLO, R.R.Jr. and YADEN, E.L. (1984).
Existence of spare $\alpha_1$-adrenoceptors, but not $\alpha_2$-adrenoceptors, for respective vasopressor effects of cirazoline and B-HT 933 in the pithed rat.
J. Cardiovasc. Pharmacol. 6 : 1011-1019.

RUFFOLO, R.R.Jr., and ZEID, R.L. (1985)a.
Relationship between $\alpha_2$-adrenoceptor occupancy and response for B-HT 933 in canine saphenous vein.
Eur. J. Pharmac. 111 : 267-271.

RUFFOLO, R.R.Jr. and ZEID, R.L. (1985)b.
Relationship between alpha adrenoceptor occupancy and response for the alpha-1 adrenoceptor agonist, cirazoline, and the alpha-2 adrenoceptor agonist, B-HT 933, in canine saphenous vein.
J. Pharmac. Exp. Ther. 235 : 636-643.

SCHÜMANN, H.J. and LUES, I. (1983).
Postjunctional α-adrenoceptors in the isolated saphenous vein of the rabbit. Characterization and influence of angiotensin.
Naunyn-Schmied. Arch. Pharmacol. 323 : 328-334.

SHIPLEY, R.E. and TILDEN, J.H. (1947).
A pithed rat preparation suitable for assaying pressor substances.
Proc. Soc. Exp. Biol. Med. 64 : 453-455.

SHOJI, T., TSURU, H. and SHIGEI, T. (1983).
A regional difference in the distribution of postsynaptic alpha-adrenoceptor subtypes in canine veins.
Naunyn-Schmied. Arch. Pharmacol. 324 : 246-255.

SINGER, H.A. and PEACH, M.J. (1982).
Calcium- and endothelial-mediated vascular smooth muscle relaxation in rabbit aorta.
Hypertension. 4 (Suppl. II): II-19-II-25.

SNEDDON, P. and BURNSTOCK, G. (1985).
ATP as a co-transmitter in rat tail artery.
Eur. J. Pharmac. 106 : 149-152.

STARKE, K. (1977).
Regulation of noradrenaline release by presynaptic receptor systems.
Rev. Physiol. Biochem. Pharmacol. 77 : 1-124.

STARKE, K. (1981).
α-adrenoceptor subclassification.
Rev. Physiol. Biochem. Pharmacol. 88 : 119-236.

STARKE, K., MONTEL, H. and SCHÜMANN, H.J. (1971).
Influence of cocaine and phenoxybenzamine on noradrenaline uptake and release.
Naunyn-Schmied. Arch. Pharmacol. 270 : 210-214.

STARKE, K., TAUBE, H.D. and BOROWSKI E. (1977).
Presynaptic receptor systems in catecholaminergic transmission.
Biochem. Pharmacol. 26 : 259-268.

STEEN, S., SJÖBERG, T., SKÄRBY, T., NORGREN, L. and ANDERSSON, K.E. (1984).
The postjunctional α-adrenoceptors of the human saphenous vein.
Acta Pharmacol. et Toxicol. 55 : 351-357.

STEPHENSON, R.P. (1956).
A modification of receptor theory.
Br. J. Pharmac. 11 : 379-393.

STEVENS, M.J. and MOULDS, R.F.W. (1980).
Are neuronal uptake mechanisms responsible for the difference in sensitivity to noradrenaline between human arteries and veins?
Clin. Exp. Pharmacol. Physiol. 7 : 139-145.

THOM, S., CALVETE, J., HAYES, R., MARTIN, G. and SEVER, P. (1985).
Human vascular smooth muscle responses mediated by $\alpha_2$-mechanisms in vivo and in vitro .
Clin. Sci. 68 (Suppl. 10): 147s-150s.

TIMMERMANS, P.B.M.W.M., BECKERINGH, J.J., Van ZWIETEN, P.A. and THOOLEN, M.J.M.C. (1987).
Sensitivity of alpha-1 adrenoceptor-mediated pressor responses to inhibition by $Ca^{++}$ entry blockers in the pithed rat: Arguments against the role of receptor reserve.
J. Pharmac. Exp. Ther. 240 : 864-870.

TIMMERMANS, P.B.M.W.M., KWA, H.Y., KARAMAT, A.F. and Van ZWIETEN, P.A. (1980).
Prazosin and its analogues UK-18,596 and UK-33,274: A comparative study on cardiovascular effects and α-adrenoceptor blocking activities.
Arch. Int. Pharmacodyn. 245 : 218-235.

TIMMERMANS, P.B.M.W.M., MATHY, M.J., THOOLEN, M.J.M.C., De JONGE, A., WILFFERT, B. and Van ZWIETEN, P.A. (1984).
Invariable susceptability to blockade by nifedipine of vasoconstriction to various $\alpha_2$-adrenoceptor agonists in pithed rats.
J. Pharm. Pharmacol. 36 : 772-775.

TIMMERMANS, P.B.M.W.M., MATHY, M.J., WILFFERT, B., KALKMAN, H.O., THOOLEN, M.J.M.C., De JONGE, A., Van MEEL, J.C.A. and Van ZWIETEN, P.A. (1983).
Differential effect of calcium entry blockers on $\alpha_1$-adrenoceptor-mediated vasoconstriction in vivo .
Naunyn-Schmied. Arch. Pharmacol. 324 : 239-245.

TIMMERMANS, P.B.M.W.M., QIAN, J.Q., RUFFOLO, R.R. and Van ZWIETEN, P.A. (1984).
A study of the selectivity and potency of rauwolscine, RX781094 and RS21361 as antagonists of alpha-1 and alpha-2 adrenoceptors.
J. Pharmac. Exp. Ther. 228 : 739-748.

TIMMERMANS, P.B.M.W.M., THOOLEN, M.J.M.C., MATHY, M.J., WILFFERT, B., De JONGE, A. and Van ZWIETEN, P.A. (1983).
Sgd 101/75 is distinguished from other selective $\alpha_1$-adrenoceptor agonists by the inhibition of its pressor responses by calcium entry blockade and vasodilatation in pithed rats and cats.
Eur. J. Pharmac. 96 : 187-192.

TIMMERMANS, P.B.M.W.M., THOOLEN, M.J.M.C., MATHY, M.J., WILFFERT, B., De JONGE, A. and Van ZWIETEN, P.A. (1985).
Effects of the irreversible α-adrenoceptor antagonists phenoxybenzamine and benextramine on the effectiveness of nifedipine in inhibiting $\alpha_1$- and $\alpha_2$-adrenoceptor mediated vasoconstriction in pithed rats.
Naunyn-Schmied. Arch. Pharmacol. 329 : 404-413.

TIMMERMANS, P.B.M.W.M. and Van ZWIETEN, P.A. (1980)a.
Postsynaptic $\alpha_1$- and $\alpha_2$-adrenoceptors in the circulatory system of the pithed rat: Selective stimulation of the $\alpha_2$-type by B-HT 933.
Eur. J. Pharmac. 63 : 199-202.

TIMMERMANS, P.B.M.W.M. and Van ZWIETEN, P.A. (1980)b.
Vasoconstriction mediated by postsynaptic $\alpha_2$-adrenoceptor stimulation.
Naunyn-Schmied. Arch. Pharmacol. 313 : 17-20.

TIMMERMANS, P.B.M.W.M. and Van ZWIETEN, P.A. (1982).
$\alpha_2$-adrenoceptors: Classification, localization, mechanisms, and targets for drugs.
J. Med. Chem. 25 : 1389-1401.

Van BREEMEN, C., FARINAS, B.R., CASTEELS, R., GERBA, P., WUYTACK, F. and DETH, R. (1973).
Factors controlling cytoplasmic $Ca^{++}$ concentration.
Phil. Trans. R. Soc. (Lond.) B. 265 : 57-71.

Van BREEMEN, C., HWANG, O. and MEISHERI, K.D. (1981).
The mechanism of inhibitory action of diltiazem on vascular smooth muscle contractility.
J. Pharmac. Exp. Ther. 218 : 459-463.

VANHOUTTE P.M. and RIMELE, T.J. (1982).
Calcium and α-adrenoceptors in activation of vascular smooth muscle.
J. Cardiovasc. Pharmacol. 4 (Suppl. 3): s280-s286.

Van MEEL, J.C.A., De JONGE, A., KALKMAN, H.O., WILFFERT, B., TIMMERMANS, P.B.M.W.M. and Van ZWIETEN, P.A. (1981)a.
Vascular smooth muscle contraction initiated by postsynaptic $\alpha_2$-adrenoceptor activation is induced by an influx of extracellular calcium.
Eur. J. Pharmac. 69 : 205-208.

Van MEEL, J.C.A., De JONGE, A., KALKMAN, H.O., WILFFERT, B., TIMMERMANS, P.B.M.W.M. and Van ZWIETEN, P.A. (1981)b.
Organic and inorganic calcium antagonists reduce vasoconstriction *in vivo* mediated by postsynaptic $\alpha_2$-adrenoceptors.
Naunyn-Schmied. Arch. Pharmacol. 316 : 288-293.

Van MEEL, J.C.A., De JONGE, A., TIMMERMANS, P.B.M.W.M. and Van ZWIETEN, P.A. (1981).
Selectivity of some alpha-adrenoceptor agonists for peripheral alpha-1 and alpha-2 adrenoceptors in the normotensive rat.
J. Pharmac. Exp. Ther. 219 : 760-767.

Van ROSSUM, J.M. (1963).
Cumulative dose response curves II. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters.
Arch. Int. Pharmacodyn. 143 : 299-330.

Van ZWIETEN, P.A., Van MEEL, J.C.A. and TIMMERMANS, P.B.M.W. M. (1982).
Calcium antagonists and $\alpha_2$-adrenoceptors: Possible role of extracellular calcium ions in $\alpha_2$-adrenoceptor-mediated vasoconstriction.
J. Cardiovasc. Pharmacol. 4 (Suppl. 3): s273-s279.

Van ZWIETEN, P.A., Van MEEL, J.C.A. and TIMMERMANS, P.B.M.W. M. (1983).
Functional interaction between calcium antagonists and the vasoconstriction induced by the stimulation of postsynaptic $\alpha_2$-adrenoceptors.
Circ. Res. 52 (Suppl. 1): 77-80.

WAUD, D.R. (1969).
On the measurement of the affinity of partial agonists for receptors.
J. Pharmac. Exp. Ther. 170 : 117-122.

WEISS, R.J. (1981).
In: Vasodilatation. Vanhoutte, P.M. and Leusen I. (eds.). New York: Raven Press.

WICK, P.F., KEUNG, A.C., BOWLER, J.J. and DETH, R.C. (1987).
Alpha-1 adrenergic coupling events induced by full and partial agonists in the rabbit aorta.
J. Pharmac. Exp. Ther. 241 : 458-464.

WILFFERT, B., GOUW, M.A.M., TIMMERMANS, P.B.M.W.M. and Van ZWIETEN, P.A. (1983)a.
Interaction between $\beta_2$-adrenoceptor-mediated vasodilation and $\alpha_2$-adrenoceptor-mediated vasoconstriction in the pithed normotensive rat.
J. Cardiovasc. Pharmacol. 5 : 822-828.

WILFFERT, B., GOUW, M.A.M., TIMMERMANS, P.B.M.W.M. and Van ZWIETEN, P.A. (1983)b.
Interaction between $\beta_2$-adrenoceptor-mediated vasodilation and $\alpha_1$-adrenoceptor-mediated vasoconstriction in the pithed normotensive rat.
J. Cardiovasc. Pharmacol. 5 : 829-835.

YAMAGUCHI, I. and KOPIN, I.J. (1980).
Differential inhibition of alpha-1 and alpha-2 adrenoceptor mediated pressor responses in pithed rats.
J. Pharmac. Exp. Ther. 214 : 275-281.