# The effect of Aminoglycoside antibiotics on erythrocyte membrane potassium ion transport.

Charles Mark Lazenby.

Master of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

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## Summary

The aminoglycoside antibiotics, gentamicin and neomycin have been demonstrated to inhibit $Ca^{2+}$-dependent $^{86}Rb$ uptake into inside-out vesicles derived from human erythrocyte membranes at concentrations of $2x10^{-4}M$ and $10^{-3}M$ respectively. Both drugs were also however able to stimulate $^{86}Rb$ uptake into the vesicles when added to the incubation buffer in the absence of $Ca^{2+}$ ($4x10^{-4}$ M EGTA). Inhibition of $Ca^{2+}$-dependent $^{86}Rb$ uptake by neomycin ($10^{-4}M$) was more clearly revealed after subtraction of the $^{86}Rb$ uptake in the presence of drug alone.

This aminoglycoside stimulated uptake varied in magnitude according to the source of the blood from which the vesicles were prepared. For vesicles prepared from blood drawn from one particular subject a dose response was determined for aminoglycoside stimulated $^{86}Rb$ uptake and $EC50s$ of $9x10^{-6}M$ and $2x10^{-5}M$ for neomycin and gentamicin respectively were recorded.

Attempts to characterise aminoglycoside stimulated $^{86}Rb$ uptake pharmacologically were undertaken. The only instance when pharmacological intervention inhibited this effect was when the vesicles were loaded with either of the loop diuretics furosemide or bumetanide ($10^{-3}M$). Utilization of such drug loaded vesicles has allowed the demonstration of statisticaly significant inhibition of $Ca^{2+}$-dependent $^{86}Rb$ uptake by the aminoglycoside neomycin ($10^{-4}M$), where previously the co-existent drug stimulated $^{86}Rb$ uptake partially masked such an effect. The precise nature of aminoglycoside stimulated $^{86}Rb$ uptake into one-step inside out erythrocytes however remains unclear.

By the expedient of loading human erythrocytes with gentamicin, the effect of this drug on $Ca^{2+}$-dependent $K^+$ transport was further investigated. In two of the three subjects investigated 50% inhibition of the initial rate of KCl efflux was observed on loading the cells with 20 mg/ml gentamicin. In a third subject however a significant increase in the rate of KCl efflux was observed at 10 mg/ml gentamicin, whereas no such increase was recorded for the other two subjects.

Keywords; Aminoglycosides antibiotics, Human erythrocyte one-step inside-out vesicles, Human erythrocytes, Toxicology and $Ca2^{+}$-dependent $K^+$-transport.

**This thesis is dedicated to my mother and to the memory of my father.**

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Index of abbreviations used.

AGs Aminoglycoside antibiotics.

ATP Adenosine Triphosphate.

BSA Bovine serum albumin.

EGTA Ethyleneglycobis-(aminoethylether) tetra acetic acid.

GEN Gentamicin sulphate.

IOVs One-step inside-out erythrocyte vesicles.

$IP_3$ Inositol trisphosphate.

NAD Nicotinamide adenine dinucleotide.

NADH Nicotinamide adenine dinucleotide (reduced form).

NEO Neomycin sulphate.

NET Netilimicin sulphate.

OUA Ouabain

PBS Phosphate buffered saline.

PtdIns (4,5) $P_2$ Phosphatidylinositol 4, 5-bisphosphate.

VAL Valinomycin.

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# Introduction.

## [1.1] The Aminoglycoside Antibiotics.

Aminoglycoside antibiotics (AGs) are organic polycationic drugs, elaborated by actinomyces of the genus streptomyces and micromonospora. They are water soluble compounds with broad antibiotic spectra against gram negative and positive micrococci, gram negative bacilli, and the tubercular bacilli.

The usefulness of the aminoglycosides as antibiotics is blighted by their toxicities. The two major sites of adverse reaction in the human body are the kidney and the inner ear.

An example of the degree of the problem is provided by clinical data for the extensively used AG gentamicin (GEN). It has been reported that GEN caused renal disfunction in 8-37% of treated patients and auditory toxicity in 2-25% (Arcieri et al , 1970; Myers, 1970; Jackson and Arcieri, 1971; Kahlmeter and Dahlager, 1984). The variation in the incidence of AG toxicity is probably due to variations in the number of patients studied, the specific drug and the criteria used to define toxicity.

A further site of adverse reactions to AGs is the neuromuscular junction. The first experimental study describing the symptoms of acute toxicity at this site was published in 1950 (Molitor and Graessle, 1950). Subsequent studies revealed that these symptoms were the result of blockade of transmission at the neuromuscular junctions (Corrado,

1963). It has been demonstrated that the antibiotics of this group also cause neuromuscular blockade and additionally have cardiovascular effects (Pittinger and Adamson, 1972).

Another adverse reaction which surfaced in the nineteen fifties was that AGs (in this case neomycin) increased the anaesthetic effects of ether during surgery, causing respiratory paralysis (Pridgen, 1955). It has since become evident that the use of an AG in combination with an anaesthetic or a muscle relaxant causes respiratory disturbance ( Pittinger et al, 1970 ).

AGs have also been found to have hypotensive actions. This is probably as a conseqence of a myocardial, neurovascular and ganglonic depression which is reversable by $Ca2^{+}$ (Swain et al, 1956; Corrado, 1958; Leaders et al, 1960; Singh et al 1982).

To date no metabolism of the AGs has been described in any species. Thus the adverse reactions brought about by these drugs are thought to be caused by the AGs themselves and not metabolites of the drugs. A great deal of effort has been expended to the end of determining the mechanism by which AGs exert their toxicities.

## [1.2] Aminoglycoside toxicity

### [1.2.1] Ototoxicity

An irreversible loss of hearing or vestibular function is a known complication of AG therapy (Rybak, 1986). Furthermore hearing loss may progress after cessation of treatment with certain of these drugs (Supacek, 1972).

Hearing loss caused by the use of AGs in general is limited to a high frequency range initially, and associated histological changes in the inner ear originate in the outer hair cells of the basal turn of the cochlea. As the total dose is increased and the duration of administration is prolonged, these changes extend to involve low frequency ranges and the upper turns of the cochlea (Hawkins and Engstrom, 1964; Kohonen, 1965; Ward and Rounthwaite, 1978).

As in the cochlea, the sensory cells in the vestibular system are much more sensitive to damage caused by AGs than are the supporting cells. Degeneration of hair cells in the cristae ampullaris precedes that in the maculae of the utricle and saccule. Of the two types of hair cells, the type 1 cells are more vunerable than the type 2 cells.

AGs have the potential to damage both the cochlea and the vestibular systems, however individual AGs have different toxic effects. For example, neomycin (NEO) is almost exclusively cochleotoxic, whereas streptomycin causes vestibular damage (Hawkins, 1958; Duvall and Wersall, 1963; Hybels, 1979). The cochlea and the vestibular epithelia are both damaged by GEN and tobramycin but to different degrees

(Smith et al , 1980; Fee, 1980). The reasons for this heterogeneity of action between different AGs is unknown.

Recently a molecular model for AG ototoxicity based on the available experimental evidence has been proposed (Schacht, 1986). It is proposed that the ototoxic action of the AGs encompasses several steps.

The first step in AG ototoxicity is thought to involve binding of the drug to the plasma membrane and displacement of cations such as calcium. This action has been demonstrated directly in binding studies in inner ear tissues of the guinea pig (Orsulakova et al, 1976) as well as indirectly in studies of drug effects on the cochlear microphonic potential (Takada and Schacht, 1982). This step is thought to reversibly interfere with calcium-dependent membrane functions.

The proposed second step in Schacht's hypothetical mechanism of toxicity is the uptake of AGs into the cell. Uptake of GEN has been determined in the developing inner ear of the mouse (Schacht and Van de Water, 1986) and further characterized in the cristae ampullaris (Williams et al, 1987a) and inner hair cells of the guinea pig cochlea (Williams et al, 1987b). Energy dependence of this uptake is indicated by the drastic reduction of AG uptake into hair cells by cooling (thus eliminating metabolic activity) and the fact that the uptake occured against a concentration gradient. Competition between AGs and polyamines for uptake into hair cells has been advanced as evidence for AGs utilising polyamine transport systems for cellular uptake (Williams et al, 1987b). Thirdly AGs bind to and interact with sites intracellularly.

One such binding site is thought to be phosphatidylinositol 4, 5-bisphosphate ($PtdIns(4,5)P_2$), a physiologically important lipid involved in the inositol phosphate second messenger system. Inhibition of $PtdIns(4,5)P_2$ metabolism by AGs has been observed in vivo and in vitro (Schacht, 1976, Tachibana et al, 1983).

A correlation between ototoxic potential of the AGs and their ability to disturb the structure of $PtdIns(4,5)P_2$ containing artificial membranes (Wang et al, 1984; Au et al, 1986a-b) is said to be suggestive evidence, that AG interaction with $PtdIns(4,5)P_2$ is of importance in the development of ototoxicity. Polyamines with the exception of spermine also exhibit such a correlation (Tachibana et al, 1984).

[1.2.2] Renal Toxicity

In the kidney AGs produce tubular cell necrosis which is largely confined to the proximal tubule. Net reabsorption of AGs occurs in the proximal tubule by means of a high capacity transport system. (Pastoriza-Munoz et al , 1979; Seniekjan et al , 1981). Luminal drug uptake takes place by means of endocytic pinocytosis, after initial binding to the brush border membrane.

Luminal uptake is followed by lysosomal uptake. AGs accumulate in the lysosomes and there inhibit phospholipases resulting in phospholipidosis. The earliest morphological lesions being an increase in the number and size of secondary lysosomes (Bennet et al , 1980; Kaloyanides and

Pastoriza-Munoz, 1980). The development of phospholipidosis is accompanied by focal necrosis, tubular regeneration and interstitial proliferation even at low therapeutic doses of these agents. After being taken up by the renal tubular cell AGs reside in a poorly exchangeable pool and have a prolonged tissue half life ( Fabre et al , 1976).

AG nephrotoxicity is characterised by a variety of renal functional alterations. One such alteration is that these drugs can induce renal potassium and magnesium wasting with resulting hypokalemia and hypomagnesmia ( Cronin et al ,1980). The selective effects on the renal handling of these cations may reflect a specificity of action of the AGs, namely alterations of the plasma membrane transport of these particular ions (Humes, 1988).

The membrane phospholipids phosphatidic acid, phosphatidylinositol 4-phosphate and PtdIns(4,5)$P_2$ are the preferred binding sites for AGs (Schacht ,1978; Wang et al, 1984; Williams and Schacht, 1986). Binding between AGs and these membrane phospholipids in the renal cortex appears to involve the electrostatic interaction of the cationic AGs with the acidic phospholipids particularly the phosphoinositides (Schacht, 1979; Sastrasinh et al , 1982). Binding is immediate and strong. The resulting membrane alterations may figure importantly in the pathogenesis of AG induced nephrotoxicity.

[1.2.3] Neuromuscular Blockade.

AGs with neuromuscular blocking properties exert both pre- and post-junctional actions at the neuromuscular junction (Elmquist and Josefsson, 1962; Vital-Brazil and Prado-Franceschi, 1969; Bushby, 1976; Wright and Collier, 1977; Singh, 1982; Fiekers, 1983a , 1983b ).

The mechanism of presynaptic neuromuscular blockade by AGs is thought to involve the inhibition of $Ca2^{+}$-mediated acetylcholine release. According to the competitive hypothesis the cationic AGs compete with $Ca2^{+}$ for anionic binding sites on the external surface of the presynaptic junctional membrane at or near the point of $Ca2^{+}$ entry (Prado et al, 1978; Uchiyama et al , 1981).

The postsynaptic component of AG induced neuromuscular blockade can be antagonised by anticholinesterases such as neostigimine but not by $Ca2^{+}$ (Elmquist and Josefsson, 1962).

Postsynaptic blockade by AGs is thought to be due to the interaction of the drug with the acetylcholine receptor (Torda, 1980) or with the ionic channel of the receptor (Vital-Brazil and Corrado, 1957; Fiekers, 1983b).

Of the two proposed sites of action at the neuromuscular junction, the presynaptic effect is thought to predominate. Evidence for which is that $Ca2^{+}$ is more effective than neostigmine in reversing neuromuscular blockade by the AGs (Vital-Brazil and Corrado, 1957; Corrado, 1963; Pittinger and Adamson, 1972; Sokoll and Gorgis, 1981; Farely et al, 1982; Fiekers, 1983a).

The neuromuscular blockade produced by the AGs is similar

to that produced by high concentrations of $Mg2^{+}$ in that both are associated with a large decrease in evoked release of acetylcholine and with a smaller decrease in postjunctional receptor sensitivity. (Vital Brazil and Corrado, 1957; Corrado and Ramos, 1958, 1960; Elmquist and Josefsson, 1962; Singh et al , 1979, 1982; Caputy et al, 1981).

## [1.3.] The effect of aminoglycoside antibiotics on ion transport across the plasma membrane.

It is thought that the intial events of AG toxicity occur at the plasma membrane. The biochemical composition of the plasma membrane includes receptor sites, transport functions and selective ion channels and pumps. The sensitivity of certain tissues to the AGs may be based on differences in the composition of plasma membrane of different types of cell.

AGs can produce an acute reduction in cochlear microphonic potentials (Nuttal et al , 1977) and receptor potentials from cochlear hair cells (Wersall and Flock, 1964; Hudspeth and Corey, 1979; Kroese and Van den Bercken, 1980; Hudspeth, 1982 ). It has been shown that the AG, dihydrostreptomycin may block poorly selective cationic channels of the hair cells of the sacculus of bullfrog (Hudspeth and Kroese, 1983). Furthermore, co-administration of the loop diuretic furosemide and GEN to the guinea pig abolishes the negative endocochlear potential, which is thought to be a $K^+$ diffusion potential. (Lee and Harpur, 1985 ).

The neuromuscular toxicity of AGs may be reversed with calcium. (Vital-Brazil and Corrado, 1957; Singh et al , 1978 a&b). Early changes induced by AGs in the ear may also be reversed with calcium. Furthermore AGs are able to compete with and displace $Ca2^+$ ions at binding sites on lipid monolayers and cell membranes (Elmquist and Josefsson, 1962; Just and Habermann, 1977; Weiss, 1981; Williams et al, 1981b; Lullmann and Vollmer, 1982; Sastrasinh et al, 1982). The binding affinity of AGs and some of their membrane effects

correlate with their net cationic charge (Vital-Brazil and Prado-Franceschi, 1969; Feldman et al , 1982; Josepowitz et al, 1982; Johansson et al, 1984 ).

The binding of AGs to phosphoinositides and their ability to displace $Ca2^+$ would certainly effect membrane transport. It is the aim of this project to investigate the interaction between AGs and plasma membrane ion transport systems.

In recent years a degree of information has accumulated regarding the interaction between AGs and ion channels and pumps in the plasma membrane; AGs are known to inhibit $Ca2^+$ currents and action potentials in a variety of tissues (Hino et al , 1982; Suarez-Kurtz, 1974; Suarez-Kurtz and Reuben , 1987; Wagner et al , 1987; Adams and Durnett, 1978). Inhibition of such ion channels would prevent a rise in intracellular free $Ca2^+$ and thereby AGs could indirectly prevent the activation of $Ca2^+$-dependent $K^+$ channels. This has been proposed as the mechanism whereby AGs inhibit $Ca2^+$-activated $K^+$ channels in nerve terminals of the frog neurohypophysis (Parsons et al , 1987).

The abolition of the receptor potential in bullfrog sensory hair cells and the negative endocochlear potential by AGs (Kroese and Van den Bercken, 1980; Hudspeth, 1982; Lee and Harpur, 1985) originates from a change in $K^+$ permeability. AGs would thus appear to be able to block $K^+$ transport in the inner ear. Inhibition of $K^+$ channel conductance by AGs has been shown also to occur in the sarcoplasmic reticulum (Oosawa and Sokabe, 1986).

NEO has been demonstrated to have a depressive effect upon the discharge activity of an isolated invertebrate sensory

neuron. This effect is associated with changes in membrane electrical properties suggestive of an increased membrane potassium conductance (Nation and Roth, 1988).

Another membrane effect of AGs on excitable membrane ion channels is that the action potentials are blockable by AGs, indicating that the drugs have blocking effects on these particular membrane ion channels (Sokoll and Dieke, 1969; Dieke et al, 1971; Singh et al, 1982). The concentrations required were however greater than those known to cause neuromuscular blockade and it is therefore unlikely that such an action will contribute to the neuromuscular blocking actions of the AGs.

In the mouse postsynaptic endplate ion channel blockade has been demonstrated for AGs and is thought to have a role to play in the mechanism of postsynaptic blockade produced by these drugs (Pennefather and Quastel , 1980). However such an effect was not seen in the snake (Fiekers and Parsons, 1980).

The AGs NEO and GEN inhibit renal Na,K-ATPase activity in vitro and in vivo (Lipsky and Lietman, 1980; Williams et al 1981a-b; Cronin et al , 1982; Aramaki et al, 1986) and this effect is specifically antagonised by phophatidylinositol. This antagonism is of interest as the activity of the Na,K -ATPase is dependent on its surrounding phospholipid environment (Dahl and Hokin, 1974). The inhibition of Na,K-ATPase in the kidney basolateral membrane denotes a functional change in an enzyme intimately involved in cellular electrolyte balance and fluid reabsorption in the kidney.

Na,K-ATPase is found in virtually all mammalian cells including those of the stria-vascularis and spiral ligaments of the guinea

pig inner ear (Ilnuma et al, 1967). It was thought that interference by AGs with the ATPases in the inner ear would effect processes changing the ionic content of the endolymph, (Mendelsohn and Konishi, 1969; Konishi and Mendelsohn, 1970 ) leading to a fall in the normally high concentration of potassium in the endolymph
and a rise in the concentration of sodium ions (Mendelsohn and Katzenberg, 1972; Neu and Bendush, 1976). Chronic kanamycin toxicity has been reported to alter potassium ion permeability of the cochlear partitions (Konishi and Salt, 1980) but not sodium ion permeability (Konishi and Mori, 1984 ). Recently Komune et al (1987) have reported no change in potassium ion activity in either the endolymph or the perilymph after administration of chronic amounts of kanamycin, only cochlear microphonics were depressed. They postulated from this finding that injury to the hair cells may not be secondary to damage to the stria vascularis, but a primary event in AG ototoxicity.

Direct competitive inhibition of a $Ca2^{+}$-regulated $K^{+}$ channel by AGs has been demonstrated by Sokabe and his co-workers (1982), in an in vitro membrane model. They utilised a bilayer membrane formed from lysotriphosphoinositide, an oxidised product of triphosphoinositide. The AGs tested in this model system showed a similar effect of varying severity closely paralleling their ototoxic actions.

$Ca2^{+}$-dependent $K^{+}$ conductance has been demonstrated in target tissues for AGs. This $K^{+}$ transport system is an important component of the transduction process, maintaining the resting potential in mammalian cochlear hair cells

(Ashmore and Meech, 1986). It has also been shown to exist in the neuromuscular junctions of the mouse (Mallart, 1985) and the apical (brush border) membrane of amphibian kidney proximal tubule (Hunter, 1986).

Active cellular uptake mechanisms for AGs have been established for the kidney (Kluwe and Hook, 1978; Pastoriza-Munoz et al, 1979; Silverblatt and Kuehn, 1979; Bennett et al, 1982; Lipsky et al, 1980; Wedeen et al, 1983), crista ampularis (Williams et al , 1987a) and the cochlear hair cells of the guinea pig (Williams et al , 1987b )

It was in the light of the distribution of the $Ca2^{+}$- dependent $K^{+}$ channel in the body, the known interaction with $Ca2^{+}$ by AGs and the existence of cellular uptake systems for AGs that this study was undertaken to examine what effect AGs might have on $Ca2^{+}$-dependent $K^{+}$ transport.

The difficulties involved in isolating sufficent viable inner ear or neuromuscular material has precluded their use in this study and has led to the utilisation of a model system. The human erythrocyte has been widely used as a model for the study of $Ca2^{+}$-dependent $K^{+}$ transport in vitro (Schwarz and Passow, 1983) and was selected for use in this study. An advantage of using an erythrocyte model system is that these cells lack internal organelles. This allows the examination of drug effects on the plasma membrane in isolation from possible effects on such organelles.

## [1.4] Potassium transport in the human erythrocyte plasma membrane.

### [1.4.1] Calcium-dependent potassium channels in erythrocytes

In a wide range of cell membranes studied to date, elevation of the intracellular free $Ca2^+$ increases the ion permeability, the most frequently observed pathway consists of $K^+$ selective channels and was first documented in human erythrocytes (Gardos, 1958). The role of $Ca2^+$ on the activation of specific $K^+$ channels of the cell membrane has been extensively documented in human erythrocytes and other animal cells. (Meech, 1978; Lew and Ferreira, 1978; Putney, 1979, Schwarz and Passow, 1983; Peterson and Maruyamay, 1984; Latorre et al , 1984).

The most widely used system to investigate $Ca2^+$-dependent $K^+$ transport whether as a whole cell or a membrane preparation has been the erythrocyte. Although the function of this ion transport system in the erythrocyte remains unclear much detailed information has been collected characterising this transport system in this particular cell;

It is known that $Ca2^+$ acts on the intracellular side of the cell membrane and that the magnitude of the potassium ion movement is related to the concentration of free internal $Ca2^+$ (Blum and Hoffman, 1971; Lew and Ferreira, 1976 ). In the erythrocyte the relationship between intracellular $Ca2^+$ concentration and effect is sigmoidal. It is suggested that this is due to two or more $Ca2^+$ ions being required to stimulate one $K^+$ channel (Simons, 1976; Porzig, 1977). This may

account for the rather narrow range of $Ca2^+$ concentrations between zero and maximal response (Schwarz and Passow,1983).

The presence of $Ca2^+$ inside the cell does not suffice to open the $K^+$ channels unless extracellular $K^+$ is also present (Heinz and Passow, 1980; Knauf et al, 1975 ).

Erythrocyte $Ca2^+$-dependent channels are thought to be aqueous pores (Glynn and Warner, 1972) with a conductance of 20 ps (Hamill , 1981) . They are very selective; only $K^+$, $Rb^+$ and to some extent $Cs^+$ ions move through them (Kregenow and Hoffman, 1962; Passow , 1963; Simons, 1976; Porzig, 1977). Substitution of $K^+$ with $^{86}Rb^+$ is commonly used as a means of monitoring $K^+$ movements across erythrocyte membranes.

The divalent cation $Pb2^+$ is able to substitute for $Ca2^+$ in this transport system (Orskov, 1935) and is thought to act directly upon the $Ca2^+$ binding site (Riordan and Passow, 1973; Passow , 1981; Simons, 1985; Shields et al , 1985; Alvarez et al , 1986).

It has been put forward that individual $Ca2^+$-dependent $K^+$ channels respond in an all or nothing fashion to changes in intra-cellular $Pb2^+$ or $Ca2^+$ concentration. A pure all or nothing response is however difficult to reconcile with observations from patch clamp experiments (Lew et al, 1983) where a progressive increase of mean open time of $K^+$ channels with the increase of $Ca2^+$ concentrations has been reported (Grygorczyk and Schwarz, 1983; Grygorczyk et al , 1984). A modification of the all or nothing hypothesis has been suggested whereby each channel has a threshold for $Ca2^+$

below which no measurable activation occurs. At submaximal $Ca2^+$ concentrations a gradual activation of the individual channels may take place, with an increase of the apparent permeability on increasing $Ca2^+$ concentrations (Alvarez et al, 1986a ).

Estimates as to the number of $Ca2^+$-dependent $K^+$ channels per cell vary depending on the method of calculation. Using one-step inside out erythrocyte vesicles a mean of between 100 - 200 channels per cell has been calculated on the basis of comparison with the number of $Na^+$ pumps found in the vesicles (Lew et al , 1982 ). Employing the patch clamp technique to compare unitary conductance and tracer equilibration rate measurements, a mean of 10 heterogeneously distributed channels per cell has been calculated (Grygorczyk et al, 1984). Again using an inside-out vesicle preparation and estimating the relative frequency of vesicles having no $K^+$ channels the figure has been calculated to be 142 +\- 27 (Mean +\- S.E.M) (Alvarez and Garcia-Sancho, 1987). The recent discovery of a high-affinity toxin (charibdotoxin) against $Ca2^+$-dependent $K^+$ channels in erythrocytes (Castle and Strong 1986) may help resolve the descrepency between these estimates.

There is a certain amount of evidence that $Ca2^+$-dependent $K^+$ transport in human erythrocytes may be modulated by cytoplasmic NADH\NAD (Lindenmann and Passow, 1960; Alvarez et al , 1984). However direct evidence of redox modulation of $Ca2^+$-dependent $K^+$ transport under physiological conditions is still lacking.

Another candidate for modulation of $Ca2^+$-dependent $K^+$

transport in human erythrocytes is calmodulin. Erythrocytes contain an outwardly directed $Ca2^+$ pump which is stimulated by calmodulin (Roufogalis and Mauldin,1980). The published information on the role of calmodulin in modulation of this particular $K^+$ transport system is contradictory ( Lackington and Orrego, 1981; Plishker, 1984; Alvarez, 1986c; Pape and Kristensen, 1984; Garcia-Sancho et al, 1982) and it is unclear whether or not calmodulin has a role as a modulator of $Ca2^+$-dependent $K^+$ transport in erythrocyte plasma membranes.

The $Ca2^+$-dependent $K^+$ transport system is conserved in one-step inside out erythrocyte vesicles (IOVs) (Lew et al, 1982; Garcia-Sancho et al , 1982) as opposed to its previously reported absence in IOVs prepared by other methods (Grinstein and Rothstein, 1978). Thus it is thought that some vital component of this transport system is lost when cell contents are extremely dilute or low ionic strength solutions are used. These findings are indicative of the involvement in $Ca2^+$-dependent $K^+$ transport of a protein that is loosely bound to the membrane.

Sarkadi et al (1980) were the first group to show that a concentrated haemoglobin free cytoplasmic extract partially restored $Ca2^+$-dependent $K^+$ transport to an IOV preparation previously insensitive to $Ca2^+$. Because boiling of the extract destroyed this effect, the effect was thought to involve a protein other than calmodulin, which is heat stable.

Iodoacetic acid, which can inhibit $Ca2^+$-dependent $K^+$ transport, carboxymethylates a 23000 Dalton protein that is found in the haemolysate and in association with the erythrocyte cell membrane (Plishker et al , 1985). Furthermore

antiserum raised against this protein inhibits $K^+$ efflux from resealed ghosts and elevated $Ca2^+$ prevents the removal of this protein from the ghost, whereas without $Ca2^+$ and with EGTA added to the wash buffer the protein is easily removed. How this protein acts biochemically has not been established.

[1.4.2] Inhibition of Ca2+-dependent K+ channels in human erythrocytes.

There are known to be a range of compounds which inhibit $Ca2^+$-dependent $K^+$ channels in human erythrocytes (either in resealed ghosts or IOVs). These include quinine and its analogues (Blum and Hoffman, 1971; Gardos, 1958; Armando-Hardy et al , 1975; Simons, 1976; Burgess et al , 1981; Riechstein and Rothstein, 1981; Sanchez et al, 1980; Garcia-Sancho et al, 1982), oligomycin (Blum and Hoffman, 1972; Riordan and Passow, 1973; Porzig, 1977), the venom of the Israeli scorpion Leiurus quinquestriatus (Abia et al , 1986; Castle and Strong, 1986; Leneveu and Simonneau, 1986), iodoacetic acid (Plishker, 1985), methyl phenidate (Lackington and Orrego, 1985), probucol (Howlands et al, 1984), carbocyanine dyes (Simons, 1976, 1979) and menadione analogues, but not menadione (Fuhrmann et al, 1985).

Quinine although a cation, is lipophilic and partitions rapidly within the cell membrane (Burgess et al, 1981). It is however thought to inhibit $Ca2^+$-dependent $K^+$ transport by competitively displacing $K^+$ from external binding sites, the reported $K^+$ activation site for $Ca2^+$-dependent $K^+$ permeability

(Reichstein and Rothstein, 1981).

Oligomycin is thought to decrease affinity for $Ca2^+$ and to decrease the maximal rate of $K^+$ movement. It is thought to be a non competitive inhibitor that interacts only with $Ca2^+$ sites which are involved in activation of $K^+$ transport. (Porzig, 1977).

Inhibition of $Ca2^+$-dependent $K^+$ channels in mammalian skeletal muscle by Leiurus quinquestriatus venom is due to a minor protein component, charibdotoxin (Miller et al, 1985). This has also been shown to be the case in human erythrocytes (Abia et al, 1986). It has been suggested that the inhibitory effect is not accomplished by decreasing the affinity for external $K^+$ or internal $Ca2^+$, as seems to be the case for other inhibitors of $Ca2^+$-dependent $K^+$ channels in human erythrocytes.

The carbocyanine dyes are most effective at blocking these $K^+$ channels when the system is maximally activated, indicating that they block primarily the $K^+$ movements across the open channels rather than the opening of the channels (Simons, 1976).

### [1.4.3] Co-transport of K+ with other ions in the human erythrocyte plasma membrane.

Besides $Ca2^+$-dependent $K^+$ transport, other $K^+$ transport systems are to be found in the human erythrocyte plasma membrane. These include co-transport systems and the Na-K pump.

Co-transport systems have been extensively reviewed

recently (Lauf, 1985; Chipperfield, 1986; O'Grady et al , 1987). The concept behind co-transport is that the ions involved can be so coupled that they are carried together across the cell membrane. The ions are interdependent upon one another, if any ion is missing then transport of the remaining ions is blocked. This interdependency is a critical functional definition of co-transport.

There are two documented co-transport systems to be found in the human erythrocyte; Na-K-Cl and K-Cl co-transport. It was shown in 1974 that human erythrocytes possess a furosemide sensitive Na-K co-transport system (Willey and Cooper, 1974). Linkage of Cl to Na-K movements in human erythrocytes is difficult to establish, because $Cl^-$ fluxes via the $Cl/HCO_3$ exchanger are so fast that they swamp $Cl^-$ fluxes via co-transport (Wiater and Dunham, 1983; Duhm and Goebel, 1984; Ellory and Hall, 1984). Nevertheless furosemide-sensitive $Na^+$ and $K^+$ movements do conform to co-transport involving $Cl^-$ (Duhm and Goebel, 1984). In ferret erythrocyte where co-transport is much more prominent the linkage has been established (Ellory and Hall, 1984).

Na-K-Cl co-transport has been shown to be sensitive to pharmocological inhibition by loop diuretics such as furosemide and bumetanide (Dunham et al , 1980), of which bumetanide is the more potent (Ellory and Stewart, 1982). At concentrations greater than $10^{-4}M$ , furosemide has been reported to inhibit various $K^+$ transport systems in human erythrocytes (Palfrey et al, 1980; Ellory et al, 1983; Brazy and Gunn, 1976), including K-Cl co-transport (Lauf, 1985; Wiater and Dunham, 1983). The definition of Na-K-Cl co-transport thus requires

inhibition by loop diuretics at concentrations lower than $10^{-4}$M. To this end the more potent loop diuretic bumetanide is of particular use.

Evidence is available that K-Cl co-transport can occur in human erythrocytes under normal conditions (Kaji, 1986; Berkowitz and Orringer, 1987). Inhibitory substances specific for K-Cl co-transport at low concentrations are not available (Lauf, 1985). This form of $K^+$ transport is however Cl-dependent (as is Na-K-Cl co-transport), thus in the event of $K^+$ transport exhibiting poor sensitivity to loop diuretics, $Cl^-$ substitution may be an aid to definition.

[1.4.4] The Na-K pump in the human erythrocyte plasma membrane.

The erythrocyte plasma membrane contains a Na-K pump which utilizes metabolic energy to transport $Na^+$ out of the cell and $K^+$ into it (Schatzmann, 1953). The pump itself consists of an enzyme known as Na,K-ATPase (Skou, 1957) which requires the presence of $Mg2^+$ for activation. The Na-K pump in erythrocyte plasma membrane has recently been reviewed (Hoffman, 1987).

The pump operates so that influx of $2K^+$ is coupled to the efflux of $3Na^+$ (Post and Jolly, 1957) at the expense of one ATP (Sen and Post, 1964). The difference in $3Na^+/2K^+$ stoichiometry is the basis for the pump being electrogenic, moving net charge ($Na^+$) outward (Hoffman et al , 1979).

The average erythrocyte contains 200-300 pumps, each transporting about 150 ions per site per second (Hoffman,

1969; Joiner and Lauf, 1978).

Pharmacological inhibition of the Na-K pump occurs with ouabain and was first demonstrated by Skou in 1960.

The Na-K pump is comprised of two types of subunit (Kyte, 1971). ATP interacts with and phosphorylates the subunit on its inward facing aspect (Uesugi et al , 1971), while ouabain binds to an outward facing portion of the subunit (Ruoho and Kyte, 1974; Forbush et al, 1978) at a site that is antagonized by $K^+o$ (Glynn, 1957; Hoffman, 1966).

It is thought that Na,K-ATPase may be regulated by intracellular $Ca2^+$ (Yingst, 1988). This is of interest in the light of the AGs ability to displace $Ca2^+$ from membranes and their ability to inhibit Na,K -ATPase.

## [1.5] Aims of study

Despite extensive investigation the mechanisms by which aminoglycoside antibiotics exert their toxicities are not fully understood. One possible mode of action is that they interfere with the normal functioning of plasma membrane ion channels. It is not inconceivable that such an effect could be involved in drug toxicity.

The initial aim of this study was to determine what effect AGs might have on one particular plasma membrane ion transport system the $Ca2^+$-dependent $K^+$ channel.

That the $Ca2^+$-dependent $K^+$ channel has been well characterised in the human erythrocyte and the availability of an inside out vesicle preparation in which the channel is preserved, led to the selection of the human erythrocyte as a model system.

Employment of the human erythrocyte as a model offered the advantage that, besides being readily available, it allowed the examination of a drug effect in a human as opposed to an animal system. Thus the effects observed could be related to the known toxicology of AGs in the human body with a higher degree of confidence than would have been the case had an animal model been used.

That the plasma membrane of the human erythrocyte is not a recorded site of AG toxicity does not necessarily imply that the $Ca2^{+}$-dependent $K^{+}$ channel in the human erythrocyte is not representative of that found in tissues known to be sites of AG toxicity. The $Ca2^{+}$-dependent $K^{+}$ transport system is not known to have a major physiological role to play in the erythrocyte and thus any drug effect on it would not have any major toxicological manifestations.

An investigation of the human erythrocyte as an in vitro model system for determining the mechanisms of possible membrane related AG toxicity was pursued with the goal of improving existing methodology for the assessment of drug toxicity.

## Materials and Methods

### [2. 1] Materials

#### [2.1.1] Chemicals and drugs.

Obtained from Sigma Chemical Company, Poole, Dorset, U.K.

Bovine serum albumin (B.S.A) fraction v 96-99% albumen

Dowex-50W Hydrogen form (8% crosslinked ion exchange resin)

Furosemide

D-Glucose anhydrous (cell culture tested)

Hepes (N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid)

Ionophore A23187

Neomycin sulphate

Ouabain (G-strophanthin) octahydrate

Potassium thiocyanate

Sodium thiocyanate

Trichloroacetic acid

Tris(hydroxymethyl)aminomethane (Sigma 7-9)

Quinine (free base)

Valinomycin

Obtained from FSA, Loughborough, Leics, U.K.

Calcium chloride (AR Grade)

Potassium chloride (AR Grade)

Potassium hydroxide

Sodium chloride (AR Grade)

Sodium hydroxide

Sucrose

Obtained from B.D.H. Poole, Dorset, U.K.
EGTA (Ethyleneglycobis-(aminoethylether)tetra-aceticacid)

Bumetanide (3-butylamino-4-phenoxy-5-sulphamoyl benzoic acid) was a gift to Dr J Hickman, from Leo laboratories Ltd, Aylesbury, Bucks, U.K.

Obtained from Roussel Laboratories Ltd, Wembley, Middlesex, U.K.
Gentamicin sulphate as cidomycin sterile powder.

Obtained from the London Analytical and Bacteriological Media Ltd, Salford, U.K.
Sensitivity Test agar.

Obtained from Oxoid Ltd, Basingstoke, U.K.
Mueller Hinton assay broth.

Obtained from Imperial Laboatories Ltd.
Horse serum.

[2.1.2.] Radiochemicals

$^{86}$Rubidium chloride (1 mCi $ml^{-1}$) was obtained from Amersham International plc, Amersham, Bucks, U.K.

[2.1.3] Equipment and other materials

Obtained from Sigma Chemical Company, Poole, Dorset, U.K.
Dialysis tubing (cellulose 16 mm diameter Mwt limit >12,400 dalton)

Obtained from Luckhams Plastics, Burgess hill, Sussex, U.K.
Plastic tubes (3 ml LP3)

Obtained from Biomedical Supplies, Small Heath, Birmingham.U.K.
Plastic bijou bottles
Universal containers
Pipetman pipette tips
Pasteur pipettes
Plastic spectrophotometer cuvettes (3 ml)

Obtained from L.I.P, Dewsbury, Yorkshire, U.K.
Heparinised plastic blood collection tubes (10 ml)

Obtained from Northern Media Supply Ltd.
Culture plates (9 cm).

Spectrophotometer (Double beam, C.E. 594)
Cecil instruments, Cambridge, U.K.

Shaking water bath
Mickle Engineering, Gomshall, Surrey, U.K.

Labofuge 6000 Heraeus bench top centrifuge
Supplied by V. A. Howe, London, U.K.

Hi-Spin 21 centrifuge with 8 x 50 ml rotor
M.S.E, Fisons, Leicestershire, U.K.

Gilson pipeters
Supplied by Anachem, Luton, Bedfordshire, U.K.

Micro-haematocrit centrifuge
Haematocrit capillary tubes
Hawksley, London U.K.

Hypodermic needles 26 G
F.S.A, Loughborough, Leics, U.K.

Corning 435 flame photometer
Potassium standard 80 mM $L^{-1}$
Sodium standard 160 mM $L^{-1}$
Lithium internal standard
Corning Medical, Corning Ltd.

A culture of Klebsiella Edwardii NCTC 10896 was a gift from Dr I Farrell of the public health laboratories, East Birmingham Hospital, Birmingham, U.K.

## [2.2] Buffers and Solutions

### [2.2.1] Inside-out erythrocyte (IOVs) experiments.

Erythrocyte isotonic wash buffer A

KCl 150 mM

EGTA 0.1 mM pH 7.7 (with Tris)

Lysis buffer A

Hepes 2.5 mM

EGTA 0.1 mM pH 7.5 (with KOH)

IOV wash buffer

Hepes 16.5 mM

EGTA 0.1 mM

KCl 18.0 mM pH 7.5 (with KOH)

IOV incubation buffer

Hepes 16.5 mM

EGTA 0.4 mM

KCL 18.0 mM pH 7.5 (with KOH)

$CaCl_2$*

* The $Ca2^+$ concentration in the incubation buffer was calculated using a value of $6.5x10^{-8}$ M for the apparent dissociation constant of EGTA (Alvarez et al 1984).

For the $Cl^-$ substitution experiments (see Results 3.7) potassium chloride was replaced in all buffers with potassium thiocyanate at the same concentration.

Elution buffer

Sucrose 0.2 M

Hepes 5 mM

B.S.A. 0.5 mg $ml^{-1}$ pH 7.5 (with NaOH)

[2.2.2] Intact erythrocyte shrinkage experiments.

Erythrocyte isotonic wash buffer B

NaCl 150 mM

$CaCl_2$ 0.1-0.5 mM

Hepes 10 mM pH 7.5 (with KOH)

Incubation buffer B

NaSCN 150 mM

$CaCl_2$ 0.1-0.5 mM

Hepes 10 mM pH 7.5 (with KOH)

Erythrocyte loading wash buffer (Phosphate buffered saline)

NaCl 150 mM

$K_2HPO_4/KH_2PO_4$ 5 mM pH 7.4

Lysis buffer B (erythrocyte loading)

$K_2HPO_4/KH_2PO_4$ 5 mM pH 7.4

Resealing buffer

| | |
|---|---|
| NaCl | 150 mM |
| $K_2HPO_4/KH_2PO_4$ | 5 mM |
| Glucose | 10 mM pH 7.4 |

[2.2.3] Preparation of stock drug and ionophore solutions.

Preparation of stock solutions of furosemide and bumetanide.

Furosemide and bumetanide were both dissolved in acetone to 50 times the required final concentration in the incubation buffer. Fresh stocks were prepared daily.

Preparation of stock solutions of quinine

Quinine was dissolved in incubation buffer to 10 times the required final concentration. Fresh stocks were prepared daily.

Preparation of stock solutions of aminoglycoside antibiotics.

Aminoglycoside antibiotics were dissolved in incubation buffer to 25 mM, from which solutions were prepared as required. Fresh stocks were prepared daily.

Preparation of solutions of the divalent cation ionophore A23187.

The ionophore A23187 was dissolved in absolute ethanol to 1000 times the required final concentration. Aliquots of stock solution were stored short term at 4°C and long term at -20°C.

Preparation of solutions of the potassium ionophore valinomycin.

Potassium ionophore valinomycin was dissolved in absolute ethanol to 1000 times the required final concentration. Aliquots of stock solution were stored short term at 4 °C and long term at -20 °C.

## [2.3] Methods

### [2.3.1] Preparation of one-step inside out erythrocyte vesicles

One-step inside out human erythrocyte vesicles (IOVs) were prepared using the method described by Lew and Seymour (1982), as modified by Alvarez et al. (1984).

Fresh cells were drawn, after first obtaining informed consent (see consent form), from healthy, volunteer male subjects. Training in venepuncture was received at the phlebotomy clinic at the General Hospital, Steelhouse Lane, Birmingham, West Midlands.

The blood was collected in a 10 ml heparinised plastic tube, mixed and centrifuged at 2000 rpm (Labofuge 6000 Heraeus benchtop centrifuge ) for 10 min at room temperature. The plasma and the buffy coat were removed from the cells. The cells were washed four times at room temperature by resuspension in 40 ml isotonic KCl containing EGTA (See Buffers and Solutions) followed by centrifugation at 2000 rpm for 10 min. They were then packed by centrifugation at 4000 rpm for 10 min at room temperature. The packed erythrocytes were lysed by adding them to 40 volumes of an ice-cold lysis buffer (A) (see Buffers and Solutions), whilst being gently stirred for 10 min. Aliquots (40 ml) of lysed erythrocytes were centrifuged for 10 min at 30000 g (19000 rpm, Hi-spin 21 centrifuge) at +4°C. The pelleted ghosts from each aliquot were resuspended in 2 ml of the same lysing buffer in 5 ml plastic bijou bottles and incubated at 37°C in a gently shaking water bath for 45 min.

According to Lew and Seymour (1980), spontaneous inside out vesiculation occurs during the first 15-20 min of incubation of

## CONSENT FORM FOR VOLUNTEER SUBJECTS

PROJECT TITLE: Studies of the effects of drugs on cell membranes.

RESEARCH GROUP: MRC Mechanisms of Drug Toxicity Research Group, Department of Pharmaceutical Sciences, Aston University.

INVESTIGATORS: Dr. E. S. Harpur and Mr. C. M. Lazenby

OBJECTIVES:

1. The overall objectives of this group's research are to improve our understanding of the mechanisms by which certain drugs cause unwanted toxicity, to improve existing methodology for the assessment of drug toxicity and thereby to contribute to improvements in drug therapy.

2. The specific objectives of this project are to identify and elucidate the actions of certain antibiotics and anti-cancer agents at cell membranes.

MATERIAL: The studies are conducted using human red blood cells or membrane vesicles prepared from human red blood cells obtained from freshly-drawn blood.

To this end it is necessary to obtain by venepuncture 50 ml of blood from healthy, unmedicated subjects. No individual should donate blood more often than once every two weeks.

CONFIDENTIALITY: Samples will be coded so that it will be apparent when blood from one donor is used in more than one experiment. No information regarding the identify of the donors will be disclosed.

POSSIBLE HAZARDS FOR DONORS: Infection or haematoma (leakage of blood into the tissues causing bruising and discomfort).

These are rare events and are minimised by appropriate training of the investigators.

## STATEMENT OF VOLUNTEER

I have read and understand the attached explanation. I have had the opportunity to discuss it with the investigators and to ask any questions. I agree to take part in the above project and I understand that I am free to withdraw at any time.

Signed.....

Dated.....

erythrocyte ghosts at 37·C. Membrane elasticity increases with time (Lew and Seymour, 1982) allowing subsequent fragmentation and resealing of a fraction of the vesicles by vigourous passage through small gauge needles (needling).

Following incubation at 37·C the erythrocyte ghosts were fragmented by forcefully passing them through a 26 gauge needle four times. By measurement of $Ca2^{+}$-ATPase it has been demonstrated that at least 25% of the original erythrocyte membrane area surrounds IOV-sealed vesicular space (Lew and Seymour, 1982).

The vesicles were washed once by resuspension in 40 volumes of IOV wash buffer (see Buffers and Solutions) followed by centrifugation for 10 min at 30000 g at 4·C (High-spin centrifuge). The vesicles were collected from the centrifuge tube by decanting the supernatant and resuspending them in the same buffer by disaggregating the pellet by drawing it through a 1 ml Gilson pipette. The vesicles were further disaggregated by a single passage through a 26 gauge needle and stored on ice until used.

[2.3.2] Determination of the activity of Ca2+-dependent K+ transport

The activity of $Ca2^{+}$-dependent $K^{+}$ transport in the one-step IOVs was assessed by the measurement of the uptake of $^{86}Rb$, which behaves similarly to $K^{+}$ in this transport system (Simons, 1976).

Experiments were initiated by mixing in a 3 ml Luckhams plastic tube (by means of a vortex mixer) one volume of IOV suspension with four volumes of incubation buffer (see Buffers and Solutions)

containing 10 μCi $^{86}Rb$ per ml of incubation buffer. The relative $K^+$, $Cl^-$, and Hepes concentrations within the vesicles have been stated to be about the same as that within the incubation buffer, so that experiments were performed at or near equilibrium-exchange conditions (Alvarez et al. 1986c). After the desired incubation period 0.1 ml aliquots of the incubation mixture were added to 1ml columns of $Na^+$-activated Dowex 50-X8-100 resin, and were eluted with 1.8 ml of ice cold elution buffer (see Buffers and Solutions).

The columns were prepared from glass wool plugged pasteur pipettes, to which 1 ml of the Dowex resin was added. Sodium activation of the Dowex resin was achieved by running 20 ml of 20% NaCl through the columns, followed by 2 ml of single distilled water. The columns were further treated with 0.9 ml of 1% BSA and were washed with 1.8 ml of eluting buffer immediately prior to use.

The eluted IOVs were collected in 3 ml plastic tubes. The $^{86}Rb$ uptake into the IOVs was then determined by counting on an ICN Tracerlab Gamma Set 500 gamma detector, set with minimum threshold and maximum window gain 32, for 5 min. Empty tubes were counted so as to obtain a value for background radiation, this value was subseqently subtracted from all the sample counts.

[2.3.3]Time course experiments ( see Results 3.1)

To 0.5 ml of incubation buffer containing 10 μCi $^{86}Rb$ per ml, with or without added aminoglycoside antibiotics (AGs) and or $Ca2^+$, 0.125 ml of IOVs were added to start the experiment (final volume 0.625 ml). The mixture was vortexed immediately after the addition of the IOVs and incubated at room temperature.

Aliquots (0.1 ml) were taken at the desired time points and were added to the Dowex columns to determine $^{86}Rb$ uptake into the IOVs as previously described (2.3.2).

[2.3.4] Calcium and aminoglycoside antibiotic dose response experiments (see Results 3.2-3.7)

To 0.1 ml of incubation buffer containing $^{86}Rb$ (10 $\mu Ci\ ml^{-1}$) with or without aminoglycoside antibiotics (AGs) and or $Ca2^{+}$, 0.025 ml of IOVs were added to start the experiment (total final volume 0.125 ml). The vesicles were separated from free $^{86}Rb$ by adding 0.1 ml of the mixture to the Dowex columns after a 10 min incubation period. Drugs were added to the incubation buffer as required from concentrated stock solutions (see Buffers and Solutions).

[2.3.5] Loading of IOVs with loop diuretics (see Results 3.4)

To investigate the effect of the presence of either gentamicin (GEN), neomycin (NEO), quinine, furosemide, bumetanide or ouabain in the external incubation buffer these drugs were incorporated in the buffer prior to the addition of $^{86}Rb$ or the IOVs.

Loop diuretics (furosemide or bumetanide) were loaded into IOVs by addition of drug to the hypotonic lysate buffer, prior to the 45 minute 37·C incubation (2.3.1). The drugs were dissolved in acetone, which was added to a control sample of IOVs run in parallel to the loaded vesicles.

[2.3.6] Panel of blood donors

Blood samples used in this investigation were obtained from a pool of eight healthy male volunteer subjects aged between 20 and 40 years. Informed consent was obtained from the volunteers prior to venepuncture (see Consent Form).

The source of the blood used in any one particular experiment depended to a certain extent on the availability of a subject and the length of time elapsed since previous venepuncture.

It was initially intended to pool the data obtained from blood drawn from different donors, however as the study progressed it became clear that intersubject variation in response to drugs was occurring. The source of the blood used in each experiment was therefore recorded, each subject being identified by a number (1-8).

[2.3.7] Effects of aminoglycoside antibiotics on Ca2+-dependent K+ transport in intact erythrocytes.

The activity of the $Ca^{2+}$-dependent $K^+$ channel was estimated from the loss of KCl observed on incubation of erythrocytes in low-K buffer after the increase of intracellular $Ca^{2+}$ levels by the addition of divalent cation ionophore A23187 to the incubation buffer in the presence of $Ca^{2+}$ (Alvarez et al, 1986c).

Blood was drawn from healthy volunteers as described previously (2.3.1) and was placed in a 10 ml heparinised tube which was subsequently centrifuged at 2000 rpm for 10 min at room temperature. The plasma and the buffy coat were removed and the cells were washed twice by resuspension in isotonic wash buffer B (see Buffers and Solutions) followed by centrifugation at 2000 rpm for 10 min at room temperature. The erythrocytes were finally resuspended to 10% haematocrit in the wash buffer.

The measurement of KCl loss was recorded as follows; Immediately prior to the experiment 2 ml of the cell suspension were mixed in a 3 ml spectrophotometer cuvette with 1 ml of incubation buffer containing 150 mM NaSCN in place of NaCl (see Buffers and Solutions). The addition of $SCN^-$ to the medium speeds up the loss of $K^+$ since the electrogenic permeability of the membrane to this anion is so large in comparison to $Cl^-$ that it does not limit the $K^+$ loss after maximal activation of $Ca^{2+}$-dependent $K^+$ transport (Alvarez et al, 1986). The activation of the $K^+$ channels was accomplished by the addition of the ionophore A23187 to the cell suspension to give a final concentration of 3 $\mu$M. As the $K^+$ efflux is accompanied by changes in cell volume and thus density, it is possible to follow the rate and extent of $K^+$ efflux by recording

the change in transmittance at 650 nM. In certain experiments as a control the loss of $K^+$ was initiated by the addition of valinomycin (final concentration 3 μM ) instead of A23187. To investigate the effect of AGs, the drugs were added to the cell suspension immediately prior to the ionophore.

[2.3.8] Loading of erythrocytes with gentamicin.

Loading of human erythrocytes with GEN was achieved by the method described by Eichier et al (1986) for the preparation of GEN loaded erythrocytes for use as a slow drug release system.

Blood was drawn from volunteer donors, added to 10 ml heparinised tubes and centrifuged at 2000 rpm at room temperature for 10 min. The plasma and the buffy coat were removed and the erythrocytes were washed by resuspension in isotonic phosphate buffered saline (PBS) (see Buffers and Solutions) followed by centrifugation at 2000 rpm for 10 min at room temperature. The cells were packed by centrifugation at 4000 rpm for 10 min at room temperature and resuspended in PBS to give a haematocrit of 60%. Aliquots (2 ml) of the erythrocytes were mixed with GEN to the required concentration. The mixture was then placed in dialysis bags and dialysed against 20 ml of hypotonic phosphate buffer (see Buffers and Solutions) for 90 min at 4°C. Resealing was obtained by subsequent dialysis against 20 ml of isotonic PBS, containing glucose ( see Buffers and Solutions) for 15 min at 37°C. To ensure efficient loading GEN was also included in the resealing buffer during this dialysis step at the same concentration as in the hypotonic buffer. The erythrocytes were removed from the dialysis bags and were then washed twice by

resuspension in the resealing buffer without GEN and centrifuging them at 2000 rpm for 10 min at room temperature. The cells were then packed by resuspending them in isotonic saline buffered with Hepes and containing $CaCl_2$ (see Buffers and Solutions) and centrifuging them at 2000 rpm at room temperature.

The cells were resuspended to 10% haematocrit and were treated as before (2.3.6) to monitor the loss of KCl.

[2.4] Analytical determinations

[2.4.1] Determination of calcium, sodium and potassium concentrations in the incubation buffer.

Sodium and potassium concentrations in the incubation buffer were determined using a Corning 435 flame photometer. Distilled water was used to obtain a zero sodium or potassium reading and the photometer was calibrated using standards as described in the materials section (2.1.3). Calcium concentration was measured by atomic absorption spectrophotometry in an air : acetylene flame. The wavelength for Ca determination was 422.7nm, the sensitivity of the determination was 0.08 μg/ml and the relationship between Ca concentration and absorbance was linear up to a Ca concentration of 5 μg/ml. Distilled water was used to provide a zero Ca reading and a one point calibration of 5μg/ml was used. The standard used to give this calibration was made up from a combined Ca/Mg stock solution containing $Ca(NO_3)_2$ atomic absorption standard (1mg/ml) diluted in distilled water or lanthanum chloride. All atomic absorption standards were freshly made up in bottles which were washed, rinsed in distilled water and thoroughly dried before use.

[2.4.2] Determination of potassium concentrations in gentamicin loaded erythrocytes.

The internal potassium concentration of erythrocytes (whether loaded or unloaded with GEN) was determined by the following method. The resealed erythrocytes were washed twice by

resuspending them in isotonic tris buffered saline and centrifuging them at 2000 rpm for 10 min at room temperature. The cells were then packed by centrifugation at 4000 rpm for 10 min at room temperature. A 0.5 ml aliquot of cells was added to a 10 ml glass test tube, these cells were then lysed by addition of 2 volumes of single distilled water (1 ml) followed by mixing by vortex. The cells were then extracted by addition of 2.5 ml of 10% trichloroacetic acid followed by vortexing and centrifugation at 4000 rpm for 10 min at room temperature. The potassium concentration of the resulting supernatant was then determined using a Corning 435 flame photometer as described above (2.4.1).

[2.4.3] Assay of gentamicin

The efficiency of loading of the erythrocytes ( see Materials and Methods 2.3.7 ) was determined by means of a bioassay.

Plates were prepared by pouring 20 ml of sensitivity test agar into 9 cm diameter plastic plates, using an automatic agar dispenser. The agar was allowed to set and the plates were stored at 4°C until required.

On the day of the assay the plates were allowed to dry by inverting them for 45 min on a rack in a warm room (37°C). The plates were then flooded with 5 ml of a 1 in 500 dilution of a culture of Klebsiella NCTC 10896 in Mueller Hinton assay broth. The plates were drained and were allowed to stand for 15 min at room temperature.

Using the blunt end of a sterile glass pasteur pipette three wells were cut in each plate. The plugs were then removed with the tip of the pipette. Using an adjustable Gilson pipette 0.04 ml of standard

or sample was added to each well.

The loaded erythrocytes were lysed by repeated freezing and thawing. A range of GEN standards (0-10μg/ml) was prepared in horse serum.

The plates were incubated overnight and the diameter of the zone of inhibition of bacterial growth around each well was determined. A standard curve was constructed and the GEN concentration of the samples was determined from this.

## Results

### [3.1] Effect of aminoglycoside antibiotics on the time-course of $Ca2^+$-dependent $Rb^+$ uptake into one-step inside out erythrocyte vesicles.

#### [3.1.1] Effect of neomycin$10^{-4}$ M

The time course of $Ca2^+$-dependent $^{86}Rb^+$ uptake into one-step inside out erythrocyte vesicles (IOVs) prepared from various blood donors was assessed at a $Ca2^+$ concentration of $10^{-6}$ M, with and with-out the addition of neomycin (NEO) $10^{-4}$ M to the incubation buffer.

The time course for $Ca2^+$-dependent $^{86}Rb^+$ uptake ($Ca2^+10^{-6}$ M) was rapid with a mean half time to equilibrium of 1.3 min. (The half time to equilibrium being based on the $Ca2^+$-dependent $^{86}Rb^+$ uptake equilibrating after 10 min(arbitary determination)) (Fig 1a). The rate of $^{86}Rb^+$ uptake was only slightly slower on addition of NEO $10^{-4}$ M ( mean half time to equilibrium= 1.5 min). These data show a trend (not statistically significant) for inhibition of $Ca2^+$-dependent $^{86}Rb^+$ uptake by NEO $10^{-4}$ M.

Fig 1a shows the pooled data from 5 experiments using IOVs prepared from 3 different blood donors (subjects 1,2, and 6). Data were also obtained from subject 4, this data has been analysed separately (see Fig 1b) because there was an unusually large $^{86}Rb^+$ uptake produced by AGs alone, into vesicles prepared from blood drawn from this subject (see Results 3.5) .

The uptake of $^{86}Rb^+$ in the absence of both AGs and $Ca2^+$ ($4x10^{-4}$ M EGTA) has been subtracted from all the values, as it has from all other data in this section ( Mean value 88.2 c.p.m).

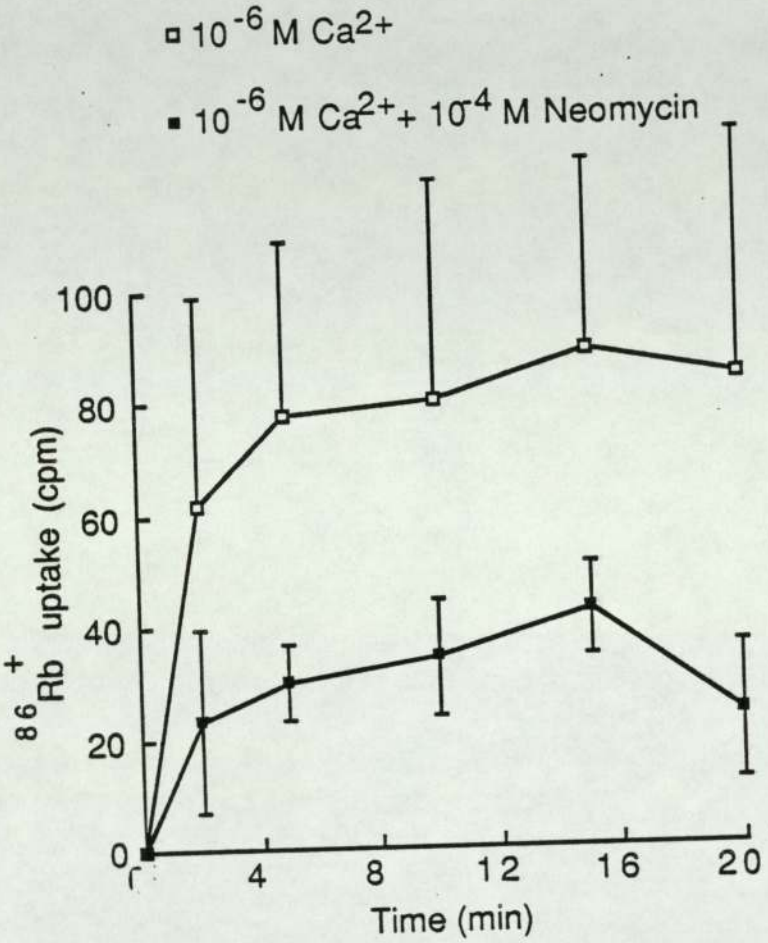


Fig 1a Effect of neomycin on the time-course of Ca-dependent Rb uptake into one-step IOVs. Pooled data from 5 experiments using IOVs prepared from blood drawn from subjects 1,2 and 6. Mean data +/- SE. The uptake in the absence of both AGs and Ca has been subtracted from all the values.

Previous studies of the kinetics of $Ca2^+$-dependent $^{86}Rb^+$ uptake into one-step IOVs by other groups have shown that in addition to being rapid they cannot be described by a single exponential (Garcia-Sancho et al. 1982). Further analysis of the kinetics of $Ca2^+$-dependent $^{86}Rb^+$ uptake has therefore been confined to single populations of vesicles (i.e vesicles prepared from one donor). Calculation of the half equilibration time has allowed comparison of the rates of uptake by the same population of vesicles under different conditions i.e with or without the presence of AG antibiotics.

[3.1.2] The effect of neomycin $10^{-4}$ M on the time course of Ca2+-dependent Rb+ uptake into IOVs prepared from erythrocyte drawn from subject 4.

Time course experiments were carried out using blood drawn from subject 4. $^{86}Rb^+$ uptake into IOVs has been assessed at a $Ca2^+$ concentration of $10^{-6}$ M with and without the addition of NEO $10^{-4}$ M to the incubation buffer.

There was no trend for NEO $10^{-4}$ M to inhibit $Ca2^+$-dependent $^{86}Rb^+$ uptake into vesicles prepared from blood drawn from this subject. The pooled data from 3 experiments along with the data from the individual experiments are shown in Fig 1b.

Table 1 Comparison of half times to equilibrium for $Ca2^+$-dependent $^{86}Rb^+$ uptake for individual time course experiments, using IOVs prepared from blood drawn from subject 4, with and without addition of NEO $10^{-4}$ M to the incubation buffer.

| Experiment | (T1\2 ) |
|---|---|
| 1. $Ca2^+$ $10^{-6}$ M | 1.6 min |
| $Ca2^+$ $10^{-6}$ M + NEO $10^{-4}$ M | 1.0 min |
| 2. $Ca2^+$ $10^{-6}$ M | 1.0 min |
| $Ca2^+$ $10^{-6}$ M + NEO $10^{-4}$ M | 1.25 min |
| 3. $Ca2^+$ $10^{-6}$ M | 1.0 min |
| $Ca2^+$ $10^{-6}$ M + NEO $10^{-4}$ M | 0.9 min |

Addition of NEO $10^{-4}$ M causes no large overall change in the rate of $^{86}Rb^+$ uptake into IOVs, prepared from blood drawn from subject 4. The mean half time to equilibrium with and without NEO are shown below (Table 2).

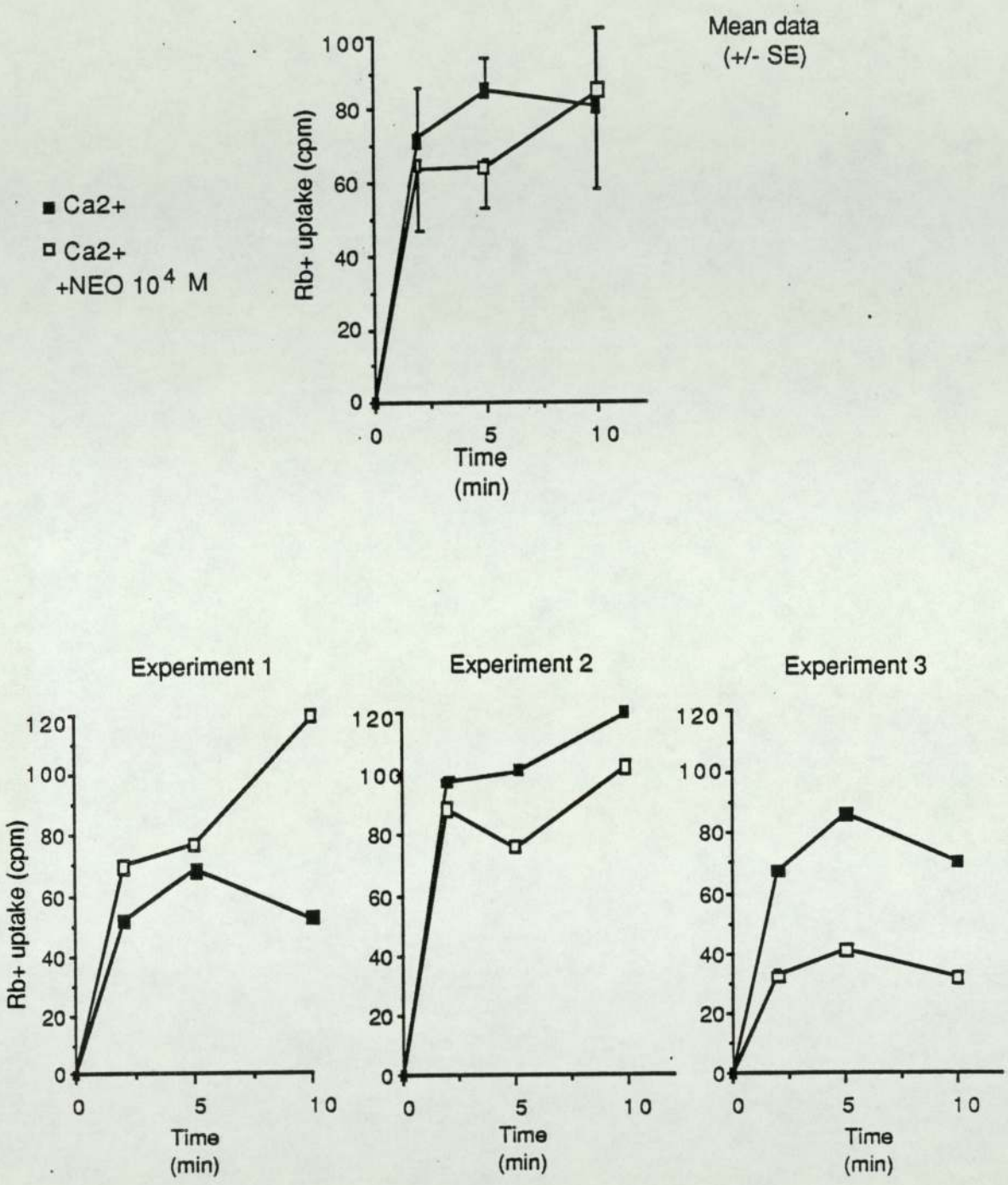


Fig 1b The effect of NEO $10^{-4}$ M on the time-course of Ca-dependent Rb uptake into IOVs prepared from erythrocytes drawn from subject 4 Ca concentration $10^{-6}$ M. The pooled data from 3 experiments along with the data from the individual experiments are shown. The uptake in the absence of Ca or NEO has been subtracted from all the values.

Table2 Comparison of mean half times to equilibrium from time course experiments 1-3 (subject 4). With and without NEO $10^{-4}$ M.

| Sample. | ( mean t1\2 ) |
|---|---|
| Ca2+ $10^{-6}$ M | 1.20 min |
| Ca2+ $10^{-6}$ M + NEO $10^{-4}$ M | 1.05 min |

[3.1.3] Effect of gentamicin 2x10-4 M

The time course of $Ca2^{+}$-dependent $^{86}Rb^{+}$ uptake into IOVs prepared from subject 4 and subject 2 erythrocytes was assessed at a $Ca2^{+}$ concentration of $10^{-6}$ M, with and without the addition of GEN $2x10^{-4}$ M to the incubation buffer. Furthermore the addition of GEN $2x10^{-4}$ M alone was also been investigated.

GEN $2x10^{-4}$ M inhibited maximal $Ca2^{+}$-dependent $^{86}Rb^{+}$ uptake in both experiments (Fig 1c). The percentage inhibition at 10 min is shown below in Table 3. Little if any reduction in the initial rate of $Ca2^{+}$-dependent $^{86}Rb^{+}$ uptake was recorded in the presence of GEN (Table 4).

Table 3 Inhibition of $Ca2^{+}$-dependent $^{86}Rb^{+}$ uptake into IOVs by GEN $2x10^{-4}$ M.

Subject 2; 76.3% at 10 min.

Subject 4; 47.0% at 10 min.

Table 4 Comparison of half time to equilibrium from individual time course experiments; with and without addition of GEN $2x10^{-4}$ M to the incubation buffer.

| | | |
|---|---|---|
| Subject 2; | $Ca2^{+}$ $10^{-6}$ M | 1.5 min |
| | $Ca2^{+}$ $10^{-6}$ M + $2x10^{-4}$ M GEN. | 1.6 min |
| Subject 4; | $Ca2^{+}$ $10^{-6}$ M | 1.25 min |
| | $Ca2^{+}$ $10^{-6}$ M + $2x10^{-4}$ M GEN. | 1.0 min |

GEN $2x10^{-4}$ M alone produced an uptake of $^{86}Rb^{+}$ into the IOVs in both of these experiments. As with $Ca2^{+}$-dependent $^{86}Rb^{+}$ uptake equilibrium was achieved by 10 min (Fig 1a).

The initial rate of $^{86}Rb^{+}$ uptake stimulated by GEN , for subject 4's vesicles was faster than that observed for subject 2's vesicles (see Table 5).

Table 5 Comparison of half time to equilibrium from individual time course experiments for GEN stimulated $^{86}Rb^{+}$ uptake. (Equilibrium =10 min ).

| | |
|---|---|
| Subject 2; | 3.5 min |
| Subject 4; | 0.75 min. |

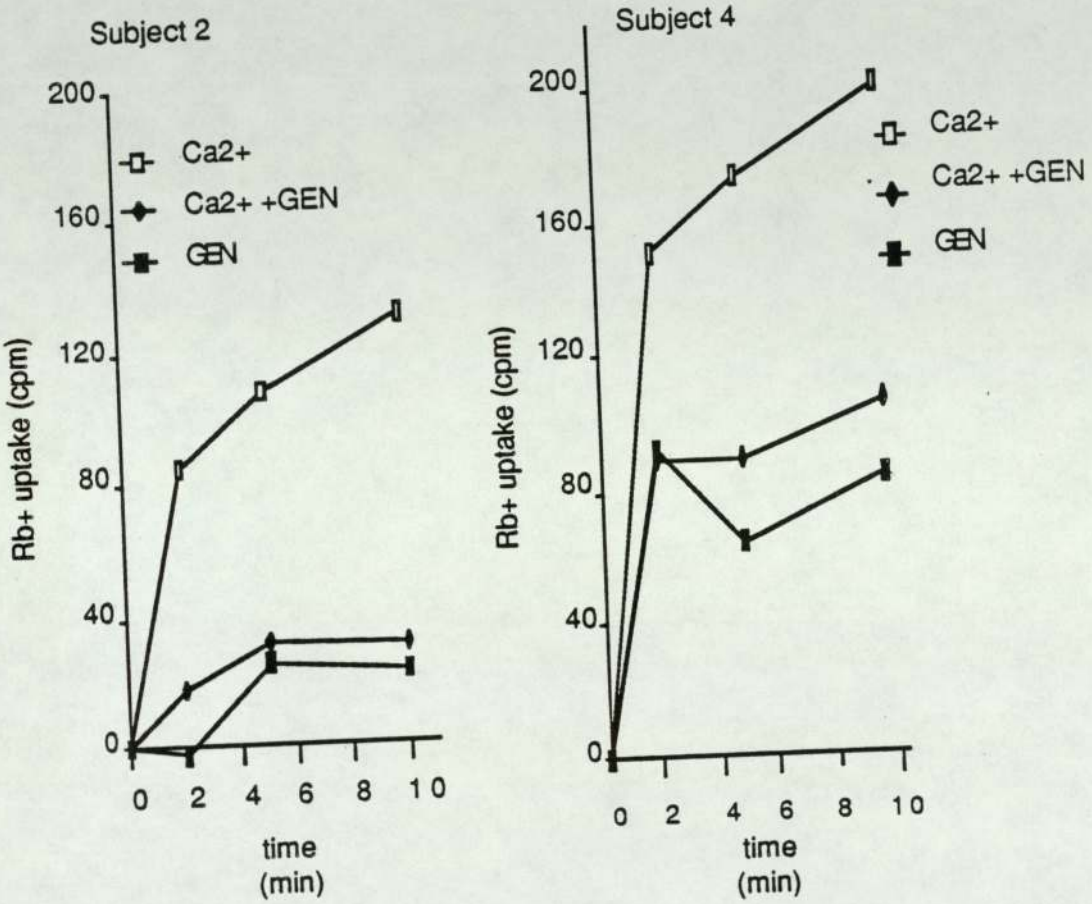


Fig 1c Effect of gentamicin on the time course of Ca-dependent Rb uptake into IOVs prepared from subjects 2 and 4.

Ca concentration $10^{-6}$ M, GEN concentration $2x10^{-4}$ M

The time course of the Rb uptake brought about by GEN alone has also been assessed.

## [3.2] Effect of aminoglycoside antibiotics on $Ca2^+$-dependent $Rb^+$ uptake into erythrocyte IOVs at equilibrium.

### [3.2.1] Effect of $10^{-4}$ M neomycin

The effect of neomycin (NEO) on $Ca2^+$-dependent $^{86}Rb^+$ uptake into erythrocyte IOVs at equilibrium (10 min, see 3.1.1) was examined at a drug concentration of $10^{-4}$ M. Figure 2a shows the effect of NEO $10^{-4}$ M on the dose response curve for $Ca2^+$-dependent $^{86}Rb^+$ uptake into erythrocyte IOVs

NEO $10^{-4}$M alone produced an uptake of $^{86}Rb^+$ into the IOVs (35% of that seen with $5x10^{-5}$ M $Ca2^+$). Despite this effect of the AG alone, partial inhibition of $Ca2^+$-dependent $^{86}Rb^+$ uptake was seen when the $Ca2^+$ response was greater than that seen with NEO alone. At concentrations of $Ca2^+$ producing a maximal response this inhibition appears to be overcome.

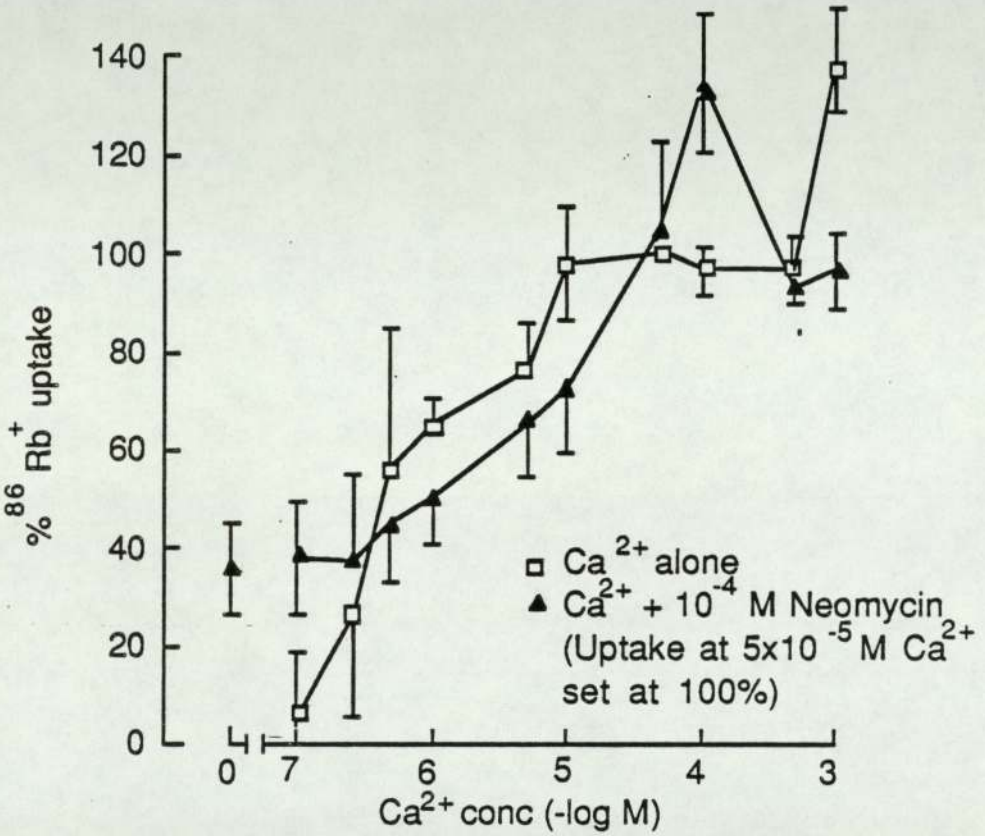


Fig 2a The effect of NEO $10^{-4}$ M on the dose response curve for Ca-dependent Rb uptake into IOVs. This figure is composed of data pooled from 8 experiments and IOVs prepared from 3 different donors (subjects 1-3) Mean data +/- SE. The Rb uptake at $5x10^{-5}$ M Ca has been set at 100%.

[3.2.2] Effect of $10^{-3}$ M neomycin.

The effect of NEO on $Ca2^+$-dependent $^{86}Rb^+$ uptake into erythrocyte IOVs at equilibrium was examined at a drug concentration of $10^{-3}$ M ( Fig 2b). The antagonism of $Ca2^+$-dependent $^{86}Rb^+$ uptake produced by NEO was more evident at this concentration of drug. Significant inhibition of $Ca2^+$ dependent uptake was observed at $Ca2^+$ concentrations known to produce a maximal response in addition to those giving a submaximal response in the absence of AGs ( Table 6).

Table 6 Percentage inhibition of $Ca2^+$-dependent $^{86}Rb^+$ uptake into one-step IOVs by NEO $10^{-3}$ M. P values indicate significance of inhibition by drug (paired t-test)

| $(Ca2^+)$ | % inhibition | P |
|---|---|---|
| $10^{-6}$ M | 82.1 | < 0.05 |
| $10^{-5}$ M | 81.5 | < 0.01 |
| 5 x $10^{-5}$ M | 58.2 | < 0.05 |
| $10^{-4}$ M | 56.6 | < 0.05 |

In these experiments NEO alone produced little stimulation of $^{86}Rb^+$ uptake allowing the inhibitory effect of the drug on this transport system to be clearly seen. Figure 2b is composed of data pooled from 3 different blood donors ( subjects 1, 2 and 7).

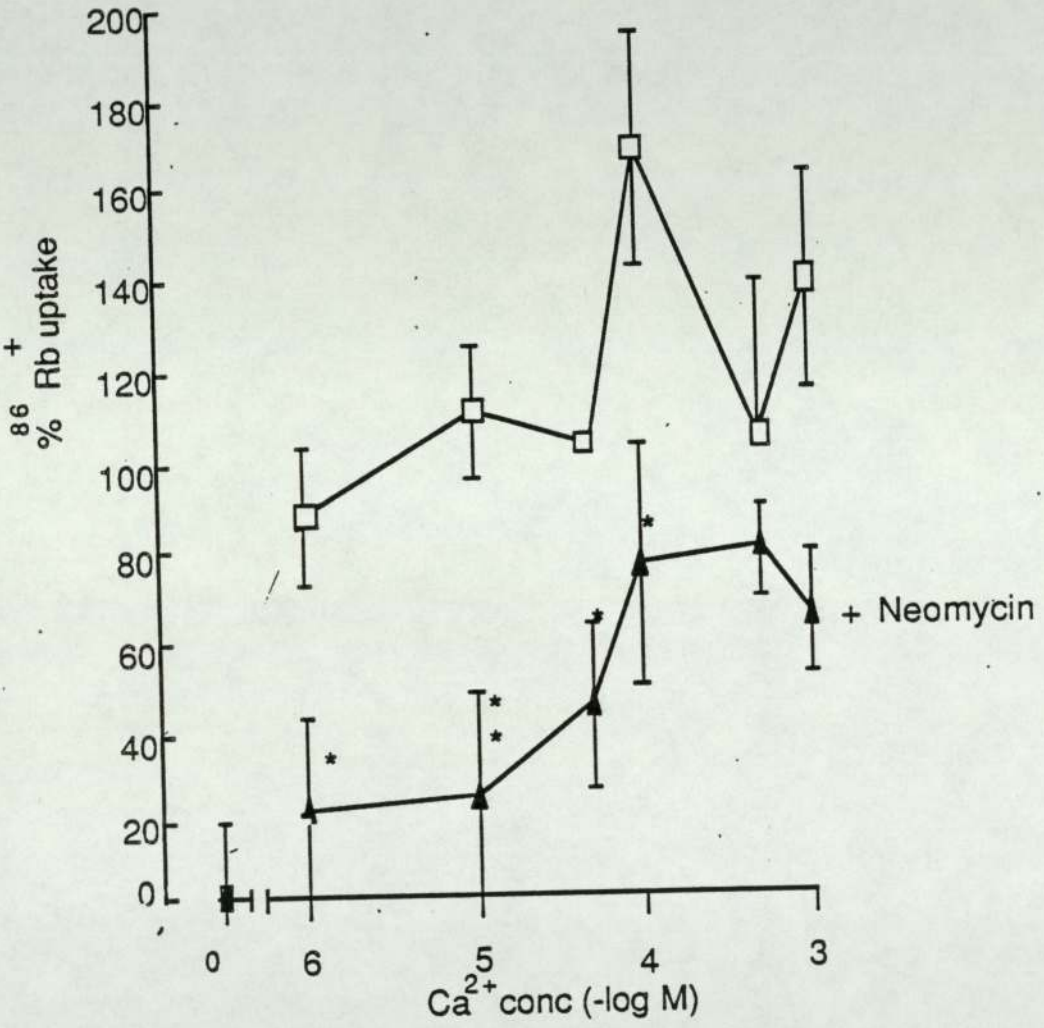


Fig 2b The effect of NEO $10^{-3}$ M on dose response curve for Ca-dependent Rb uptake into IOVs. This figure is composed of data pooled from 3 different blood donors. (subjects 1,2 and 7) Mean data +/- SE.
The uptake in the absence of Ca or NEO has been subtracted from all values.
* indicates significant reduction in Rb uptake.
(paired t test, for p values see Table 6).

[3.2.3] Effect of 10-4 M neomycin on Ca2+-dependent Rb+ uptake into IOVs prepared from blood drawn from subject 4.

The effect of NEO $10^{-4}$ M on the dose response curve for $Ca2^{+}$-dependent $^{86}Rb^{+}$ uptake into IOVs at equilibrium was examined using IOVs prepared from blood drawn from subject 4 (Fig 2c).

NEO $10^{-4}$M alone produced a large $^{86}Rb^{+}$ uptake into IOVs prepared from blood drawn from subject 4 (93% of that seen with $5x10^{-5}$ M $Ca2^{+}$). Any inhibition of $Ca2^{+}$-dependent $^{86}Rb^{+}$ uptake into the IOVs was totally masked by this large NEO-stimulated $^{86}Rb^{+}$ uptake. As this response was so different from that observed for vesicles prepared from subjects 1-3 erythrocytes ( Fig 2a), the data from subject 4 have been considered separately.

To determine whether this large NEO stimulated uptake was due to calcium contamination, calcium concentrations in the incubation buffer were measured (Table 7) by means of atomic absorption spectroscopy ( see Methods 2.4).

Table 7 Calcium concentrations in the incubation buffer, with and without NEO $10^{-4}$M.

| | [Ca] ppm |
|---|---|
| IOV incubation buffer | 1.14 |
| IOV incubation buffer + NEO $10^{-4}$ M | 1.03 |

Addition of NEO $10^{-4}$ M to the incubation buffer produced no measurable increase in calcium concentration.

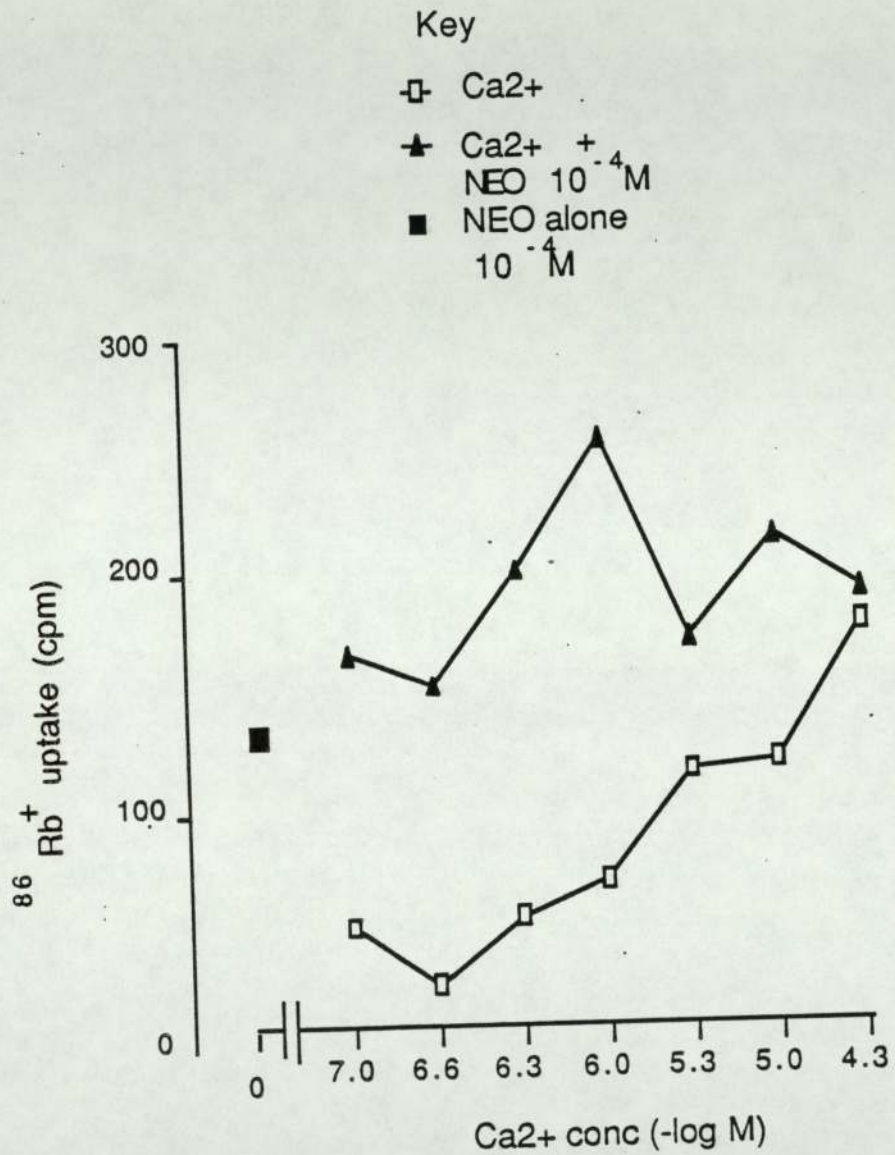


Fig 2c The effect of NEO $10^{-4}$ M on dose response curve for Ca-dependent Rb uptake into IOVs. This figure shows the data from one experiment using IOVs prepared from erythrocytes drawn from subject 4. The uptake in the absence of Ca or NEO has been subtracted from all values.

[3.2.4] Effect of 2x10-4 M gentamicin.

As with NEO (3.2.1-3.2.3) the effect of GEN $2x10^{-4}$ M on the dose response curve for $Ca2^{+}$-dependent $^{86}Rb^{+}$ uptake into IOVs at equilibrium was investigated (Fig 2d).

GEN $2x10^{-4}$ M antagonised the effect of $Ca2^{+}$ in the concentration range $5x10^{-7}$-$5x10^{-6}$ M (Table 8). This effect was overcome at high $Ca2^{+}$ concentrations.

Table 8 Percentage inhibition of $Ca2^{+}$-dependent $^{86}Rb^{+}$ uptake into one-step IOVs by $2x10^{-4}$ M GEN. P values indicate the significance of inhibition by $2x10^{-4}$ M GEN (paired t-test).

| [Ca2+] | % Inhibition | p |
|---|---|---|
| $5x10^{-7}$ M | 51.1 | < 0.05 |
| $10^{-6}$ M | 81.0 | < 0.05 |
| $5x10^{-6}$ M | 57.1 | < 0.05 |

No statistically significant inhibition of $Ca2^{+}$-dependent $^{86}Rb^{+}$ uptake was observed at any other concentration of $Ca2^{+}$ tested.
GEN $2x10^{-4}$ M alone stimulated $^{86}Rb^{+}$ uptake into the vesicles. A mean $^{86}Rb^{+}$ uptake of 25.2% (S.E +/-9.1) of that obtained with $5x10^{-5}$ M $Ca2^{+}$ was recorded.

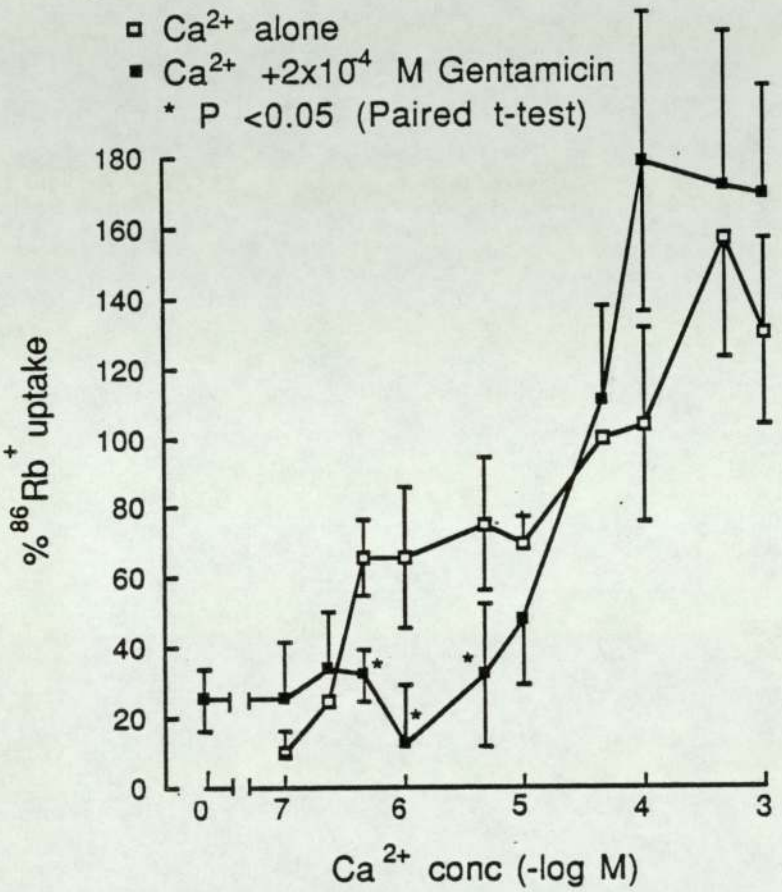


Fig 2d The effect of GEN $2x10^{-4}$M on dose response curve for Ca-dependent Rb uptake into IOVs. This figure is composed of data pooled from 6 experiments and IOVs prepared from 4 different blood blood donors (subjects 1,2,5 and 6). Each point represents the mean (+/- SE) of at least 3 experiments. The uptake of Rb in the absence of Ca or GEN ($4x10^{-4}$M EGTA present) has been subtracted from all values. The Rb uptake at $5x10^{-5}$M Ca has been set at 100%, as this Ca concentration was used in each experiment. * Indicates significant reduction in Rb uptake (Paired t test, for p values see Table 8).

[3.2.5] Effect of gentamicin on Ca2+-dependent Rb+ uptake into IOVs prepared from blood drawn from subject 4 .

The effect of GEN $2x10^{-4}$ M on the dose response curve for $Ca2^+$-dependent $^{86}Rb^+$ uptake into IOVs prepared from blood drawn from subject 4 was examined (Fig 2e). GEN $2x10^{-4}$ M alone produced a large $^{86}Rb^+$ uptake into the IOVs (89% of that seen with $5x10^{-5}$ M $Ca2^+$). As this response was so different to that observed for the vesicles prepared from erythrocytes drawn from subjects 1,2,5 and 6. (see Fig 2d) the data were not pooled with the data from these other subjects. No inhibition of $Ca2^+$-dependent $^{86}Rb^+$ uptake was observable in this case, in fact the effects of GEN and $Ca2^+$ appeared as if they may be additive.

[3.2.6] Effect of subtraction of AG-stimulated Rb+ uptake into IOVs on Ca2+ dose response curves at equilibrium (fig 2f-g)

Inhibition of $Ca2^+$-dependent $^{86}Rb^+$ uptake into IOVs by AGs appeared to be masked by AG-stimulated $^{86}Rb^+$ uptake. In order to estimate the degree of inhibition, AG-stimulated $^{86}Rb^+$ uptake has been subtracted from each value for Figs 2a and 2d, the resulting dose response curves are shown in Figs 2f and 2g. In both cases inhibition of $Ca2^+$-dependent $^{86}Rb^+$ uptake can clearly be seen below $Ca2^+$ concentrations of $10^{-5}$ M.

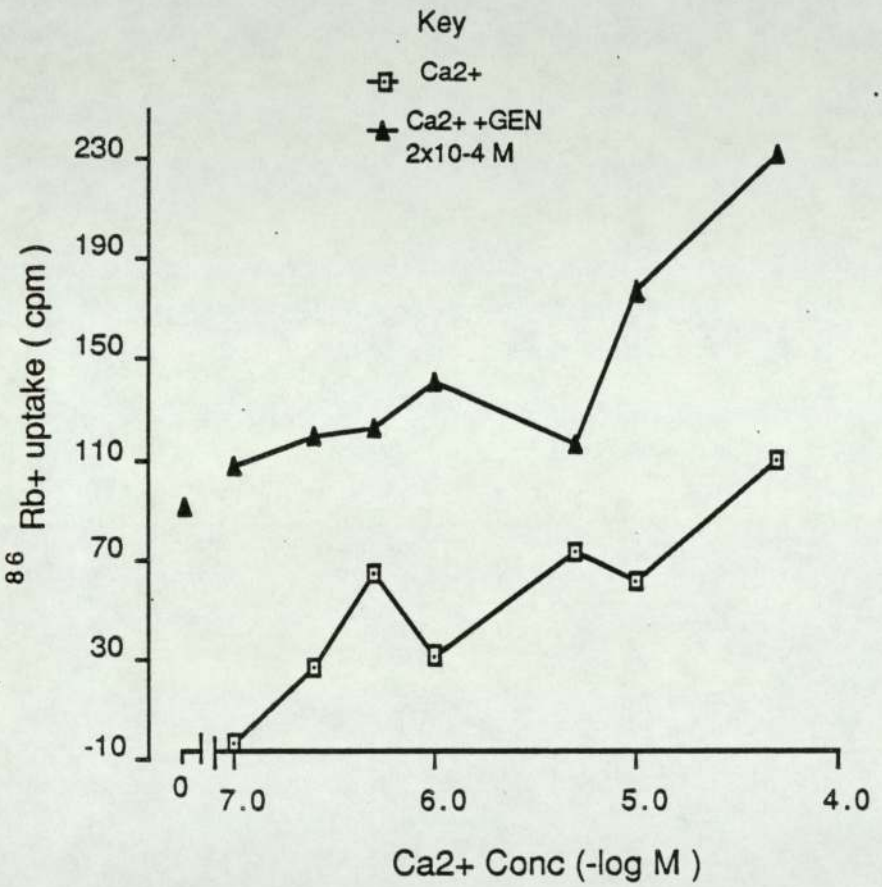


Fig 2e The effect of GEN on Ca-dependent Rb uptake into IOVs prepared from blood drawn from subject 4. GEN concentration $2x10^{-4}$ M. This figure shows the data from one experiment. The uptake in the absence of Ca or GEN has been subtracted from all values.

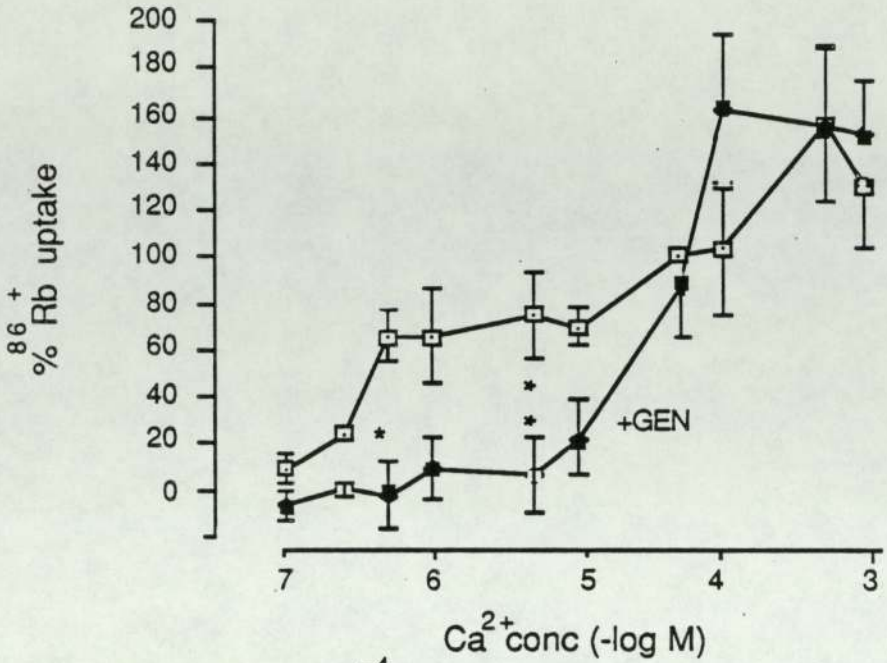


Fig 2f Effect of GEN 2x $10^{-4}$ M on Ca-dependent Rb uptake after subtraction of uptake due to GEN alone. (Mean data +/- SE).
Significant inhibition of Ca-dependent Rb uptake by GEN
* p<0.05, ** p<0.01 (Paired t test).

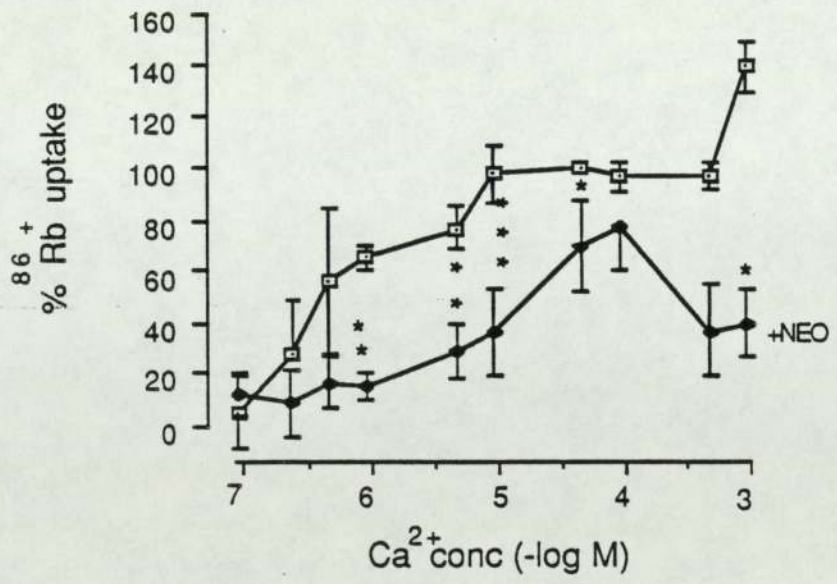


Fig 2g Effect of NEO $10^{-4}$ M on Ca -dependent Rb uptake following subtraction of uptake due to NEO alone. (Mean data +/- SE).
Significant inhibition of Ca-dependent Rb uptake by NEO
* p<0.05, ** p<0.01, P<0.001 (Paired t test).

## [3.3] Effect of aminoglycoside antibiotics on valinomycin induced $Rb^+$ uptake into one-step inside-out erythrocyte vesicles.

### [3.3.1] Effect of neomycin

In order to determine whether inhibition of $Ca2^+$-dependent $^{86}Rb^+$ uptake into IOVs at equilibrium by AGs was specific, the effect of NEO on $^{86}Rb^+$ uptake into IOVs induced by valinomycin (VAL) (a $K^+$ ionophore) was investigated.

NEO, even at a concentration of $10^{-3}$ M had little or no effect on the uptake of $^{86}Rb^+$ induced by VAL $4x10^{-6}$ M (Fig 3a). This concentration of VAL has previously been reported by other workers to bring about maximal uptake of $^{86}Rb^+$ into IOVs (Alvarez et al, 1986c).

$Ca2^+$ $5x10^{-5}$ M produced $^{86}Rb^+$ uptake into the IOVs that was 43.9% of that observed with VAL alone. This gives an estimation of the proportion of the total vesicular space which is stimulatable by $Ca2^+$ i.e. everted vesicles with $Ca2^+$-dependent $K^+$channels. There was no additive effect between $5x10^{-5}$ M $Ca2^+$ and $4x10^{-6}$ M VAL.

The data obtained in these experiments for NEO-stimulated $^{86}Rb^+$ uptake into IOVs was of the same order as previously obtained (see Results 3.2.1-3.2.2). Both sets of results are shown for comparison in Table 9.

Table 9 Comparison of NEO-stimulated $^{86}Rb^{+}$ uptake into IOVs with that observed in previous experiments (3.2.1 and 3.2.2), expressed as a percent of $^{86}Rb^{+}$ uptake by $5x10^{-5}$ M $Ca2^{+}$.

| | |
|---|---|
| NEO $10^{-4}$ M | 30.3 % |
| NEO $10^{-4}$ M ( 3.2.1 ) | 35.0 % |
| NEO $10^{-3}$ M | 4.5 % |
| NEO $10^{-3}$ M ( 3.2.2 ) | 0.6 % |

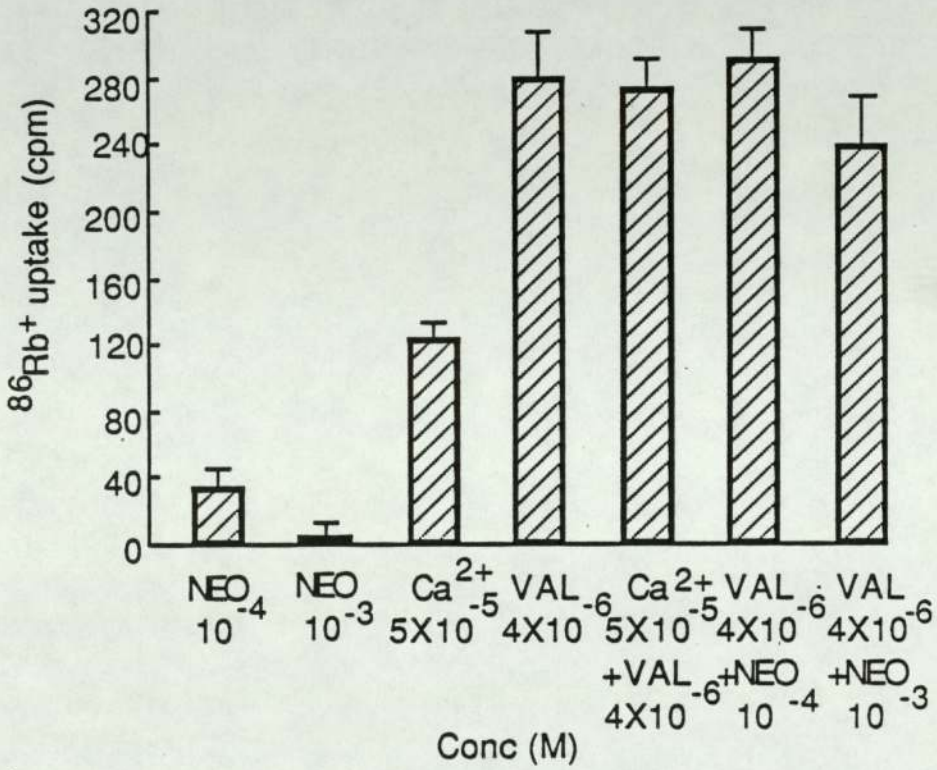


Fig 3a Effect of NEO on valinomycin induced Rb uptake into IOVs at equilibrium. This figure was prepared from data pooled from 6 experiments. Samples including Ca, VAL or VAL+Ca were included in each experiment. For all other samples n=3. Mean data +/- SE. Subjects used; 1,2,5 and 7. Uptake in the absence of Ca, NEO or VAL has been subtracted from all values. Incubation time = 10 min.

## [3.4] Dose response curves of AG-stimulated $^{86}Rb^{+}$ uptake into IOVs

During the investigation of the effect of AGs on $Ca2^{+}$-dependent $^{86}Rb^{+}$ uptake into IOVs at equilibrium, an AG-stimulated $^{86}Rb^{+}$ uptake into the IOVs was observed (3.2.3 and 3.2.5). It was felt that this action of the drugs merited further investigation. Dose response curves were prepared for the AGs NEO and GEN using IOVs prepared from blood drawn from subjects 2 and 4 ( subject 4's IOVs having previously been recorded as producing a larger than average response to AGs, see 3.2.3 and 3.2.5). The uptake was measured at equilibrium i.e 10 min. Previously half time to equilibrium for GEN alone ($2x10^{-4}$ M) were recorded as 3.5 min and 0.75 min for each subject respectively (3.1.3).

### [3.4.1] Neomycin ( subject 2 )

The concentration of NEO required to produce half of the maximal activation ($EC_{50}$) was $9x10^{-6}$ M (Fig 4a).

### [3.4.2] Neomycin ( subject 4 )

The concentration of NEO required to produce half of the maximal activation ($EC_{50}$) was $3.8x10^{-6}$ M (Fig 4b).

NEO-stimulated $^{86}Rb^{+}$ uptake at $10^{-4}$ M =91.8% of that produced by $10^{-5}$ M $Ca2^{+}$ ( see Results 3.5). It thus appears that not only does the magnitude of the response vary between IOVs prepared from blood drawn from different subjects but so does the dose required to produce a half maximal response.

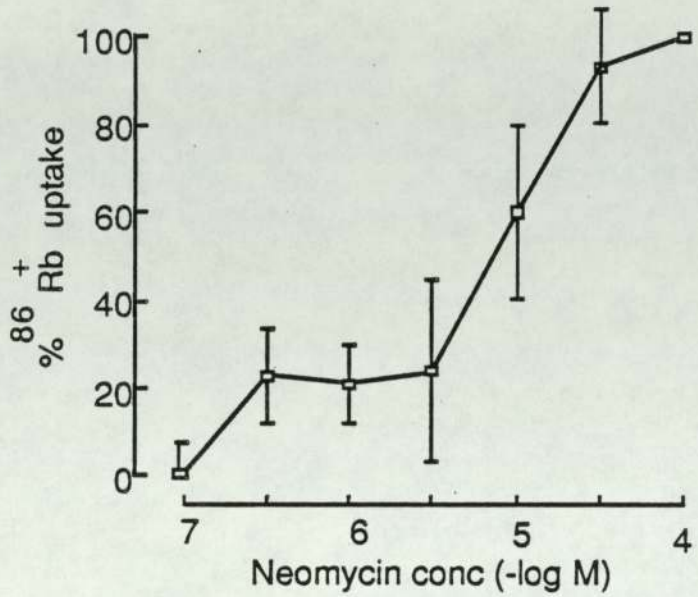


Fig 4a dose response of NEO-stimulated Rb uptake in subject 2 ( n=3; mean +/-SE).

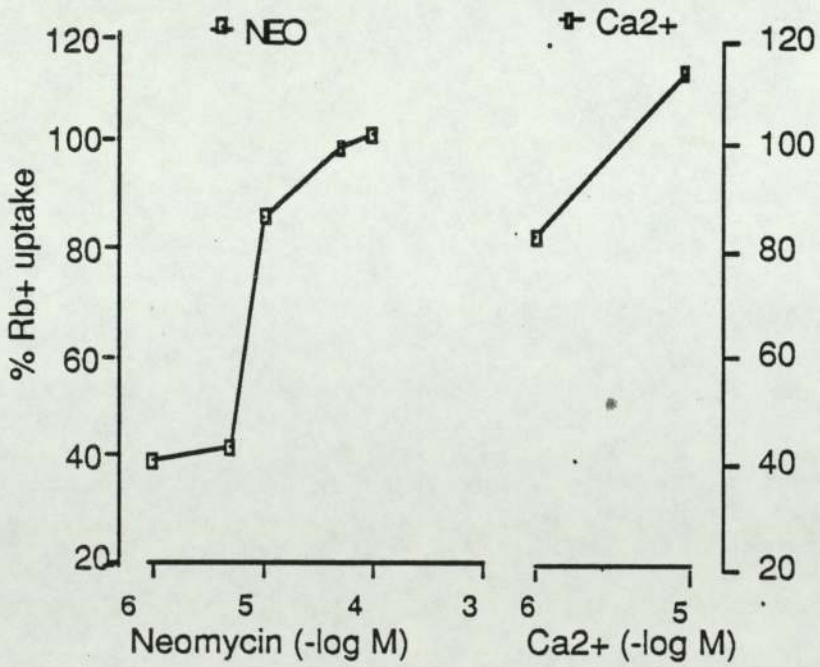


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Fig 4b Dose response of NEO-stimulated Rb uptake in subject 4 (n=1), Ca-stimulated responses are shown for comparison. NEO $10^{-4}$ M = 100% uptake.

[3.4.3] Gentamicin ( subject 2 )

The concentration of GEN required to produce half of the maximal activation ($EC_{50}$) was $2x10^{-5}$ M.

Thus there appears to be a moderate difference in potency between NEO and GEN with regard to the stimulation of $^{86}Rb^{+}$ uptake into IOVs prepared from blood drawn from subject 2, NEO being the more potent by a factor of 2.2.

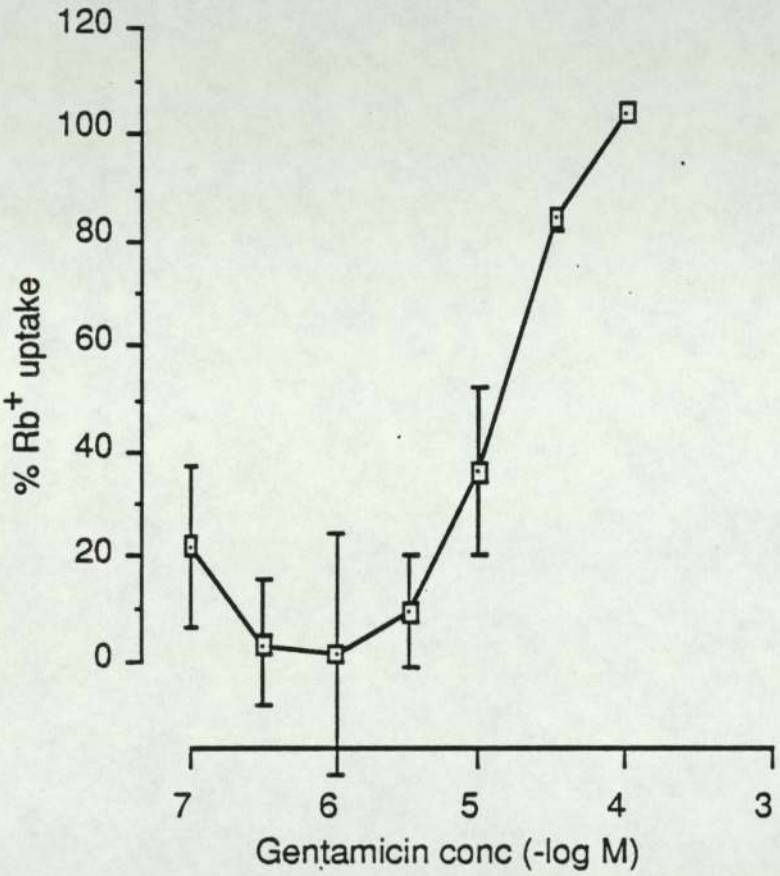



Fig 4c
Dose response curve for GEN-stimulated Rb uptake into IOVs prepared from subject 2 after 10 min, (n=3; mean +/- S.E)

## [3.5] Effect of aminoglycoside antibiotics compared with that of $Ca2^+$ on $^{86}Rb^+$ uptake into IOVs prepared from erythrocytes drawn from different donors.

### [3.5.1] Comparison of IOVs prepared from blood drawn from subjects 2 and 4

As previously stated (3.2.3 and 3.2.5) the magnitude of the AG-stimulated $^{86}Rb^+$ uptake into IOVs varied depending on the source of the blood from which the IOVs were prepared. The object of the experiments described in this section was to examine this phenomenon more closely. The magnitude of the AG-stimulated response was compared with that of the $Ca2^+$ response for subjects 2 and 4 (Fig 5a).

Figure 5a shows that the uptake of $^{86}Rb^+$ stimulated by GEN was maximal at a drug concentration of $10^{-4}$ M and varied depending on the source of erythrocytes from which the IOVs were prepared. The GEN-stimulated $^{86}Rb^+$ uptake into IOVs prepared from blood cells drawn from subject 4 was much greater than it was into those IOVs prepared from blood cells drawn from subject 2. $Ca2^+$-dependent $^{86}Rb^+$ uptake did not show the same inter-subject variability. This indicates that the variability in the magnitude of the AG-stimulated response is not due to a difference in the proportion of inside-out to rightside-out vesicles between different preparations of IOVs. If this were the case the magnitude of the $Ca2^+$ response would also show intersubject variability.

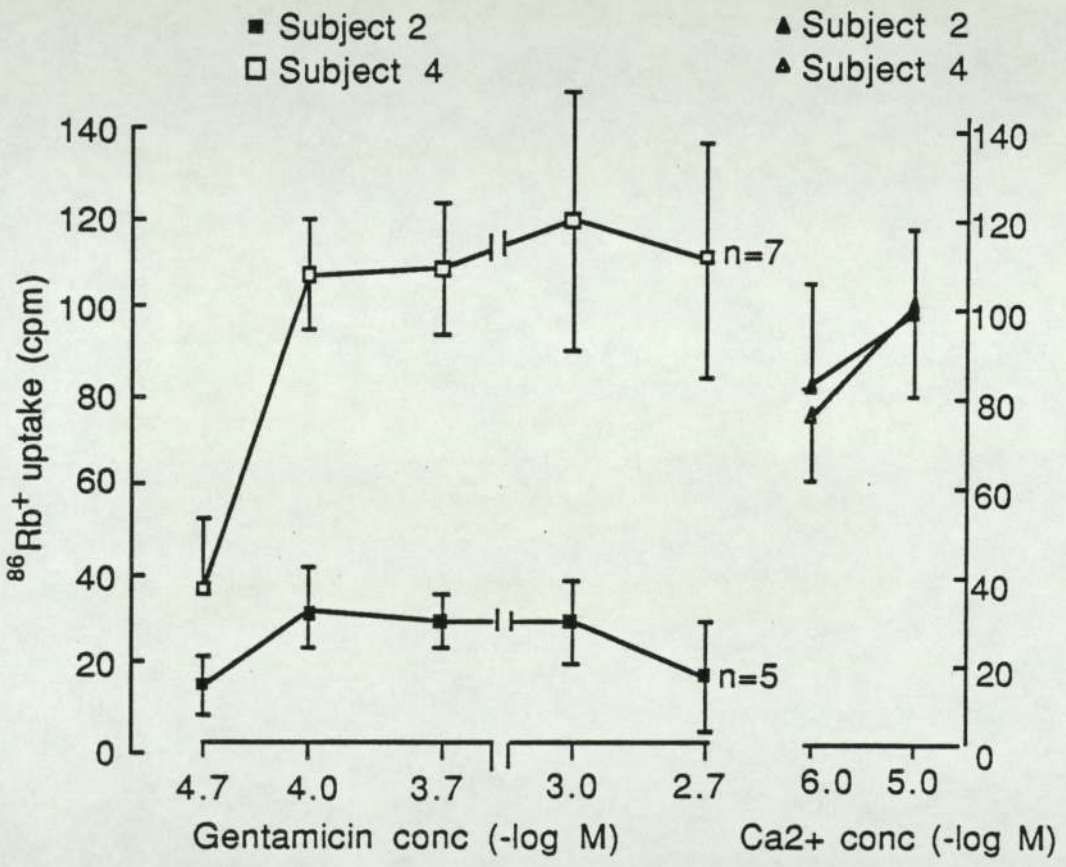



Fig 5a. GEN-stimulated Rb uptake compared with Ca-dependent Rb uptake for vesicles prepared from subject 2 and subject 4 erythrocytes. Incubation time =10 min. (Mean +/- S.E).

Inter-individual variation in AG-stimulated $^{86}Rb^{+}$ uptake into IOVs has been further investigated by retrospectively examining AG-stimulated $^{86}Rb^{+}$ uptake. Maximal AG-stimulated $^{86}Rb^{+}$ uptake was compared with maximal $Ca2^{+}$-dependent $^{86}Rb^{+}$ uptake (Fig5b-c).

[3.5.2] Gentamicin-stimulated Rb+ uptake compared with Ca2+-dependent Rb+ uptake.

GEN ($2x10^{-4}$ M )-stimulated $^{86}Rb^{+}$ uptake was compared with $Ca2^{+}$ ($10^{-5}$M )-dependent $^{86}Rb^{+}$ uptake into IOVs prepared from blood drawn from 3 individual donors ( subjects 1, 2, and 4 ) (Fig 5b).

Variability between subjects in the maximal response to Ca (range 99.1-145.8 cpm) was less marked than variability in the maximal response to GEN (range 20.0-107.1 cpm). Most striking was the variability between subjects in the relative magnitude of the Ca and GEN responses.

[3.5.3] Neomycin-stimulated Rb+ uptake compared with Ca2+-dependent Rb+ uptake .

NEO ($10^{-4}$ M)-stimulated $^{86}Rb^{+}$ uptake was compared with $Ca2^{+}$ ($10^{-5}$ M)-dependent $^{86}Rb^{+}$ uptake into IOVs prepared from blood drawn from 5 individual donors (subjects1-5) ( Fig 5c).

As for GEN (see Results 3.5.2) there was little variability between subjects in the maximal response to Ca ( range 121.0 -167.5 cpm). A greater degree of inter-individual variation was however observed for NEO-stimulated $^{86}Rb^{+}$ uptake( range 31.0-160.0 cpm).

Once again the largest AG-stimulated uptake was observed with IOVs prepared from subject 4's erythrocytes.

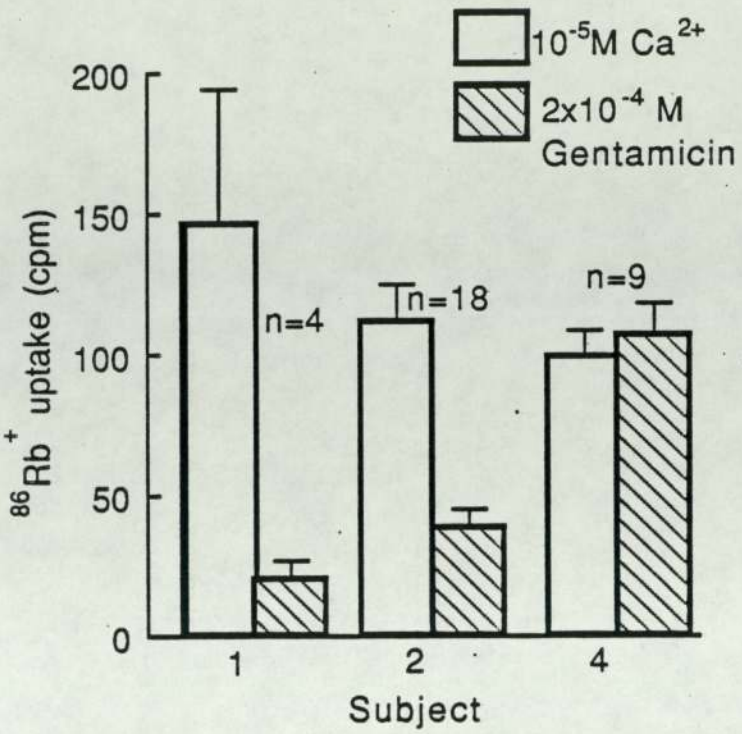



Fig 5b Intersubject variation in GEN-stimulated Rb+ uptake into IOVs after a 10 min incubation in comparison with that seen with a concentration of Ca known to cause maximal Rb+ uptake.

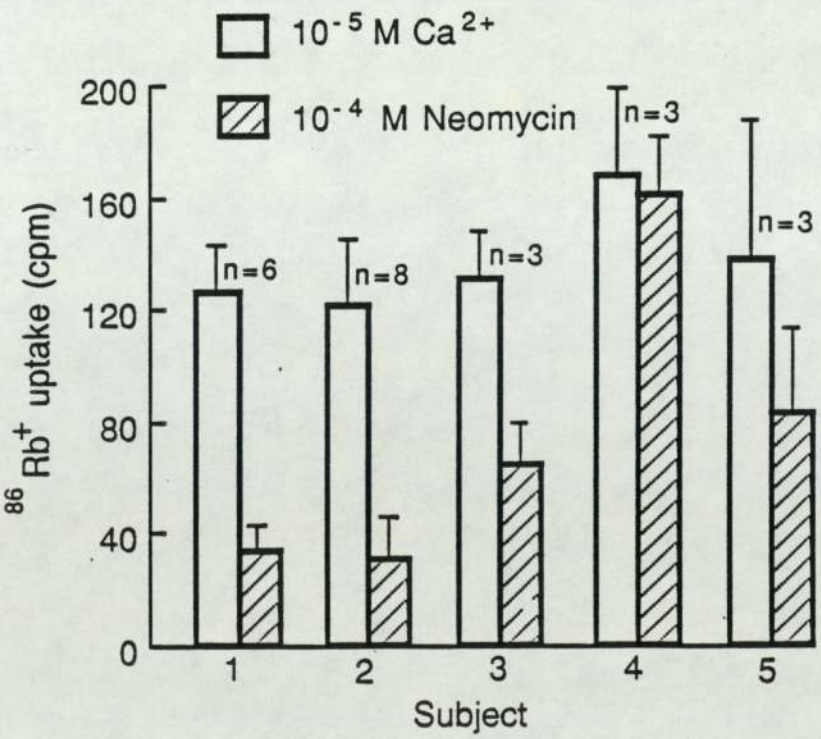



Fig 5c Inter subject variation in NEO-stimulated Rb+ uptake into IOVs after 10 min in comparison with Ca-dependent Rb+ uptake.

[3.5.4] Netilimicin-stimulated Rb+ uptake into one-step IOVs compared with Ca2+-dependent Rb+ uptake

Netilimicin (NET) ($10^{-5}$-$10^{-3}$ M )-stimulated $^{86}Rb^{+}$ uptake was compared with $Ca2^{+}$ ($10^{-5}$ M)-dependent $^{86}Rb^{+}$ uptake into IOVs prepared from blood drawn from subject 4 (Fig 5d). As with NEO and GEN, NET ($10^{-4}$ M) stimulated $^{86}Rb^{+}$ uptake into the IOVs of an order similar to that produced by $Ca2^{+}$ $10^{-5}$ M (maximal response).

To ensure NET-stimulated $^{86}Rb^{+}$ uptake was not due to calcium contamination the calcium concentration in the incubation buffer was measured by means of atomic absorption. The results are shown in the Table 10.

Table 10 Measurement of calcium concentration in incubation buffer

| | [Ca] ppm |
|---|---|
| No $Ca2^{+}$ or NET added. | 0.81 |
| $Ca2^{+}$ $10^{-5}$ M | 10.27 |
| NET $10^{-5}$ M | 0.79 |
| NET $10^{-4}$ M | 0.84 |
| NET $10^{-3}$ M | 1.57 |

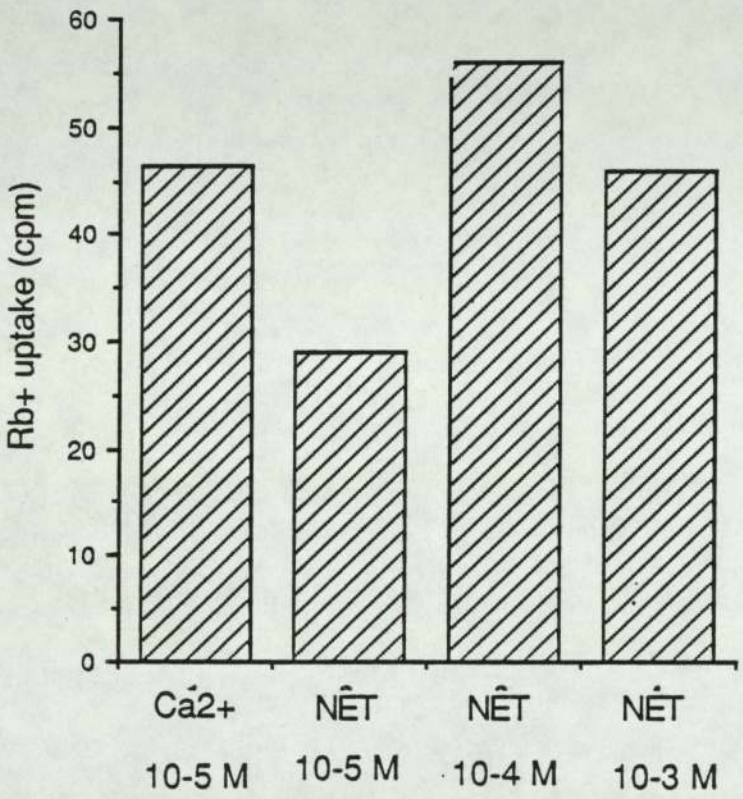



Fig 5d NET-stimulated Rb uptake, subject 4. n=1

## [3.6] Effect of pharmacological inhibitors of membrane ion transport on $Ca2^+$-dependent $Rb^+$ uptake and AG-stimulated $^{86}Rb^+$ uptake into IOVs.

In an effort to characterise AG-stimulated $^{86}Rb^+$ uptake into IOVs various inhibitors of $K^+$ transport in erythrocyte plasma membranes (see Introduction 1.4.2) were utilised.

### [3.6.1] Effect of quinine ($10^{-3}$ M)

Quinine is a well documented inhibitor of $Ca2^+$-dependent $K^+$ transport in a variety of erythrocyte preparations, including both whole cell and membrane preparations (see Introduction 1.4.2). This drug was used to investigate the relationship of AG-stimulated $^{86}Rb^+$ uptake into IOVs with $Ca2^+$-dependent $K^+$ transport.

Quinine ($10^{-3}$ M) when present in the incubation buffer prior to addition of the IOVs, inhibited $Ca2^+$-dependent $^{86}Rb^+$ uptake into IOVs irrespective of the donor of the erythrocytes from which the vesicles were prepared (Figs 6 a-c). This is in agreement with the data published by Garcia-Sancho et al , (1982).

It has previously been shown that IOVs prepared from the blood of subject 4 produced a relatively large AG-stimulated $^{86}Rb^+$ uptake in comparison with the other subjects in this study ( see Results 3.5 ) The data for GEN-stimulated $^{86}Rb^+$ uptake shown in Figs 6b and 6c confirms this observation.

Quinine ($10^{-3}$ M) did not inhibit AG-stimulated $^{86}Rb^+$ uptake whether stimulated by NEO ( Fig 6a ) or GEN. The source of the blood from which the IOVs were prepared had no bearing on the issue, as no inhibition by quinine of GEN-stimulated $^{86}Rb^+$ uptake into IOVs

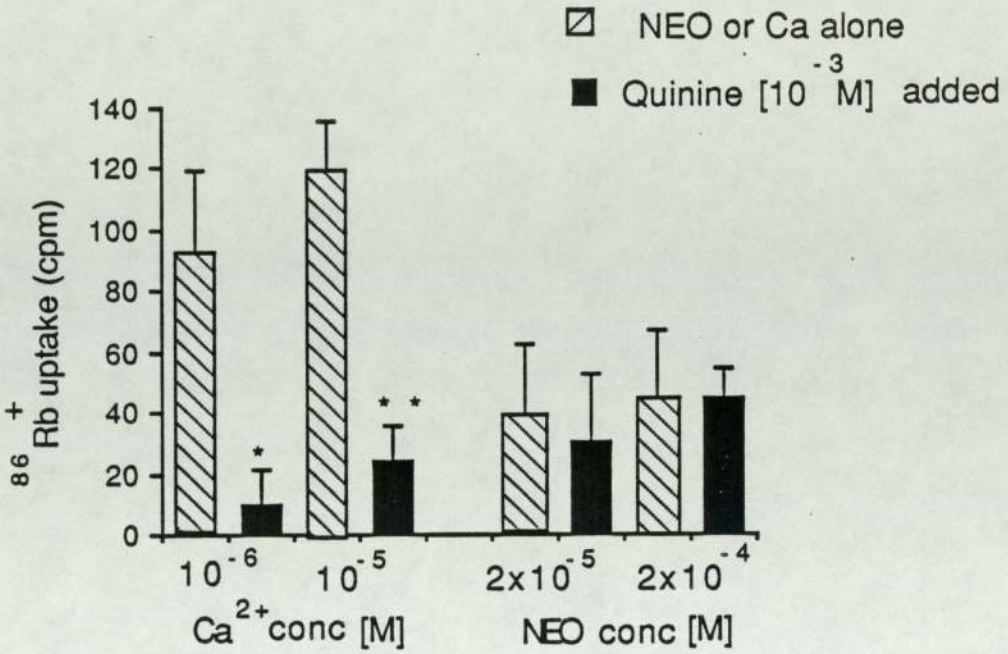



Fig 6a The effect of quinine on Ca-dependent and NEO-stimulated Rb uptake into IOVs at equilibrium. The IOVs were prepared from blood drawn from subject 2, the mean data from three separate experiments is shown (+/- S.E.).** P<0.01, * P<0.05 (Paired t-test) Statisticaly significant reduction in Rb uptake with quinine added compared to control.

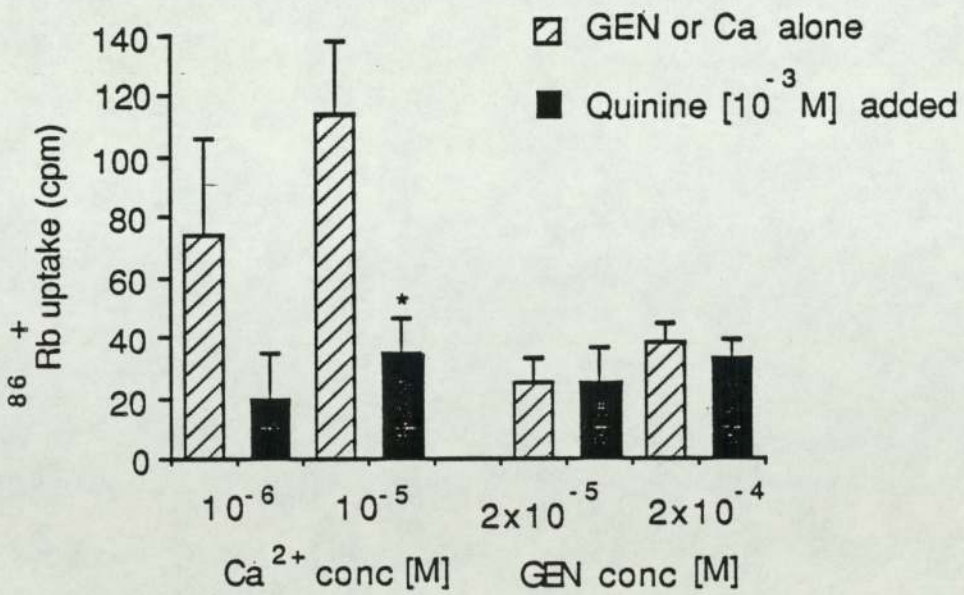



Fig 6b The effect of quinine on Ca-dependent and GEN-stimulated Rb uptake into IOVs at equilibrium. Mean data is shown from 3 separate experiments using IOVs prepared from blood drawn from subject 2 (+/- S.E.).* P< 0.05 (Paired t-test) statisticaly significant reduction in Ca-dependent Rb uptake with quinine added to incubation buffer compared to controls.

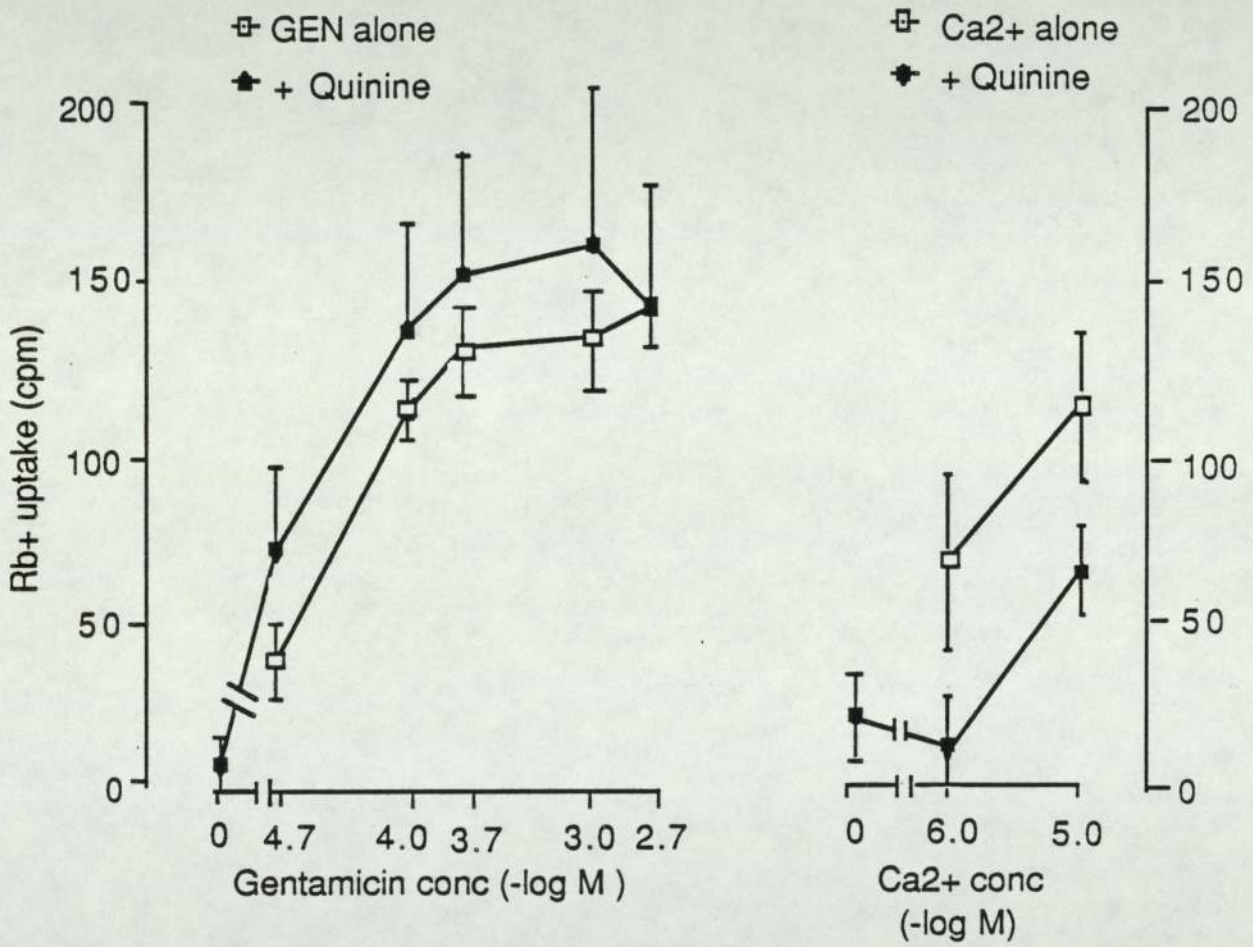



Fig 6c The effect of quinine on Ca-dependent and GEN-stimulated Rb uptake into IOVs prepared from blood drawn from subject 4. (Mean data, n=3, +/- S.E.).

from either subject 2 or subject 4 was observed (Figs 6b and 6c). These results argue against AG-stimulated $^{86}Rb^{+}$ uptake into IOVs being due to activation of $Ca2^{+}$-dependent $K^{+}$ channels.

[3.6.2] Effect of ouabain ($10^{-3}$ M) and furosemide ($10^{-3}$ M) on gentamicin-stimulated and Ca2+-dependent Rb+ uptake into IOVs.

Ouabain (OUA) and furosemide (FUR) have been documented as inhibiting a range of $K^{+}$ transport systems in the human erythrocyte plasma membrane (see Introduction 1.4.3-1.4.4).

When added to the incubation buffer prior to addition of the IOVs neither OUA($10^{-3}$ M) (Fig 6d) nor FUR ($10^{-3}$) (Fig 6e) produced any inhibition of GEN-stimulated $^{86}Rb^{+}$ uptake. Furthermore no significant inhibition of $Ca2^{+}$-dependent $^{86}Rb^{+}$ uptake was observed. Neither OUA ($10^{-3}$ M) or FUR ($10^{-3}$ M) alone produced any $^{86}Rb^{+}$ uptake into IOVs.

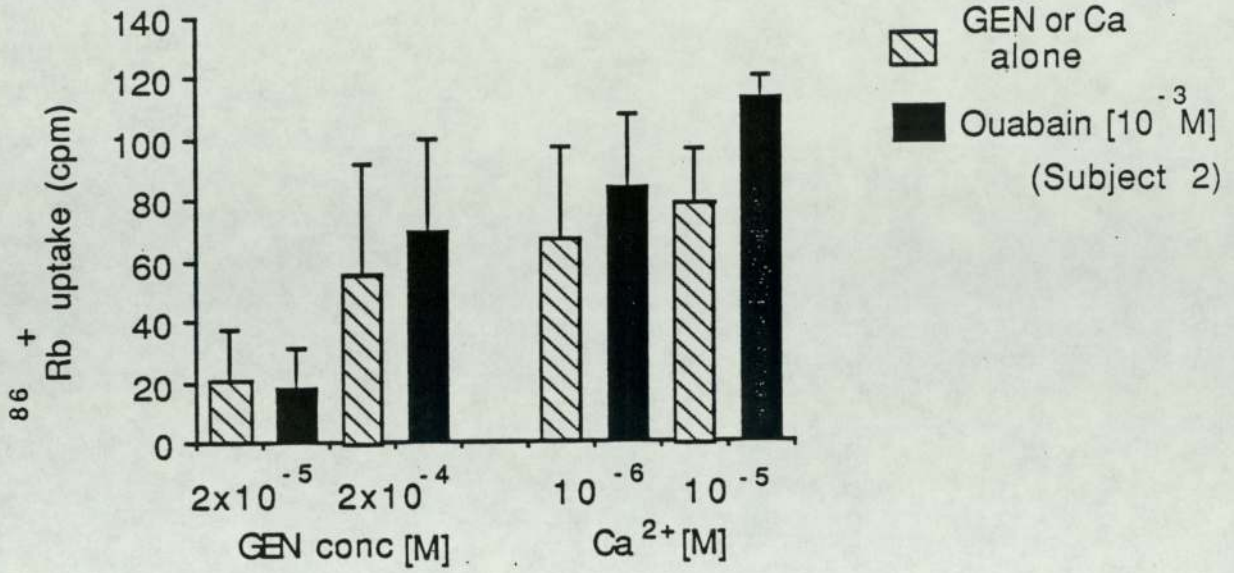



Fig 6d The effect of the addition of ouabain to the external incubation buffer on GEN stimulated and Ca-dependent Rb uptake into IOVs at equilibrium. The mean data from three separate experiments using IOVs prepared from blood drawn from subject 2 are shown (+\- S.E.).

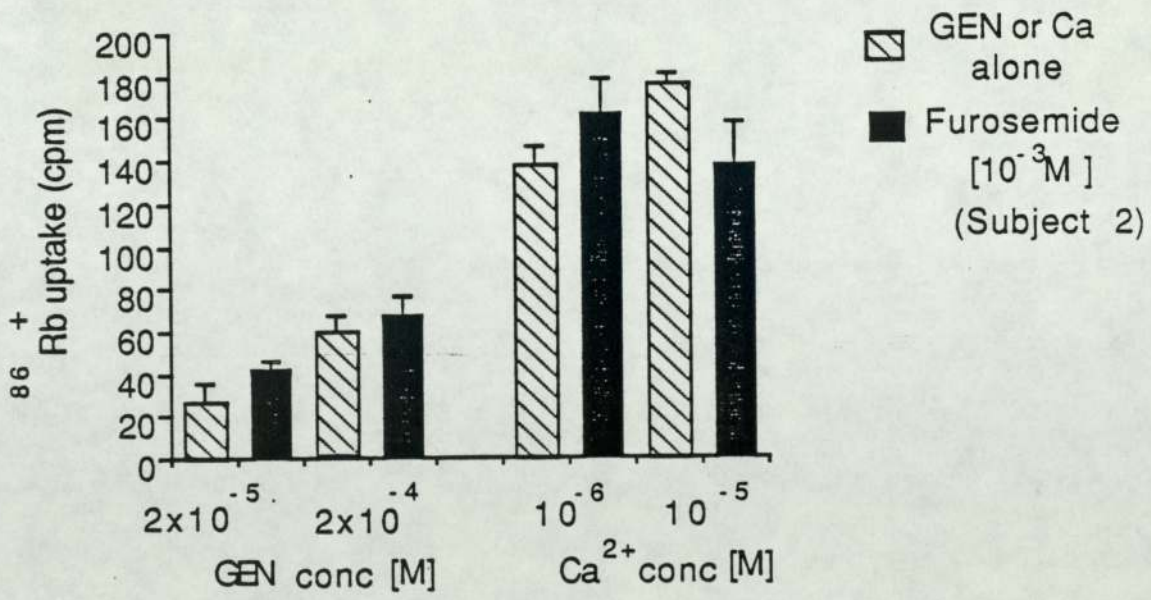



Fig 6e Effect of the addition of furosemide to the incubaton buffer, on GEN-stimulated and Ca-dependent Rb uptake into IOVs at equilibrium. The mean data from three separate experiments using IOVs prepared from blood drawn from subject 2 are shown. (+/- S.E.).

[3.6.3] Effect of loading IOVs with furosemide ($10^{-3}$ M) on GEN -stimulated and Ca2+-dependent Rb+ uptake into IOVs .

Loading IOVs with FUR ($10^{-3}$ M) (see Materials and Methods 2.3.5) produced significant inhibition of GEN-stimulated $^{86}Rb^{+}$ uptake. (Fig 6f) Paired t test for GEN-$2x10^{-4}$ M, $P<0.05$. Little if any $^{86}Rb^{+}$ uptake was produced by incubation of the loaded IOVs with GEN, whereas incubation with $Ca2^{+}$ produced a characteristic response. No GEN-stimulated $^{86}Rb^{+}$ uptake was observed into FUR loaded IOVs whether prepared from subject 2 (Fig 6f) or subject 4 erythrocytes (fig 6h).

As a control experiment IOVs were preincubated with FUR. This was done as loading with FUR involved a longer exposure to the drug and it was thought that the longer exposure rather than the loading may have been the cause of any effects.

Preincubation with FUR ($10^{-3}$ M ) for 2 hr prior to incubation with $^{86}Rb^{+}$ (in addition to its inclusion in the incubation buffer) failed to produce any inhibition of either GEN-stimulated or $Ca2^{+}$-dependent $^{86}Rb^{+}$ uptake into IOVs (Fig 6f).

Inhibition of AG-stimulated $^{86}Rb^{+}$ uptake into IOVs was only achieved by loading the vesicles with FUR and did not occur when the drug was included in the incubation buffer or after preincubation with the drug. FUR is reported not to be soluble in hydrocarbons (Bach et al, 1988), it thus appears that when added externally, FUR is not able to equilibrate with the intravesicular space. This is contradictory to data obtained with dog kidney membranes, where $^{3}H$-bumetanide was shown to equilibrate with the intravesicular environment (Forbush and Palfrey, 1983).

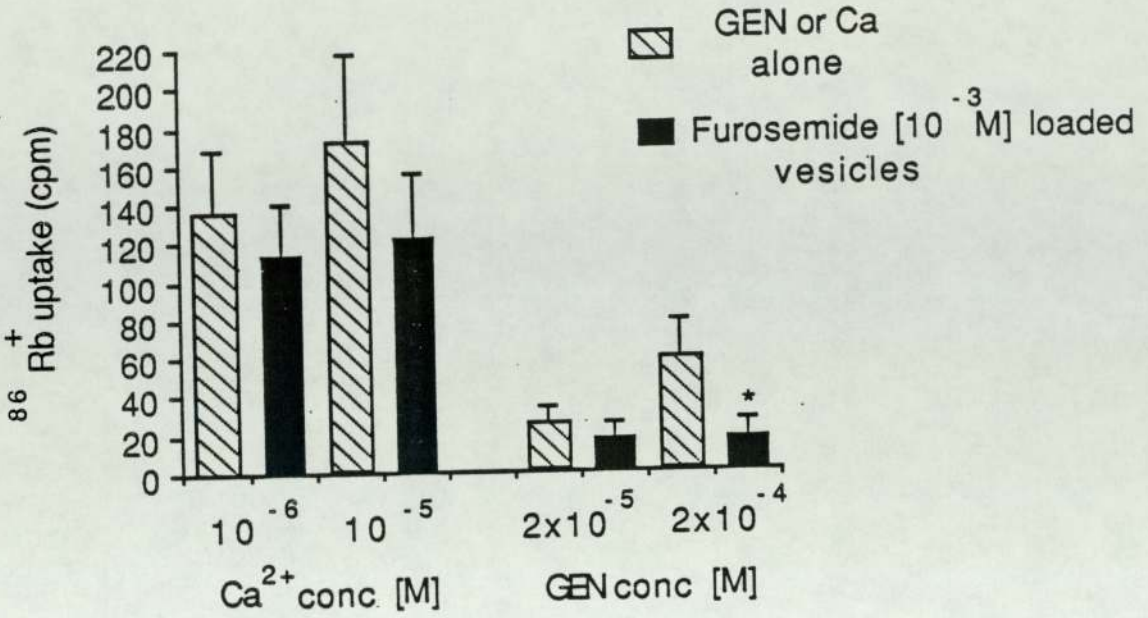



Fig 6f Effect of loading IOVs with furosemide on GEN-stimulated and Ca-dependent Rb uptake into the vesicles at equilibrium. IOVs prepared from blood drawn from subject 2. (Mean data, n=3, +/- S.E).
* Paired T test, P< 0.05, i.e furosemide loading caused significant reduction of GEN-stimulated Rb uptake.

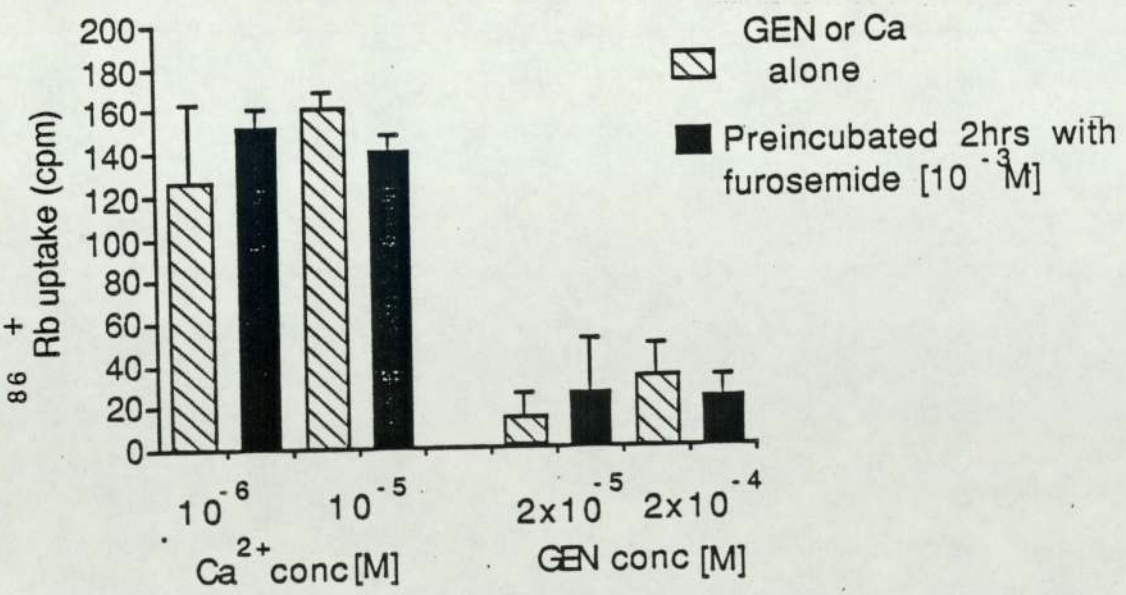



Fig 6g Effect of a 2 hr preincubation with furosemide on GEN-stimulated and Ca-dependent Rb uptake into IOVs at equilibrium. IOVs prepared from blood drawn from subject 2. (Mean data, n=3, +/- S.E.).

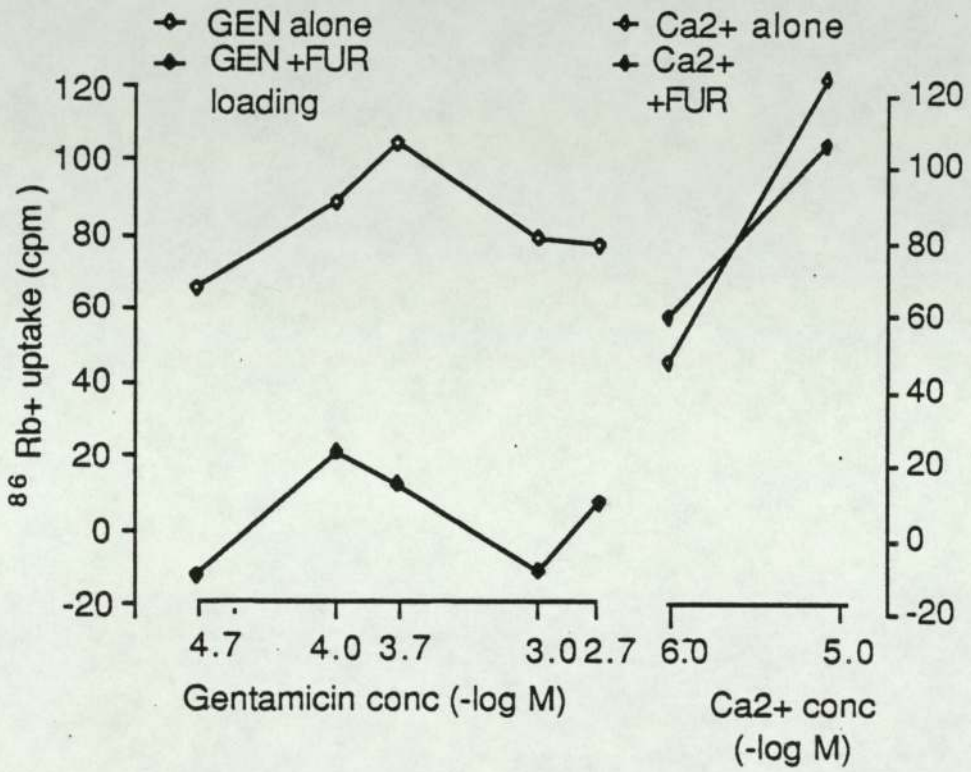



Fig 6h The effect of loading IOVs with furosemide on GEN-stimulated and Ca-dependent Rb uptake into the vesicles at equilibrium.
IOVs prepared from blood drawn from subject 4. (n=1).

IOVs (unloaded) prepared from subject 4 blood once more took up the most $^{86}Rb^+$ in comparison with vesicles prepared from other sources (see Results 3.5). This large AG-stimulated $^{86}Rb^+$ uptake has allowed, in these particular IOVs at least, the unequivocal demonstration of the absence of this ion movement in FUR loaded IOVs (Fig 6h).

[3.6.4] Effect of neomycin ($10^{-4}$ M) on Ca2+-dependent Rb+ uptake into IOVs loaded with furosemide ($10^{-3}$ M ).

In the Figs 6f and 6h it has been demonstrated that loading IOVs with FUR blocks GEN-stimulated $^{86}Rb^+$ uptake. In order to demonstrate inhibition of $Ca2^+$-dependent $^{86}Rb^+$ uptake into IOVs at concentration of NEO of $10^{-4}$ M in the absence of NEO-stimulated uptake of $^{86}Rb^+$, FUR loaded IOVs were employed.

Fig 6i shows the effect of NEO ($10^{-4}$ M) on $Ca2^+$-dependent $^{86}Rb^+$ uptake into IOVs loaded with FUR ($10^{-3}$ M). The range of $Ca2^+$ concentrations used is comparable to Fig 2a (effect of NEO $10^{-4}$ M on $Ca2^+$-dependent $^{86}Rb^+$ uptake into IOVs). In Results 3.2.1 it was shown that antagonism of $Ca2^+$-dependent $^{86}Rb^+$ uptake into IOVs by NEO $10^{-4}$ M was apparently partially masked by co-existing stimulation of $^{86}Rb^+$ uptake by the drug (35% of that seen with $5x10^{-5}$ M $Ca2^+$, mean of 8 experiments, Fig 2a ).

NEO-stimulated $^{86}Rb^+$ uptake into FUR loaded IOVs was observed, but was of a lower magnitude than previously recorded (14.9% of that seen with $5x10^{-5}$ M $Ca2^+$, mean of 3 experiments ).

Significant inhibition of $Ca2^+$-dependent $^{86}Rb^+$ uptake into these FUR loaded IOVs by NEO $10^{-4}$ M was observed at $10^{-6}$ M $Ca2^+$

($p < 0.01$, paired t test ). This data is comparable to that seen after the subtraction of the NEO-stimulated $^{86}Rb^{+}$ uptake from the $Ca2^{+}$dose response curve (see Fig 2g).

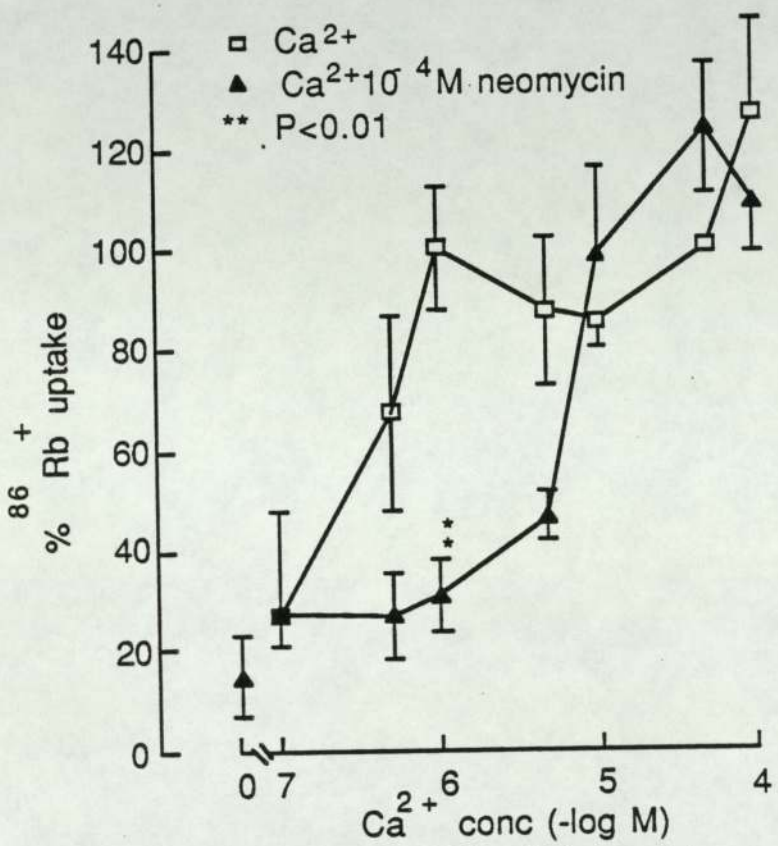



Fig 6i The effect of loading IOVs with furosemide on the inhibition of Ca-dependent Rb uptake at equilibrium by NEO ($10^{-4}$ M). Data pooled from three experiments, blood drawn from donors 2,5 and 7. The Rb uptake at $5x10^{-5}$ M Ca has been set at 100% uptake. The uptake of Rb in the absence of NEO and Ca has been subtracted from all the values. (Mean +/- S.E). ** Paired t test, P<0.01, significant reduction of Ca-dependent Rb uptake ($10^{-6}$ M Ca ) in furosamide loaded IOVs compared to unloaded controls.

[3.6.5] Effect of loading IOVs with bumetanide ($10^{-4}$ M-$10^{-3}$ M) on GEN-stimulated and Ca2+-dependent Rb+ uptake into IOVs.

As FUR loading of the IOVs abolished AG-stimulated $^{86}Rb^{+}$ into IOVs at equilibrium and in the knowledge that this loop diuretic is able to inhibit Na-K-Cl co-transport, it appeared possible that Na-K-Cl co-transport may be involved in AG-stimulated $^{86}Rb^{+}$ uptake into IOVs. At a concentration of $10^{-3}$ M however, FUR is thought to inhibit a range of transport systems in the erythrocyte plasma membrane, in addition to Na-K-Cl co-transport (Chipperfield, 1986). The effect of loading the vesicles with lower concentrations of the more potent loop diuretic bumetanide was therefore investigated.

Loading IOVs with bumetanide only produced inhibition at a drug concentration of $10^{-3}$ M (Fig 6j). No inhibition was recorded at the lower concentration of bumetanide employed ($10^{-4}$ M). It thus appears that AG-stimulated $^{86}Rb^{+}$ uptake into IOVs may involve a loop diuretic sensitive $^{86}Rb^{+}$ uptake other than Na-K-Cl co-transport.

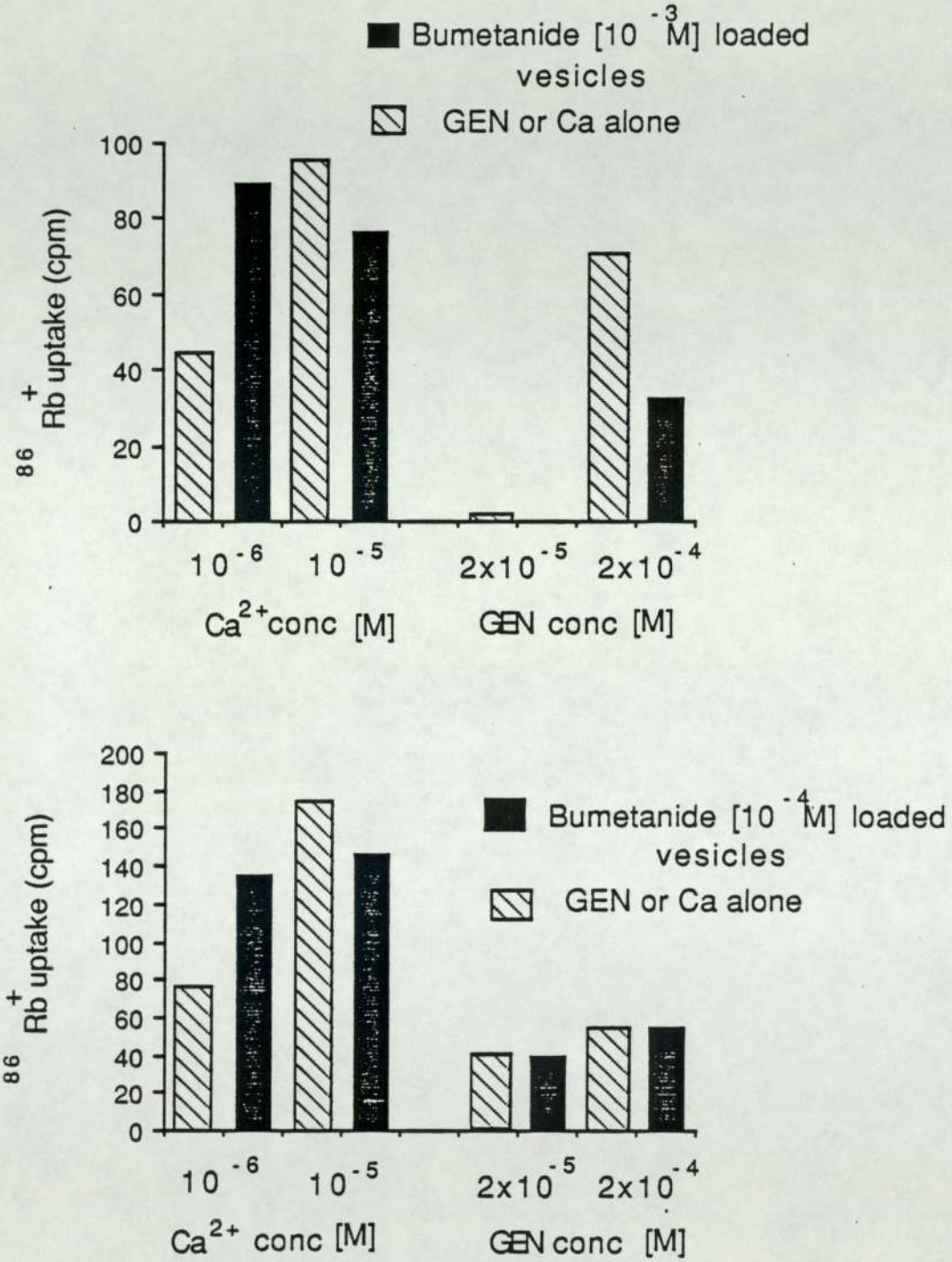



Fig 6j Effect of loading with bumetanide on GEN stimulated Rb uptake into IOVs at equilibrium. IOVs prepared from blood drawn from subject 2, n=1. The Rb uptake in the absence of GEN or Ca has been subtracted from all the values.

## [3.7] Investigation of the Relationship between AG-stimulated $Rb^+$ uptake into IOVs and $Na^+$ and $Cl^-$ ions.

### [3.7.1] Measurement of Na and K in the incubation buffer

The incubation buffer used during $^{86}Rb^+$ uptake experiments included added $K^+$ and $Cl^-$ ions, but no added $Na^+$ ions (see Buffers and Solutions). For the Na-K-Cl co-transport system to function, the presence of all three ions is required (Chipperfield, 1986). That the addition of AGs does not lead to contamination of the buffer with $Na^+$ ions, was confirmed by the analysis of sodium and potassium content of the incubation buffer by means of flame photometry (see Table 11).

Table 11 Potassium and sodium concentrations in the incubation buffer following the addition of GEN or $CaCl_2$.

| GEN (M) | Na (mM) | K (mM) |
|---|---|---|
| 0 | 1 | 28 |
| $2x10^{-5}$ | 0 | 29 |
| $1x10^{-4}$ | 0 | 29 |
| $2x10^{-4}$ | 0 | 29 |
| $1x10^{-3}$ | 0 | 29 |
| $2x10^{-3}$ | 0 | 29 |
| $5x10^{-3}$ | 1 | 27 |
| $1x10^{-2}$ | 1 | 27 |
| $2x10^{-2}$ | 1 | 28 |
| $4x10^{-2}$ | 1 | 28 |
| $Ca2^+$ (M) | | |
| $10^{-6}$ | 0 | 29 |
| $10^{-5}$ | 0 | 29 |

It thus appears that sodium was not introduced into the system as a contaminant. Therefore as sodium is absent from the incubation buffer, it would appear unlikely that the AGs are activating Na-K-Cl co-transport.

[3.7.2] Effect of addition of NaCl to the incubation buffer

Fig 7a shows that potential contaminant concentrations of NaCl, when added to the incubation buffer did not stimulate $^{86}Rb^{+}$ uptake into IOVs. Similar concentrations of GEN on the other hand did stimulate an uptake of $^{86}Rb^{+}$ into the vesicles.

These data (3.7.1-3.7.2) along with the finding, that only loading with high concentrations of loop diuretic (FUR or bumetanide) inhibits AG-stimulated $^{86}Rb^{+}$ uptake, argue strongly against the involvement of the Na-K-Cl co-transporter in AG-stimulated $^{86}Rb^{+}$ uptake into IOVs.

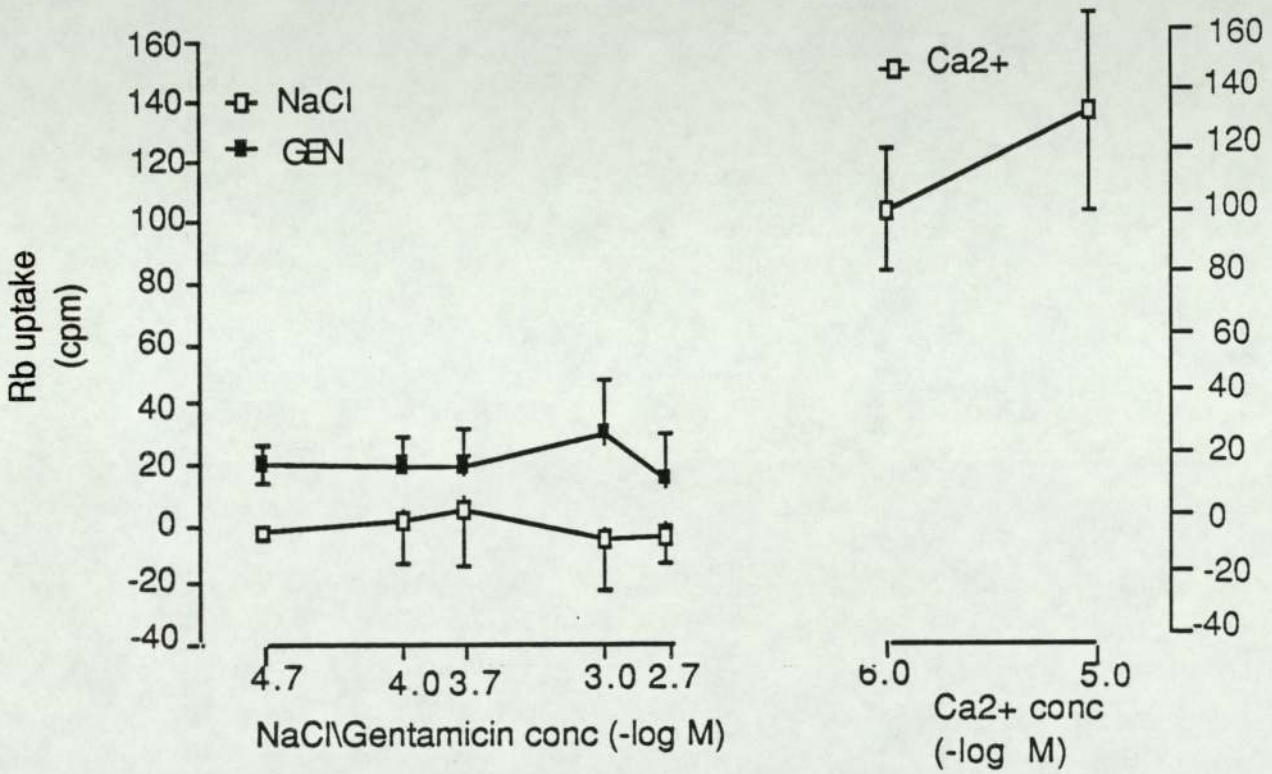



Fig 7a Effect of the addition of NaCl to the incubation buffer on Rb uptake into IOVs, in comparison to that produced by GEN. IOVs prepared from blood drawn from subject 2. (n=3, mean data +/- S.E).

[3.7.3] Effect on AG-stimulated Rb+ uptake and Ca2+-dependent Rb+ uptake of replacement of chlorine with thiocyanate in the incubation buffer.

Na-K-Cl and K-Cl co-transport both require the presence of $Cl^-$ and only $Br^-$ is able to replace $Cl^-$, while other anions of the Hofmeister series ( $NO_3^-$, $I^-$, $SCN^-$) inhibit $K^+$ movements through these systems (Chipperfield, 1986; Lauf, 1985).

To further exclude the activation of a co-transporter such as the Na-K-Cl or even the K-Cl co-transporter (both of which are Cl-dependent) by AGs, $Cl^-$ was replaced in the incubation buffer with $SCN^-$. It is clear from the data shown in Fig 7b that AG-stimulated $^{86}Rb^+$ uptake can occur in $Cl^-$ free buffer. This suggests that AG-stimulated $^{86}Rb^+$ uptake into IOVs is not the result of activation of a co-transport system. Ca-dependent $^{86}Rb^+$ uptake into the IOVs was also unaffected.

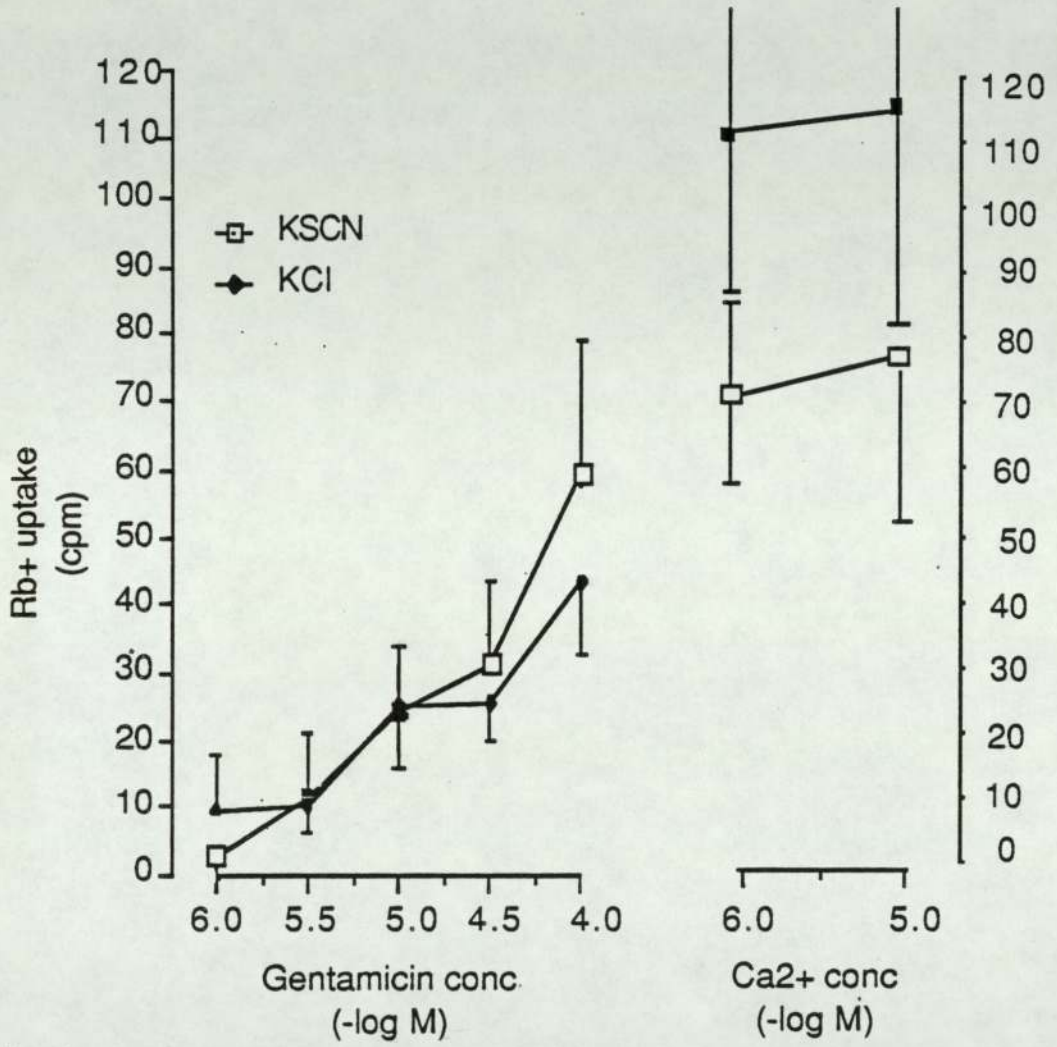



Fig 7b The effect of replacement of chlorine with thiocyanate in the incubation buffer on GEN-stimulated and Ca-dependent Rb uptake into IOVs at equilibrium.
Blood drawn from subject 2, (n=3, mean data +/- S.E).

## [3.8] Effect of aminoglycoside antibiotics on $Ca2^+$-dependent $K^+$ transport in intact erythrocytes.

### [3.8.1] Effect of calcium and A23187 ionophore on KCl efflux from erythrocytes.

As a prelude to the further investigation of the effect of AGs on $Ca2^+$-dependent $K^+$ transport, the activity of this system in intact erythrocytes was examined. An experimental protocol was used whereby the activity of the transport system was estimated from the loss of KCl observed , on incubation of erythrocytes in a low-K buffer. Intracellular $Ca2^+$ was raised by the addition of $CaCl_2$ and the ionophore A23187 to the incubation buffer and the loss of KCl monitored indirectly by following the decrease in transmittance at 650nm resulting from cell shrinkage (Alvarez et al, 1986c).

#### [3.8.1.1] Calcium dose response curve (maximal response).

As a primary step towards characterisation of this erythrocyte model a $Ca2^+$ dose response curve was constructed. Changing the $Ca2^+$ ion concentration in the final incubation buffer produced a sigmoidal dose response curve (see Fig 8a).

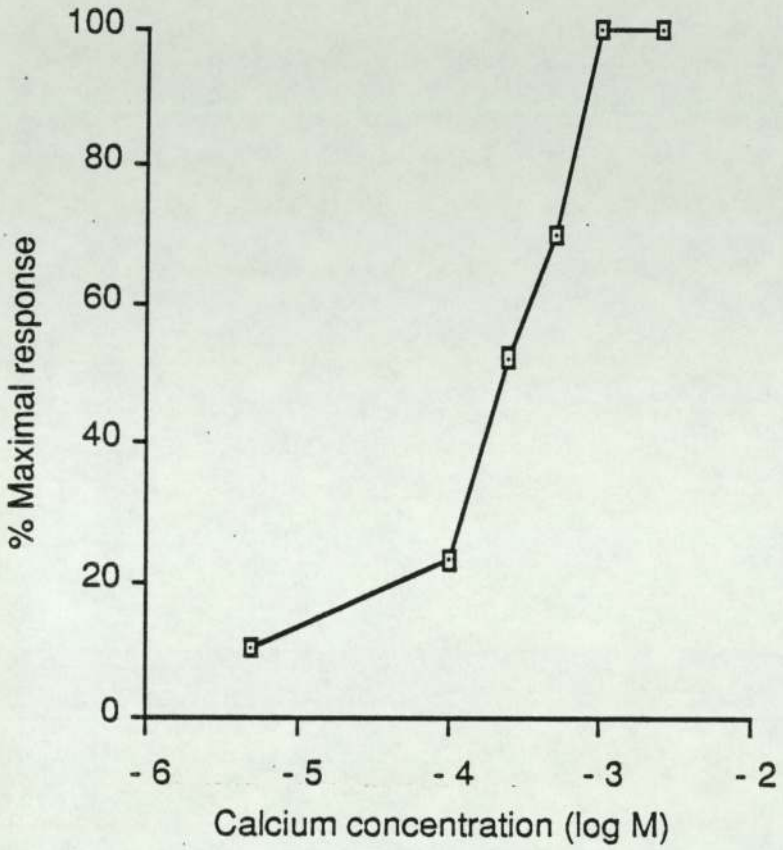



Fig 8a Effect of calcium and A23187 ionophore on KCl efflux from erythrocytes. Calcium dose response curve expressed as a percentage of the maximum response. (Mean data, n=2). 10% haematocrit.

[3.8.1.2] Calcium dose response curve (initial rate of change of transmittance 650nm).

It has been reported that inhibitors of $Ca2^+$-dependent $K^+$ transport can reduce the rate of cell shrinkage but prolonging these experiments over a long enough time course, however, resulted in the same overall change in transmittance being recorded (Alvarez et al, 1986c). It was thus decided to monitor the activity of $Ca2^+$-dependent $K^+$ transport in this system by calculating the initial rate of change of transmittance.

The initial rate of change of transmittance was determined by calculating the slope of the initial linear part of the trace (see Fig 8b).

[3.8.1.3] Effect of quinine on Ca2+-dependent K+ transport in intact erythrocytes.

To further characterise this system, the effect of addition of the $Ca2^+$-dependent $K^+$ transport inhibitor quinine was examined.

Fig 8c shows that quinine, when added to the incubation buffer immediately prior to the ionophore, caused a dose dependent inhibition of the rate of KCl efflux ($Ca2^+$ $5x10^{-4}$ M and A23187 $2x10^{-6}$ M). $EC_{50}$ $6x10^{-4}$ M.

Inhibition of $Ca2^+$/A23187 induced cell shrinkage by quinine in a dose dependent manner supports the premise that the cell shrinkage monitored by determining the transmittance at 650nM was in fact due to $Ca2^+$-dependent $K^+$ transport.

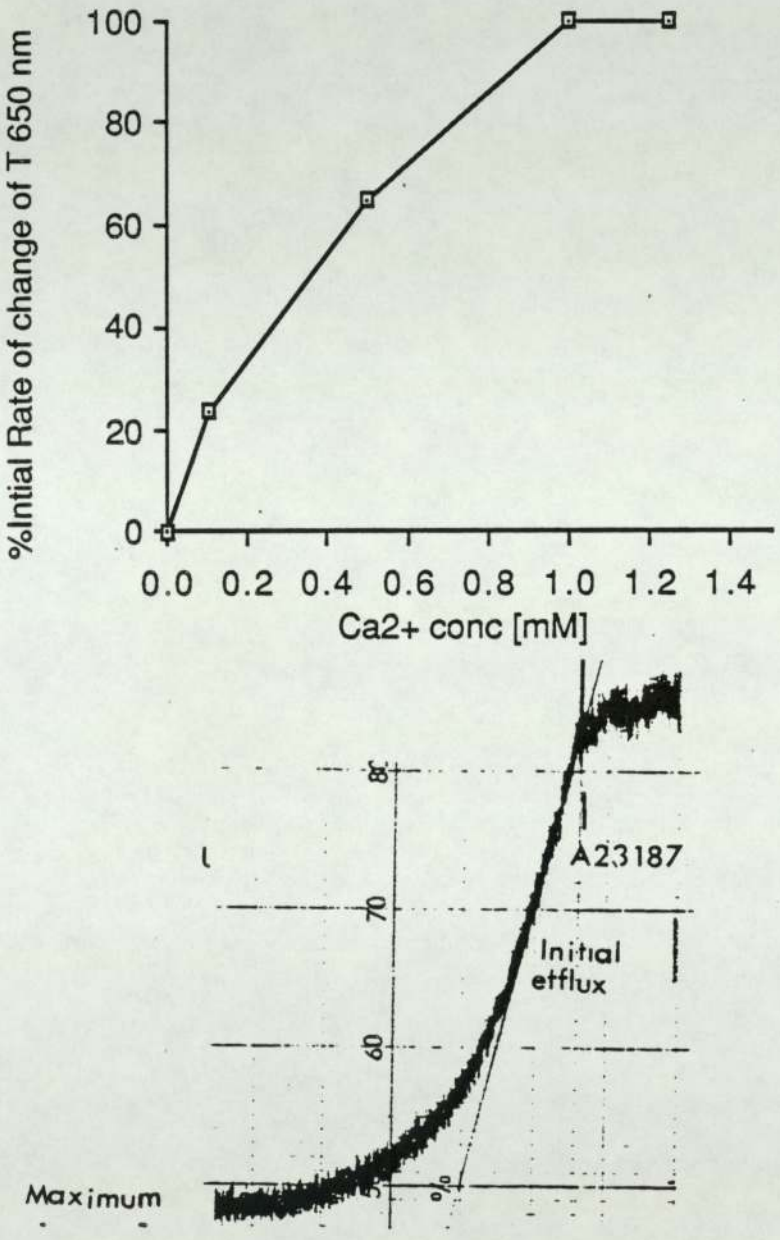



Fig 8b Effect of calcium and A23187 ionophore on KCl efflux from erythrocytes. Calcium dose response curve for initial rate of change of transmittance. n=2
Also shown is a representative trace.

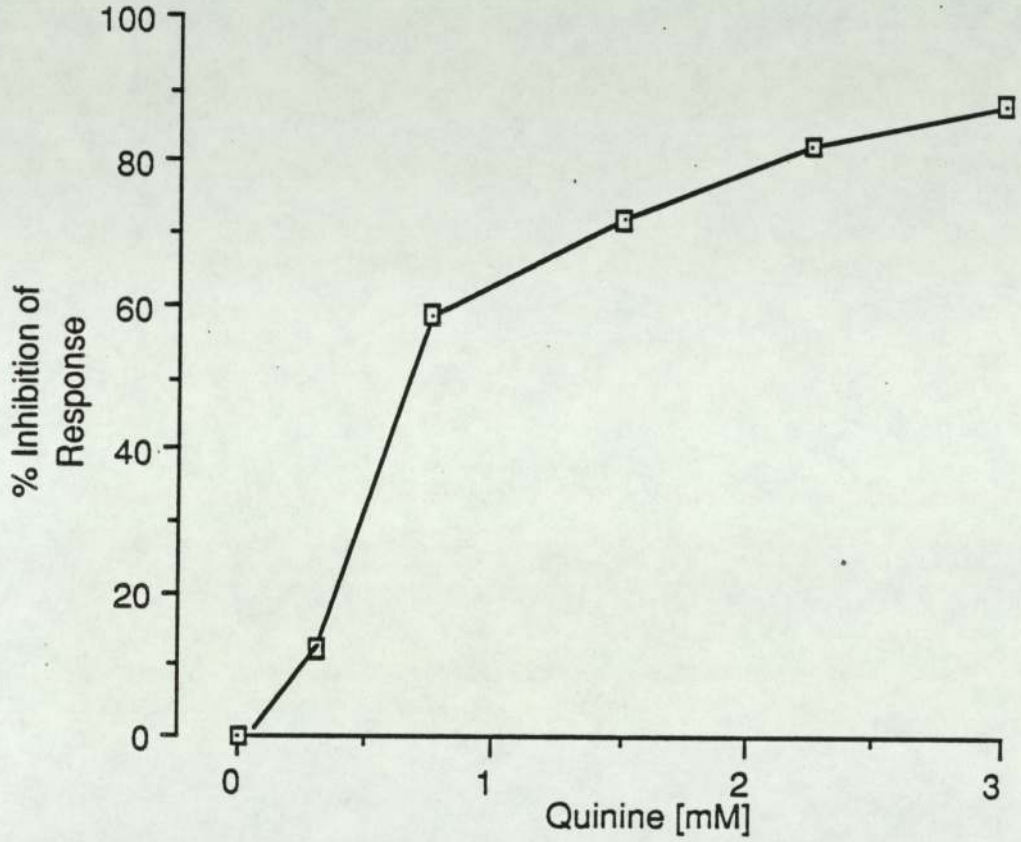



Fig 8c Effect of quinine on Ca-dependent K transport of intact erythrocytes. Inhibition is shown as a percentage of the response seen with Ca (0.5 mM) and A23187. n=1.

[3.8.2] Effect of internal gentamicin on Ca2+-dependent K+ transport in intact human erythrocytes.

[3.8.2.1] Effect of loading erythrocyte ghosts with gentamicin on calcium/A23187 induced KCl efflux.

In an attempt to compare the effects of GEN on $Ca^{2+}$-dependent $K^+$ transport of intact erythrocyte ghosts at the intra-cellular membrane surface, with that previously observed in IOVs, the cells were loaded with the antibiotic (see Materials and Methods 2.3.7). Figs 8d-f show the percentage change in the rate of KCl efflux from cells drawn from three different donors, produced by $Ca^{2+}$ ($1x10^{-4}$ M) and the ionophore A23187. A concentration of $Ca^{2+}$ known to produce a submaximal response was employed ( see Results 3.8.1.2) so as to increase the sensitivity of the system to any possible inhibition by the AG. Cells drawn from three different donors were compared (subject 2,7 and 8), the erythrocytes being loaded with varying concentrations of GEN. The three figures show the mean response from at least three experiments plotted against the concentration of GEN added to the cells.

For both subjects (2 and 8) a dose related inhibition of KCl efflux was observed (Figs 8d and 8f). It is note-worthy though that a significant increase in the rate of cell shrinkage ($p<0.05$, paired t test) was observed when cells from subject 7 were loaded by adding $2x10^{-2}$ M GEN to the erythrocytes immediatly prior to lysis, whereas addition of $4x10^{-2}$ M GEN caused significant inhibition (Fig 8e). Fig 8f also shows the percentage inhibition in the rate of VAL induced KCl efflux as a control.

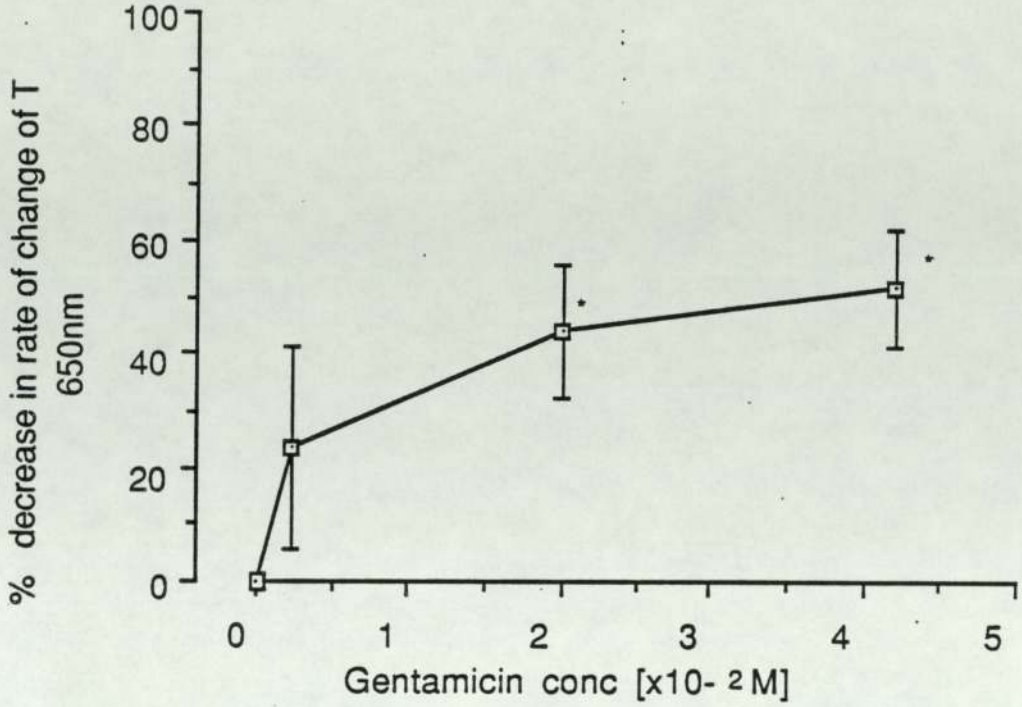



Fig 8d Effect of loading erythrocytes drawn from subject 2 with increasing concentrations of gentamicin, on the rate of efflux of KCl produced by Ca (0.1mM) and A23187. GEN is expressed as the concentration added to the cells prior to loading. * P < 0.05 Paired t test, significant decrease of KCl efflux compared to unloaded controls.

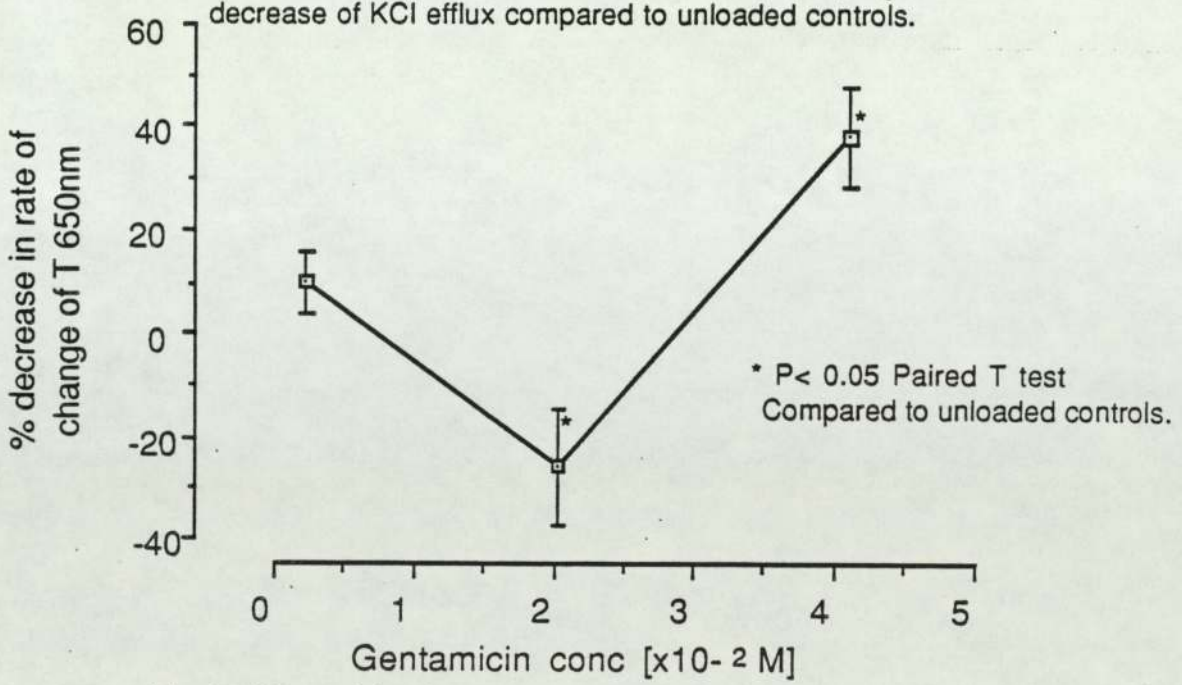



Fig 8e Effect of loading erythrocytes drawn from subject 7 with gentamicin, on the rate of KCl efflux produced by Ca (0.1mM) and A23187.

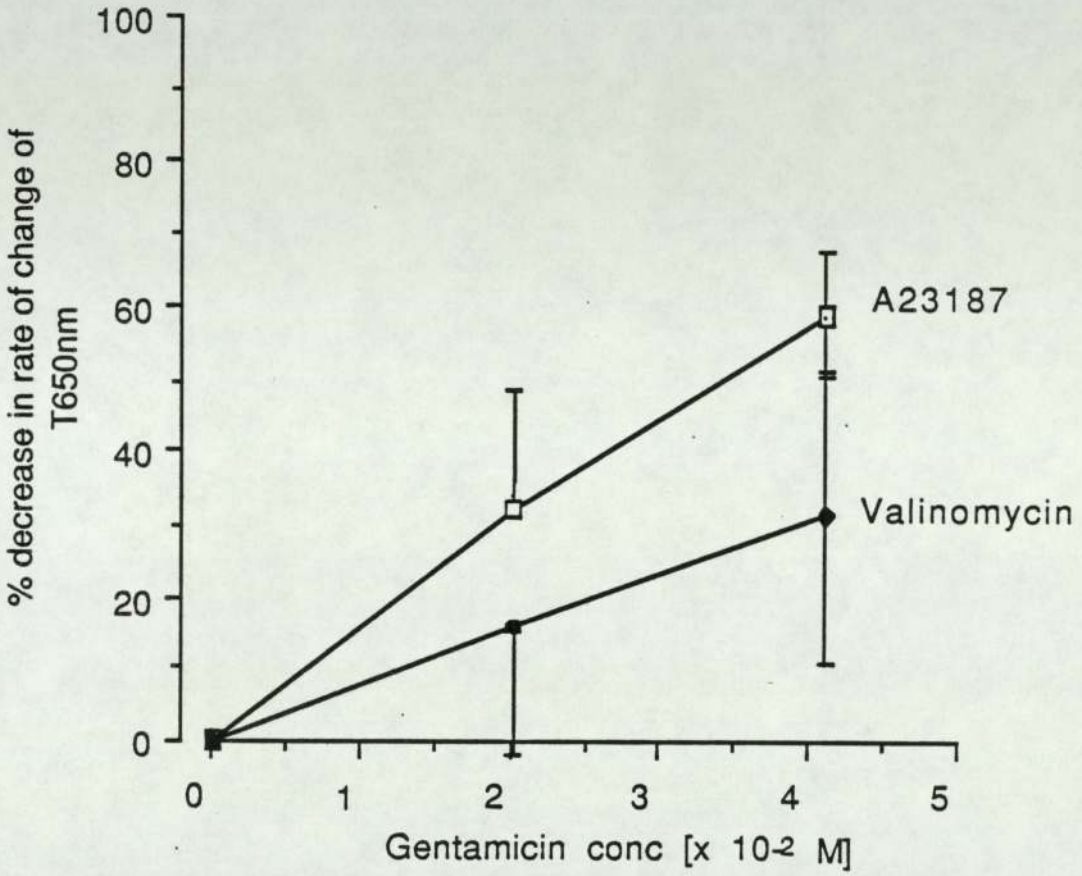



Fig 8f Effect of loading erythrocytes drawn from subject 8 with gentamicin, on the rate of KCl efflux produced by Ca (0.1mM) and A23187. The effect on valinomycin induced KCl efflux is also shown. (Mean data, +/- S.E, n =3).

[3.8.2.2] Analysis of inhibition of Ca2+-dependent K+ efflux due to the loading of erythrocytes with gentamicin.

Assay of GEN content of the loaded erythrocytes , following relysis by repeated freezing and thawing revealed loading had occured in a dose dependent manner (see Fig 8g). Addition of 0.2, 2, and $4x10^{-2}$ M of GEN to erythrocytes resulted in mean overall internal GEN concentrations of $(1.1+/-0.3)x10^{-3}$ M , $(3.2+/-0.6)x10^{-3}$ M and $(6.1+/-1.1)x10^{-3}$ M respectively. The loading efficiencies for each concentration used were therefore 55, 16, and 15.25% respectively. It would thus appear that loading efficiency decreases with the concentration of GEN added to the erythrocytes. This could be due to the saturation of a specific internal binding site i.e. PtdIns (4,5) $P_2$.

The erythrocytes have been loaded with relatively high concentrations of GEN compared with those used in the IOV experiments described previously (see Results 3.2). That inhibition of the rate of cell shrinkage by these relatively high internal concentrations of GEN did not exceed 50% of the initial rate was therefore unexpected. As a significant increase in the initial rate of cell shrinkage was observed in subject 7 on loading with GEN ($2x10^{-2}$ M) (see Fig 8e) it would seem that internal GEN may have more than one effect on ion transport across the erythrocyte plasma membrane. In the light of the results previously obtained with IOVs, where an AG-stimulated $Rb^+$ uptake was observed (see Results 3.4) it can be speculated that internal GEN is able to activate a pathway whereby $K^+$ is able to exit from the erythrocytes. Furthermore this action varys dependent on the source of the erythrocytes as was the case with the IOV experiments (see Results 3.5). Activation of KCl efflux by such a pathway may again be masking inhibition of

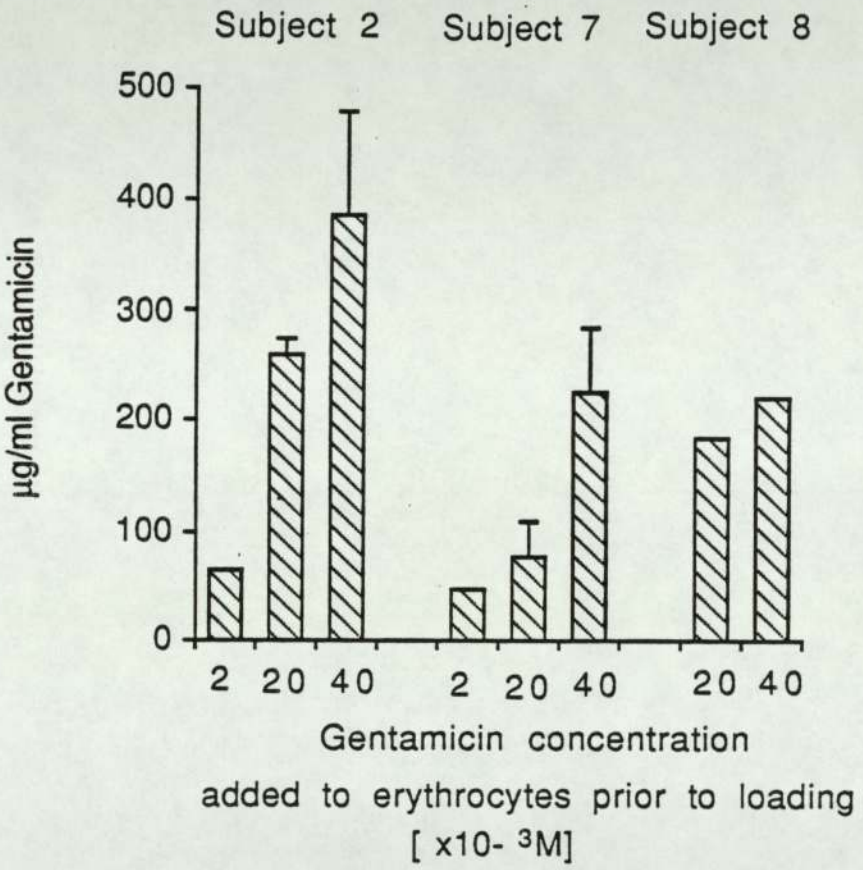


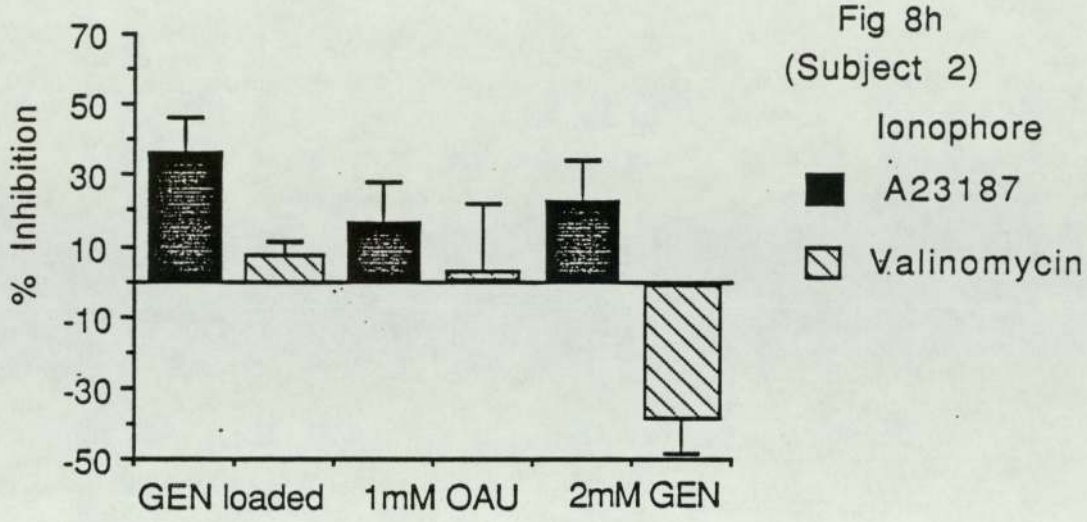

Fig 8g; Concentration of GEN loaded into erythrocytes drawn from three different subjects. The erythrocytes were assayed at 10% haematocrit. (Mean data +/- S.E.).

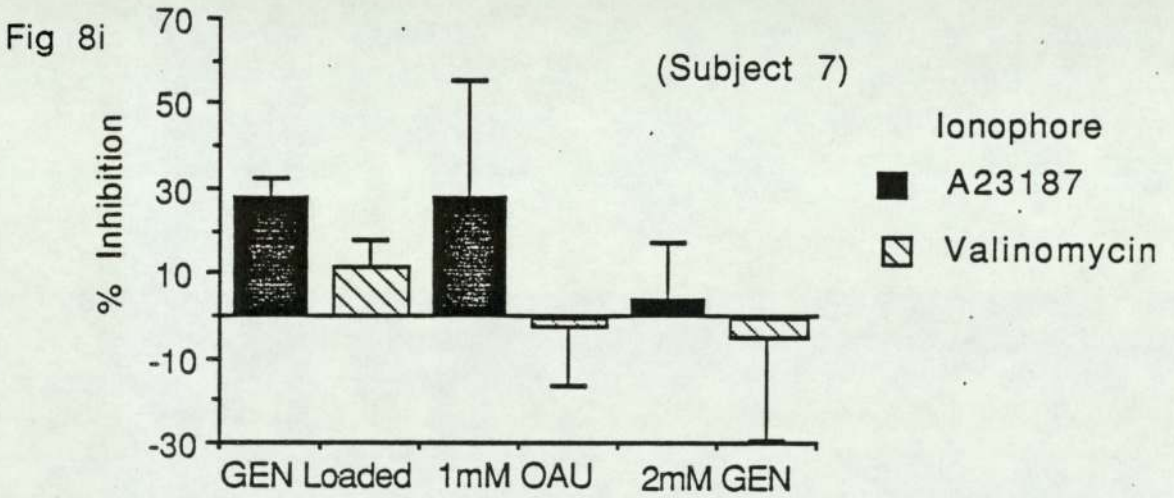
$Ca2^+$-dependent $K^+$ transport. It must however be noted that no cell shrinkage was observed in the absence of ionophore.

As a differential effect of GEN loading erythrocytes on the rate of KCl efflux from subject 7 compared with subjects 2 and 8 was observed (see Figs 8d-8f), it is of interest to note significantly ($P<0.01$ student t test) lower GEN loading of cells from subject 7, on addition of $2x10^{-2}$ M GEN to the erythrocytes prior to lysis. This differential in loading between subjects may not account for the increased cell shrinkage observed on addition of $Ca2^+$/A23187 to subject 7 erythrocytes loaded in such a manner, as no such effect was observed with subject 2 erythrocytes with a similar final internal GEN concentration.

It was considered a possibility that loading erythrocytes with GEN might be causing non-specific inhibition of KCl efflux or alternatively be interfering with the reestablishment of a normal intracellular potassium concentration ([K]i). Possible mechanisms of non-specific inhibition could include the drug acting on membrane fluidity or altering its permeability to anions. To determine whether the inhibition was non-specific or not, the effect of GEN loading of the erythrocytes on VAL induced KCl efflux was investigated (Fig 8h-j). No significant inhibition of VAL induced KCl efflux was observed in GEN loaded erythrocytes, for erythrocytes drawn from subject 2. However a moderate reduction in the initial rate of efflux was observed for cells drawn from subjects 7 and 8 (see also Fig 8f). Thus a proportion of the inhibition of the initial rate of KCl efflux observed under these conditions may be non-specific.

GEN at high concentrations has been reported to inhibit Na,K-ATPase (Chahwala and Harpur, 1982). It was thus thought possible that loading with GEN may be interfering with the restoration of a normal [K]i by blocking this membrane pump. The [K]i consequently



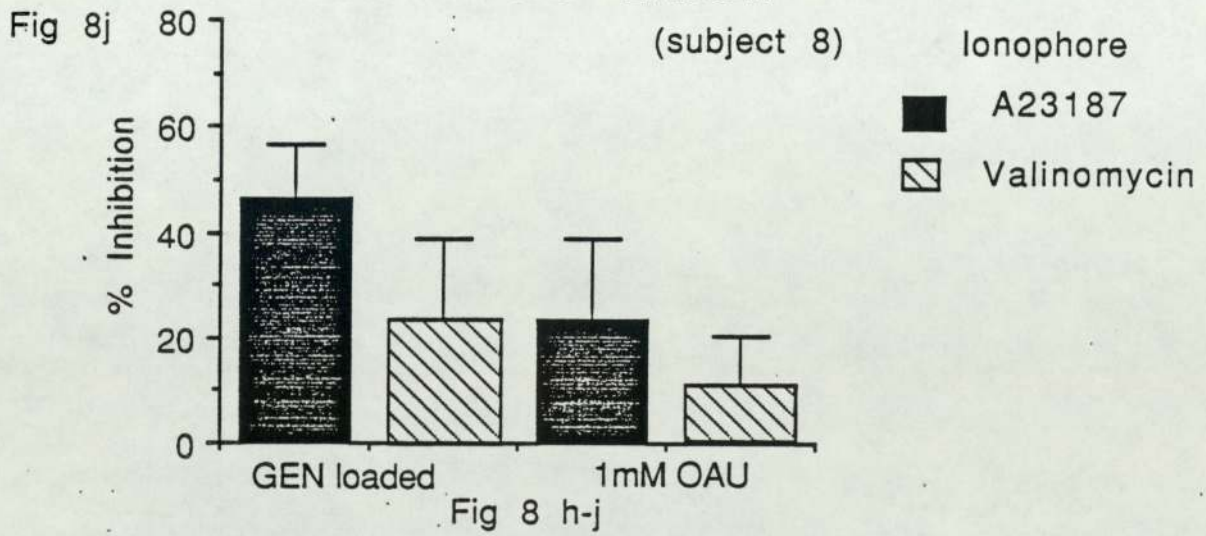




Fig 8 h-j

Inhibition of KCl efflux from erythrocytes drawn from subjects 2,7 and 8 by internal and external gentamicin and also external ouabain ie added to the reseal buffer. The cells were loaded in each case by the addition of $4 \times 10^{-2}$ M GEN to the erythrocytes prior to lysis. (Mean data +/- S.E)

would not be restored to the resting level and a slower than normal efflux of KCl would be recorded. This possibility was investigated by the employment of the Na,K-ATPase inhibitor ouabain (OUA) (Skou, 1965). Addition of OUA ($10^{-3}$ M) to the resealing buffer (see Buffers and Solutions) produced an inhibition of the KCl efflux brought about by $CaCl_2$ ($10^{-4}$ M) and A23187 to a similar degree to that seen with GEN ($4x10^{-2}$ M) loading. The VAL induced KCl efflux was not effected. Internal potassium however was not significantly altered by this treatment (see Table 12). It thus appears that loading of erythrocytes with GEN does not alter the K gradient. It would appear though that OUA is able to antagonise erythrocyte shrinkage (i.e $Ca2^{+}$-dependent $K^{+}$ transport) in this system by some other manner. A report of such an effect in erythrocytes has previously appeared (Blum and Hoffmann, 1971). OUA's mechanism of action however remains an enigma and that GEN and OUA act in a similar manner in this system cannot be ruled out. Inclusion of GEN in the reseal buffer did produce inhibition of the KCl efflux in one subject (2) but not in the other investigated (7).

Table 12 Internal potassium concentrations of erythrocytes, treated and untreated. Duplicate samples n=1.

| | Potassium concentration (mM) | |
|---|---|---|
| | Subject 2 | Subject 6 |
| Control erythrocytes | 96.0 | 74.4 |
| Dialysed erythrocytes | 66.4 | 44.8 |
| Dialysed erythrocytes ($4x10^{-2}$ M GEN loaded) | 70.4 | 60.8 |
| Dialysed erythrocytes ($10^{-3}$ M OUA in reseal buffer) | 61.2 | 60.0 |

[3.8.3] Effect of external gentamicin on Ca2+-dependent K+ efflux from intact erythrocytes

Unlike inside-out erythrocyte vesicles (IOVs) this system enables the effect of drugs (applied externally) on the outer surface of the plasma membrane to be investigated. High concentrations of GEN when added to the incubation buffer were able to reduce the initial rate of KCl efflux from erythrocytes at a $Ca2^+$ concentration known to give a submaximal response ($10^{-4}$ M). EC50 =$8x10^{-4}$ M (see Fig 8k).

A similar inhibition of the rate of efflux was also observed for erythrocytes drawn from subjects 5 and 6 ($CaCl_2$ $10^{-4}$ M), however far less inhibition was observed at a $CaCl_2$ concentration of $5x10^{-4}$ M (see Fig 8 l-m).

The mechanism of this antagonism of KCl efflux from erythrocytes by externally applied GEN (albeit at high concentrations) cannot be deduced from this experiment alone. As GEN is known not to be able to passively cross the plasma membrane (Tulkens and Trouet, 1978), but is reported to be able to interfere with $Ca2^+$ uptake into a variety of cell types (Hino et al, 1982; Suarez-Kurtz, 1974; Suarez-Kurtz and Reuben, 1987; Wagner et al 1987; Adams and Durnett, 1978), it is possible that the drug is antagonising ionophore induced $Ca2^+$ uptake into the erythrocytes. This is supported by the fact that the magnitude of cell shrinkage inhibition is related to the $Ca2^+$ concentration added to the incubation buffer (see Fig 8 l-m).

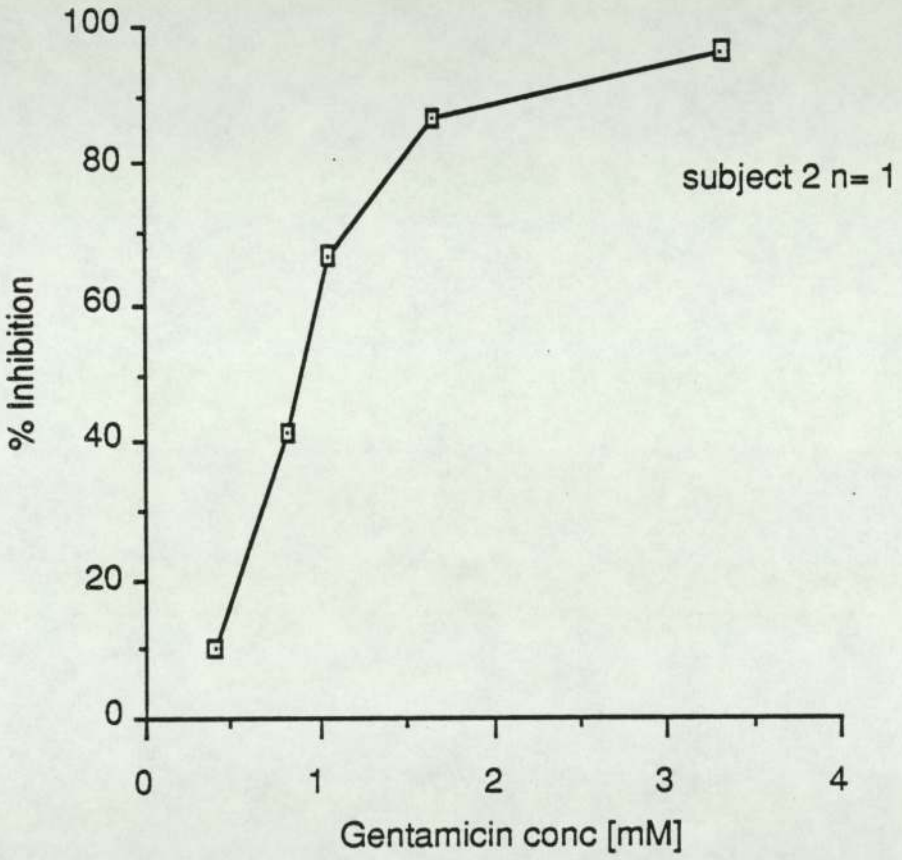



Fig 8 k The effect of external GEN on Ca-dependent K efflux from erythrocytes, expressed as percentage inhibition of initial rate of change of transmittance at GEN = 0. GEN added to incubation buffer immediately prior to the ionophore, n = 1, subject 2.

Fig 8l (subject 5) n=1

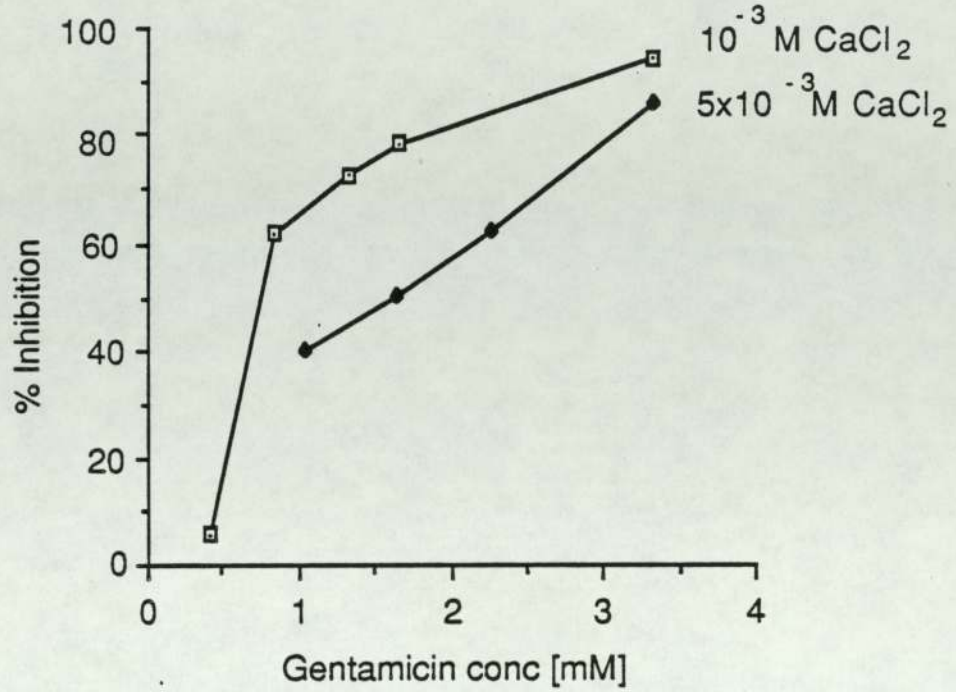



Fig 8m (subject 6) n=1

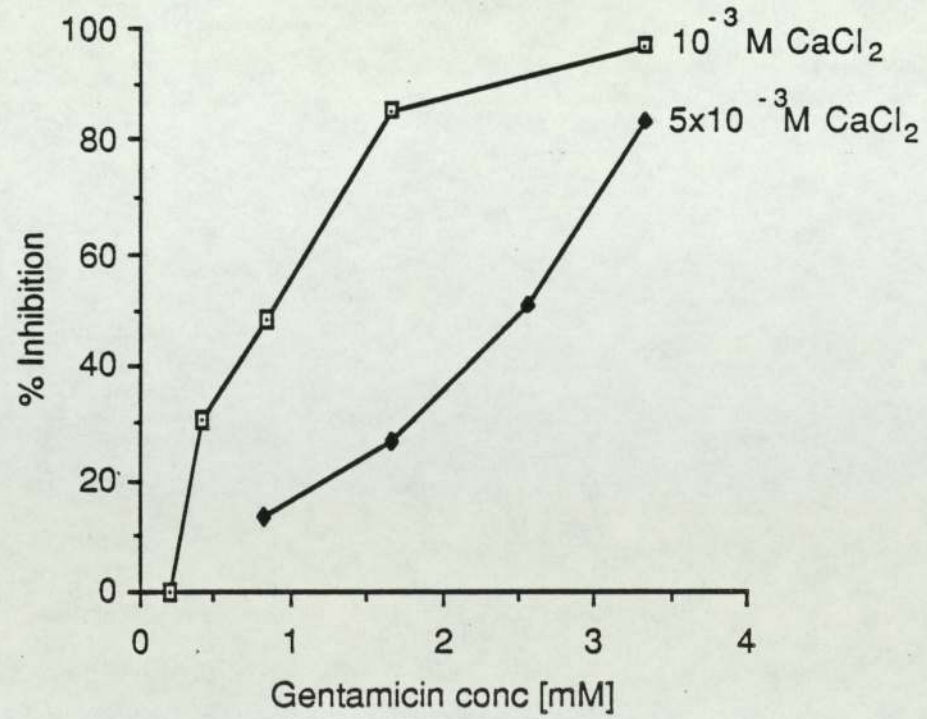



Fig l-m The effect of external GEN on Ca-dependent K efflux from erythrocytes., expressed as percentage inhibition of initial rate of change of transmittance at GEN = 0 M.

## Discussion

In recent years it has become apparent that a large number of excitable and non-excitable cells possess a $K^+$-permeability mechanism which is controlled or gated by the concentration of ionized calcium in the cytoplasm, so that increases in cytosolic $Ca2^+$ lead to increased $K^+$ efflux (Lew and Ferreira, 1978; Latorre and Miller, 1983; Marty, 1983). The human erythrocyte has been used here as a model to investigate the comparative effects of aminoglycoside antibiotics (AGs) on $Ca2^+$-dependent $K^+$ transport. The observed effects are discussed in the light of the known toxicities of the drugs in the human body.

The acute effects of AGs are thought to occur extracellularly, possibly involving competitive displacement of calcium (Schacht, 1986). Calcium displacement by cationic drugs does not necessarily however correlate with their toxicity (Lodhi et al, 1976). For instance neamine, a neomycin fragment with four positive charges, is an effective calcium antagonist yet shows little ototoxicity (Lodhi et al 1980). It has been proposed in the case of ototoxicity at least, that active uptake of the drug into the cells is an essential step in the mechanism of drug toxicity (Schacht, 1986). It was therefore the effect of AGs on the inside of the erythrocyte plasma membrane that was investigated in this study. To this end two systems enabling the exposure of the internal membrane have been employed; firstly the effect of external application of AGs to $K^+$ transport into one-step inside-out erythrocyte vesicles (IOVs) was investigated and secondly experiments to determine the effect of AG-loading on $Ca2^+$-dependent $K^+$ transport in resealed erythrocytes were conducted.

[4.1] Effect of aminoglycoside antibiotics on Ca2+-dependent Rb+ uptake into one-step inside-out human erythrocyte membrane vesicles

This study demonstrates that aminoglycoside antibiotics (AGs) antagonise $Ca2^+$-dependent $^{86}Rb^+$ uptake into one-step inside-out erythrocyte membrane vesicles (IOVs). AGs were found though to have no effect on the permeability of the $Ca2^+$-dependent $K^+$ channel itself (see Results 3.1) and thus appear to act by displacing $Ca2^+$ from its binding site on the internal face of the membrane, rather than by altering $K^+$ permeability of the channel. These results confirm the observations of Alvarez et al (1986c), that both parameters, the fraction of activated vesicles and the rate of uptake can be modified independently.

AGs are highly hydrophilic, polycationic compounds and as such do not penetrate cell membranes passively (Tulkens and Trouet, 1978). For these drugs to interact with the $Ca2^+$ binding site of the $Ca2^+$-dependent $K^+$ channel directly, they would require to be actively taken up into the target cells, the $Ca2^+$ binding site being found on the inner side of the plasma membrane. (Blum and Hoffman, 1972)

Of the known target tissues for AGs, active cellular uptake mechanisms for the drugs have been established for the kidney (Kluwe and Hook, 1978; Pastoriza-Munoz et al ,1979; Lipsky et al, 1980; Bennett et al, 1982) cristae ampularis (Williams et al, 1987a) and the cochlear hair cells of the guinea pig (Williams et al ,1987b), but not as yet into the neuromuscular junction. In the light of the role played by $Ca2^+$-dependent $K^+$ channels in determining the resting potentials in cochlear hair cells (Ashmore and Meech, 1986)

it is conceivable that the antagonism of such ion channels by AGs could have a role in the mechanism of AG ototoxicity.

$Ca2^+$-dependent $K^+$ channels have been reported to occur in mouse motor nerve terminals (Mallart, 1985). However in the absence of any evidence for active uptake of AGs into motor nerve terminals and the rapid onset and reversibility of neuromuscular blockade (Wright and Collier, 1977), it seems unlikely that AGs act intracellularly in this case, with sites such as the $Ca2^+$ binding site of the $Ca2^+$-dependent $K^+$ channel.

AGs have been demonstrated to bind to phosphatidylinositol 4, 5-bisphosphate (PtdIns(4,5)$P_2$) (Schacht, 1976). Binding of AGs to PtdIns(4,5)$P_2$, will disrupt its physiological function, by preventing its hydrolysis to inositol trisphosphate ($IP_3$) (Schacht, 1976; Van Rooijen and Agranoff, 1985) and thus prevent the liberation of calcium from internal stores. It is therefore theoretically possible, that in addition to direct interaction with the $Ca2^+$ binding site of the $Ca2^+$-dependent $K^+$ channels, AGs when taken up into cells could also inhibit these ion channels indirectly by preventing the rise in intracellular $Ca2^+$ required for activation.

Another mechanism by which AGs could prevent a rise in intracellular $Ca2^+$ and thus prevent activation of $Ca2^+$-dependent $K^+$ channels could be by blockade of plasma membrane $Ca2^+$ channels. AGs are known to inhibit $Ca2^+$ channels in a number of tissues (Adams and Durrant, 1978; Hino et al, 1982; Suarez-Kurtz annd Reuben, 1987; Wagner et al, 1987), so this particular mechanism of indirect inhibition of $Ca2^+$-dependent $K^+$ transport is at least theoretically possible. However it has been recently reported that cytosolic free calcium concentrations in a kidney epithelial cell line are in fact increased by GEN treatment

(Inui et al ,1988). It is thus unclear whether or not this particular means of indirect inhibition of this transport system has any physiological or toxicological importance in vivo at least in the kidney or the ear. However blockade of $Ca2^+$-mediated acetylcholine release by external AGs is known to occur at the neuromuscular presynaptic junction (Prado et al, 1978, Uchiyama et al 1981). To date this $Ca2^+$-mediated neurotransmitter release has not been shown to involve $Ca2^+$-dependent $K^+$ transport.

Further indirect routes (besides those already mentioned) of AG inhibition of $Ca2^+$-dependent $K^+$ channels via interference with the phosphoinositide second messenger system are possible; These could include blockade of $IP_3$ release of intracellular $Ca2^+$ stores (Williams, 1987b) or inhibition of $Ca2^+$-dependent phosphatidylinositol phosphodiesterase (Shute and Smith, 1985). Studies in tissues other than erythrocytes (which lack internal organelles) are required to determine whether indirect inhibition (via inhibition of release of intracellular $Ca2^+$ stores) of $Ca2^+$-dependent $K^+$ transport is of importance in AG toxicity.

This study by no means represents a complete investigation of the nature of AG inhibition of $Ca2^+$-dependent $K^+$ channels in IOVs. Further work is required to determine the threshold concentrations of individual AGs needed to block activation. Information as to the potency of individual AGs, in this respect would be of interest, as certain AGs are known to be more toxic than others. The toxicity of the AGs used in this study can be ranked as follows; NEO > GEN > NET (Caputy et al, 1981; Parker et al, 1982; Au et al, 1987; Davey and Harpur, 1988; Harpur, 1988). It would provide evidence for the involvement of inhibition of $Ca2^+$-dependent $K^+$ transport in AG toxicity if AGs could be ranked in similar order according to their

potency to inhibit this transport system.

The co-existence of an AG-stimulated $^{86}Rb^{+}$ uptake into the IOVs, with a $Ca2^{+}$-dependent uptake has meant that some of the results in this study initially proved difficult to interpret. This AG-stimulated uptake was not found to occur to such an extent in furosemide loaded IOVs (see Figs 6g-6h). Any investigation of the threshold of inhibition of $Ca2^{+}$-dependent $K^{+}$ channels in IOVs by AGs, would have to make use of furosemide (FUR) loaded IOVs.

Valinomycin (VAL) is an ionophore for alkali cations in biological membranes (Mueller and Rudin, 1967; Ovchinikov, 1974). It has a high selectivity for $K^{+}$ and $Rb^{+}$ over other alkali cations and transports ions across membranes via a carrier mechanism (Pressman et al, 1967; Krasne et al, 1971). Evidence that AG inhibition of $Ca2^{+}$-dependent $K^{+}$ transport into IOVs is specific, rather than the result of simple disruption of the vesicles, was provided by the data showing that even at relatively high concentrations of the toxic AG NEO ($10^{-3}$ M), VAL-induced $^{86}Rb^{+}$ uptake into IOVs is not significantly inhibited (see Results 3.3.1).If VAL induced $^{86}Rb^{+}$ uptake is assumed to reveal the entire intravesicular space, then maximal $Ca2^{+}$-activation ($5x10^{-5}$ M-$Ca2^{+}$) revealed approximately 45% of the intravesicular space. This is in agreement with the results of Garcia-Sancho et al, (1982). That maximal $Ca2^{+}$ activation does not reveal all the vesicular space can be explained in terms of not all the vesicles possessing a $K^{+}$ channel, there being only between 10 and 200 $Ca2^{+}$-dependent $K^{+}$ channels per erythrocyte (Lew et al, 1982; Grygorczyk et al, 1984; Alvarez and Garcia-Sancho, 1987). A further proportion of the IOVs will be right side out and thus quiesent to $Ca2^{+}$ activation.

$Pb2^{+}$ is known to induce a rapid efflux of $K^{+}$ from human

erythrocytes by selectively increasing the membrane permeability to this cation (Orskov, 1935). On the basis of the similarities between this action of $Pb2^+$ and $Ca2^+$-dependent $K^+$ transport, it was proposed that both calcium and lead activate the same membrane channel (Riordan and Passow, 1973; Passow, 1981; Grygorczk and Schwarz, 1983). It has also been reported that $Pb2^+$ can antagonize activation of the channel by $Ca2^+$ (Shields et al, 1985; Alvarez et al, 1986a).

AGs were found to be able to activate $^{86}Rb^+$ uptake into IOVs and inhibit $Ca2^+$-dependent $^{86}Rb^+$ uptake in this study, it is therefore of interest to compare the reported effects of $Pb2^+$ on $K^+$ uptake into IOVs (Alvarez et al, 1986a) with the effects of AGs. AG inhibition of $Ca2^+$-dependent $K^+$ transport into IOVs differs from antagonism by $Pb2^+$ in that $Pb2^+$ decreases the apparent permeability to $^{86}Rb^+$ of the channels with an increase of the half equilibrium time, whereas AGs do not (see Results 3.1). Activation of $^{86}Rb^+$ uptake into IOVs by $Pb2^+$ can be inhibited by $10^{-3}$ M quinine (Alvarez et al 1986a) as can $^{86}Rb^+$ efflux from erythrocyte ghosts containing buffered $Pb2^+$ (Simons, 1979), whereas AG-stimulated $^{86}Rb^+$ could not (see Results 3.6). It would therefore appear that the actions of AGs on IOVs documented here is different to the previously reported actions of $Pb2^+$ on such vesicles (Alvarez et al, 1986a) .

It is felt that caution should be exercised when extrapolating data obtained in erythrocyte models in relation to the toxic events brought about by a drug in other types of tissue. This may be particularly so in this case as different types of $Ca2^+$-dependent $K^+$ channels have been defined in different tissues on the basis of their unitary conductance (determined by means of patch clamp

techniques) (Latorre and Miller, 1983; Marty, 1983; Peterson and Maruyama, 1984; Hunter et al ,1986) or their sensitivity to apamin, a protein toxin of honeybee venom (Burgess et al, 1981; Lazdunski, 1983).

[4.2] Aminoglycoside-stimulated Rb+ uptake into one-step inside out human erythrocyte membrane vesicles.

The experimental conditions employed to study $^{86}Rb^+$ uptake into IOVs experiments are designed to exclude the functioning of all $K^+$ transport systems known to exist in the erythrocyte plasma membrane, with the exception of $Ca2^+$-dependent $K^+$ transport. The incubation buffer does not contain $Na^+$ ions so Na-K-Cl co-transport should not be active (Chipperfield, 1986; O'Grady et al, 1987) nor should the the Na-K pump, which in addition requires $Mg2^+$ and ATP (Post and Jolly, 1957). Despite the conditions employed, an AG-stimulated $^{86}Rb^+$ uptake into the IOVs was observed. Attempts to characterise this AG-stimulated $^{86}Rb^+$ uptake have been described. (see Results 3.4-3.7).

An intersubject variation in the magnitude of AG-stimulated $^{86}Rb^+$ uptake into IOVs prepared from blood drawn from different subjects has been identified (see Results 3.5). IOVs prepared from blood drawn from one particular subject (No 4), consistently exhibited levels of $^{86}Rb^+$ uptake when stimulated with AGs greater than that seen with vesicles prepared from blood drawn from other subjects studied.

Experimental studies have reported a variation in the ototoxic susceptibility of individual animals to AGs (Hawkins, 1959; McGee and Olszewski, 1962; Nakai-Zushi et al, 1981; Shepherd and Clark,

1985). Individual susceptibility to toxicity as a phenomenon is largely unexplained for many exogenous agents. The identification of an effect demonstrable in vitro which shows intersubject variability has important implications if it can be shown to have any relevance to the toxic effects of the compound.

As previously stated AGs vary in their toxicity and can be ranked according to their known potency as toxins. There was a moderate difference between the EC50 calculated for $^{86}Rb^+$ uptake into IOVs prepared from a single subject, for NEO and GEN (see Results 3.4). However NEO, GEN and NET all bring about large uptakes of $^{86}Rb^+$ into IOVs prepared from blood drawn from subject 4. It is felt that to relate this in vitro effect to the variation in toxic effects caused by different AGs in vivo (see Introduction 1.2.1) further investigations are required with a series of AGs of known toxicity, utilizing IOVs drawn from a panel of blood donors.

The AGs under investigation have been shown to stimulate $^{86}Rb^+$ uptake into IOVs at low concentrations (EC50 $1x10^{-5}$ M) (see Results 3.4). It would thus appear reasonable to assume that specific events may be involved. It has been reported that AGs preferentially increase the permeability of phosphoinositide-containing membranes, through their binding to PtdIns(4,5)$P_2$ (Au et al. 1987). It is suggested that such an increase in leakiness may be a critical step leading to cell death. It would be of interest in the context of this study to determine the specificity of AG-stimulated ion uptake into IOVs. If AG-stimulated ion uptake were found to be non-specific, then the effect observed in this study may prove to be due to a general leakiness of the membrane, possibly brought about by AG interaction with phosphoinositides altering the membrane structure.

If AG-stimulated $^{86}Rb^+$ uptake into IOVs were related to AG interaction with phosphoinositides such as PtdIns(4,5)$P_2$, it may be possible to explain the intersubject variation in maximal response (see Results 3.5), in terms of a difference in phophoinositide content of erythrocyte membranes between the individual subjects. It may thus be of interest in any further investigation of this effect to try and relate phosphoinositide content of the vesicles, with the magnitude of the effect.

It has not proved possible to characterise AG-stimulated $^{86}Rb^+$ uptake pharmacologicaly. Inclusion of known inhibitors of erythrocyte membrane $K^+$ transport i.e. quinine (Blum and Hoffman, 1971), furosemide (FUR), bumetanide (Palfrey et al, 1980) and ouabain (Schatzmann, 1953) in the incubation buffer has failed to significantly inhibit AG-stimulated $^{86}Rb^+$ uptake into IOVs (see Results 3.6). The only conditions under which inhibition of uptake was demonstrated, was when vesicles loaded with loop diuretics were employed.

That FUR loading reduces or completely prevents AG-stimulated $^{86}Rb^+$ uptake into IOVs, does not provide evidence that the effect involves the Na-K-Cl co-transporter for two reasons; firstly inhibition of the effect by loop diuretics did not occur at concentrations below $10^{-3}$ M (at and above which loop diuretics are able to inhibit other transport systems besides Na-K-Cl co-transport, (see Introduction 1.4.3) and secondly the Na-K-Cl co-transporter requires the presence of all three ions, for it to function (O'Grady et al, 1987) and the incubation buffer is $Na^+$ free. Addition of AGs to the incubation buffer did not introduce any $Na^+$ ions via contamination, neither did AGs seem to replace $Na^+$ in a co-transport system. Addition of NaCl to the incubation buffer

brought about no additional $^{86}Rb^+$ uptake, whereas similar concentrations of AGs did stimulate $^{86}Rb^+$ uptake (see Results 3.7).

There is no information available as to the effect of AGs on Na-K-Cl co-transport. At concentrations greater than $10^{-4}$ M, FUR inhibits various transport systems in the human erythrocyte plasma membrane besides Na-K-Cl co-transport (Palfrey et al, 1980; Ellory et al, 1983a; Brazy and Gunn, 1976). One such transport system is the K-Cl co-transporter (Lauf, 1985; Wiater and Dunham, 1983) which has been shown to exist in human erythrocytes under normal conditions (Berkowitz and Orringer, 1987; Kaji, 1986). Inhibitors specific for K-Cl co-transport at low concentrations are not available (Lauf, 1985). In the absence of a specific inhibitory substance, it is not possible to show pharmacologicaly, whether or not AG-stimulated $^{86}Rb^+$ uptake involves activation of K-Cl co-transport. This co-transport system is however chloride dependent. Substitution of $Cl^-$ with $SCN^-$, in the incubation buffer did not however prevent AG-stimulated $^{86}Rb^+$ uptake (see Results 3.7). It would therefore appear that the uptake of $^{86}Rb^+$ observed on stimulation of the IOVs with AGs did not involve K-Cl co-transport.

As a model system for the investigation of exogenous compounds on membrane transport systems one-step IOVs may have certain disadvantages; Like other IOV preparations, heterogeneity of size, sideness and permeability may occur between preparations and create ambivalence in the interpretation of ion transport kinetics (Lew and Seymour, 1982). A certain amount of variation in the rates of $^{86}Rb^+$ uptake, stimulated by $Ca2^+$ between different preparations of IOVs, even when prepared from the same source of blood has been observed in this study.

Besides being easily and rapidly prepared, the main advantage of one-step IOVs is the preservation of $Ca2^+$-dependent $K^+$ transport. This is thought to be due to the minimum amount of washing involved in the preparation of one-step IOVs, allowing the maintainance of a loosely associated membrane protein component vital to $Ca2^+$-dependent $K^+$ transport in the IOVs (Lew and Seymour, 1982). A variable loss of membrane protein components during preparation of the vesicles, may however lead to apparent differences in maximum transport rates. This, it is felt should be borne in mind when enterpreting $Ca2^+$-dependent $K^+$ uptake data, in relation to ion movements which may or may not be dependent on such membrane proteins.

As an <u>in vitro</u> model system for the investigation of the effect of AGs on $Ca2^+$-dependent $K^+$ transport one-step IOVs have been only a moderate success due to the co-existence of an AG-stimulated $^{86}Rb^+$ uptake. This uptake can however mostly be abolished by treatment with loop diuretics, thus such IOVs are the preparation of choice when investigating the effect of AGs on $Ca2^+$-dependent $K^+$ channels in the human erythrocyte plasma membrane. How treatment with loop diuretics abolishes AG-stimulated $^{86}Rb^+$ uptake into IOVs is unclear, as is the mechanism by which AGs bring about this ion movement.

[4.3] <u>Effect of aminoglycoside antibiotics on Ca2+-dependent K+ transport in intact human erythrocytes.</u>

As with one-step inside out vesicles the aminoglycoside antibiotic GEN has been demonstrated to be able to inhibit $Ca2^+$-dependent $K^+$-transport in intact erythrocytes. GEN was

shown to be able to inhibit the rate of cell shrinkage due to $Ca2^+$-dependent KCl loss, whether applied internally or externally (see Results 3.8). This is at odds with data obtained with IOVs, wereby the rate of $Rb^+$ uptake was apparently unaltered by addition of AGs to the incubation buffer.

When using whole cells, inhibition of $Ca2^+$-dependent $K^+$ transport has only been demonstrated at relatively high internal concentrations of the drug. It is difficult to imagine a situation in vivo where such a relatively large concentration of the drug could accumulate intracellularly. With regard to this particular erythrocyte ion transport model, it should however be considered whether increasing intracellular calcium by means of an ionophore genuinely represents the situation in vivo.

That GEN when applied externally was able to inhibit $Ca2^+$-dependent efflux was unexpected. AG antibiotics are unable to passively cross the plasma membrane, this effect is therefore not due to direct interaction of the drug with the calcium binding site. What is more likely, as the degree of inhibition is dependent on the amount of calcium present, is that the AG is antagonising calcium uptake brought about by the ionophore. Alternatively being cationic, GEN may be acting by blocking the external $K^+$ binding sites, external $K^+$ being a requirement for $Ca2^+$-dependent $K^+$ transport (Knauf et al , 1975; Heinz and Passow, 1980).

As when AGs were added to IOVs (AG-stimulated Rb uptake)( see Results 3.5), a variation in response according to the source of the erythrocytes used was found. This lends further credence to the possibility that variability in individual susceptibility to drug toxicity may be due to differences in cell membrane composition. Whether this be a difference in the number of a particular type of

receptor, an ion channel or even the chemical composition of the membrane warrants further investigation.

It has been reported that reduction of internal ionized magnesium in ferret erythrocytes with A23187 and EDTA approximately doubles bumetanide-resistant potassium transport (Flatman, 1988). Furthermore Ellory et al. (1983) showed that reduction of internal magnesium with EDTA and A23187 stimulated bumetanide-resistant uptake into human erythrocytes fivefold. Flatman (1988), suggested that the human erythrocyte data could be explained by removal of magnesium, activating K-Cl co-transport (which is bumetanide resistant).

The increased activation of KCl efflux seen in subject 7 could possibly be explained in terms of the activation of K-Cl co-transport. It is conceivable that as AGs compete with $Mg2^+$ in addition to $Ca2^+$, that addition of A23187 to GEN loaded erythrocytes lowers or displaces internal $Mg2^+$ and thus activates K-Cl co-transport. This effect would be superimposed on any inhibition of $Ca2^+$-dependent $K^+$ transport by the AG and would consequently mask it. This could be tested by examining the effect of A23187 on GEN loaded erythrocytes in the absence of external $CaCl_2$.

Erythrocytes contain an outwardly directed $Ca2^+$ pump stimulated by calmodulin (Roufogalis, 1979) and the possibility exists that AGs could mimic $Ca2^+$ and activate calmodulin in intact erythrocytes. If this were so they would be expected to enhance the efficiency of the $Ca2^+$-pump, which would decrease KCl efflux by reducing the intracellular concentrations of $Ca2^+$. Similarly if AGs inhibit the $Ca2^+$-pump then the inhibition of active transport out of the cell could result in a build-up of $Ca2^+$ and the activation

of $Ca2^+$-dependent KCl efflux. This is another possible explanation of the data obtained with subject 7's erythrocytes.

This particular experimental approach to studying the effects of cationic drugs (ie AGs) is beset by a number of problems which may influence the interpretation of the data. Firstly A23187 is not specific for $Ca2^+$ and may effect other divalent cations (Pfeiffer et al, 1978) and secondly loading cells with AGs may effect other membrane transport systems besides the one under investigation. It is therefore felt that caution should be exercised when interpreting and extrapolating these data to the situation in vivo. The use of the intact human erythrocyte as a model system, does however allow the complexities of drug interactions with the plasma membrane in vivo to be fully appreciated.

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