SODIUM DEPENDENT MECHANISMS REGULATING MEMBRANE POTASSIUM ($^{86}Rb^{+}$) PERMEABILITY IN MAMMALIAN EXOCRINE GLANDS *IN VITRO.*

A thesis presented to the University of Glasgow by JOHN D. PEDIANI *M. I. Biol*, a candidate for the degree of Doctor of Philosophy.

Institute of Biomedical and Life Sciences,
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Declaration

The investigations presented in this thesis were conducted by its author. No part of the work has previously been presented in fulfilment of the regulations for any degree or diploma, either at this University or any other institution. Some of the material presented has been previously published (Bovell, Elder, Pediani & Wilson, 1989b; Wilson, Pediani, Jenkinson & Elder, 1992; Pediani, McEwan, Elder & Wilson, 1994; Pediani & Wilson, 1994).

J.D.Pediani

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CONTENTS.

	PAGE.
ACKNOWLEDGEMENTS	1.
SUMMARY	2.
CHAPTER 1 : GENERAL INTRODUCTION	5.
CHAPTER 2 : MATERIAL AND METHODS	18.
CHAPTER 3 : THE EFFlUX OF POTASSIUM FROM THE RAT SUBMANDIBULAR GLAND UNDER SODIUM-FREE CONDITIONS*IN VITRO*	25.
CHAPTER 4 : Na^+-DEPENDENT K^+ EFFLUX FROM THE RAT SUBMANDIBULAR GLAND *IN VITRO* : STUDIES IN HCO_3^--CONTAINING SOLUTIONS	41.
CHAPTER 5 : Na^+-DEPENDENT MECHANISMS REGULATING K^+ ($^{86}Rb^+$) AND Ca^{2+} ($^{45}Ca^{2+}$) PERMEABILITY IN EXOCRINE GLANDS *IN VITRO*	51.
CHAPTER 6 : THE EFFECT OF THE PHORBOL ESTER TPA UPON THE CHOLINERGIC REGULATION OF MEMBRANE K^+ ($^{86}Rb^+$) PERMEABILITY IN THE RAT SUBMANDIBULAR GLAND *IN VITRO*	62.
CHAPTER 7 : GENERAL DISCUSSION	69.
REFERENCES :	78.
APPENDIX 1 :	89.
PUBLISHED WORK :	95.

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SUMMARY.

Fragments of rat submandibular gland were pre-loaded with $^{86}Rb^+$, a radioactive isotopic marker of cellular K^+ permeability, and rate constants for $^{86}Rb^+$-efflux were determined during superfusion with unlabelled experimental physiological saline solutions which were buffered with either CO_2/HCO_3^- or HEPES/NaOH.

Initial $^{86}Rb^+$-efflux experiments demonstrated that in the presence of external Na^+ ($[Na^+]_o$), acetylcholine (ACh) could evoke a rapid increase in membrane K^+ permeability. This increase could be resolved into two separate components, a Ca^{2+}-independent transient phase which was attributed to the mobilisation of Ca^{2+} from internal stores and a Ca^{2+}-dependent sustained phase which was evoked when extracellular Ca^{2+} ($[Ca^{2+}]_o$) was transported into the rat submandibular acini. This biphasic response to ACh was slightly impaired under HCO_3^--free conditions.

It has been suggested that receptor-regulated Ca^{2+}-influx in exocrine organs occurs via a Na^+-dependent mechanism (Gallacher & Morris,1987), so the effects of removing external Na^+ ($[Na^+]_o$) upon this biphasic increase in membrane K^+ permeability was investigated. The impermeant cation N-Methyl-D-Glucammonium ($NMDG^+$) or the permeant cation lithium (Li^+) were used as Na^+ substituents. It was found that the sustained response to ACh was significantly inhibited. These findings therefore supported Gallacher & Morris's (1987) hypothesis that Ca^{2+}-inflow, which supports the sustained increase in the K^+ efflux rate from rodent salivary acini, occurs via a Na^+-dependent process (Gallacher & Morris, 1987).

Although the data from the Na^+-free experiments supported Gallacher and Morris's hypothesis, the data from these experiments also suggested that $NMDG^+$ may exert an inhibitory effect on the transient response (Ca^{2+} mobilisation). This slight inhibition could be a direct effect of $NMDG^+$ or could be due to the initially very low Ca^{2+} (0.02 μmol l^{-1}) composition of these HEPES-buffered, $NMDG^+$-solutions since the salivary fragments would have been exposed to an outwardly directed Ca^{2+} gradient. The effects of $NMDG^+$ upon membrane K^+ permeability in the rat submandibular gland were therefore re-examined using HCO_3^--buffered, $NMDG^+$-solutions in which the $[Ca^{2+}]_o$ was never lower than 0.2 μmol l^{-1}. The results from these experiments demonstrated that both components of the response to ACh were significantly inhibited in the presence of $NMDG^+$. These latter findings, therefore do not support the view of Gallacher & Morris (1987) that only Ca^{2+}-inflow into rodent submandibular acini is inhibited in the absence of $[Na^+]_o$. Furthermore, these findings contrast with

analogous $NMDG^+$-experiments undertaken in the human sweat gland where only Ca^{2+} mobilisation appears to be Na^+ dependent (Wilson, Bovell, Elder, Jenkinson & Pediani, 1990). The physiological basis for this dependence upon $[Na^+]_o$ is not yet known. However, one hypothesis is that if proton (H^+) extrusion via the Na^+-H^+ exchanger is blocked in the presence of $NMDG^+$, then the resultant fall in intracellular pH (pH_i) could inhibit the mobilisation of Ca^{2+} from internal and external pools (Grinstein & Goetz, 1985; Siffert & Akkerman, 1987; Gallacher & Morris, 1987). It is thus possible that the inhibitory effects evoked by $NMDG^+$ in the rat submandibular gland and human sweat gland may also be due to inhibition of this transport system. This possibility was therefore investigated by examining the degree to which amiloride, a potent inhibitor of Na^+-H^+ exchange, could impair the cholinergic regulation of membrane K^+ permeability in the rat submandibular gland and the human sweat gland. Amiloride (1 mmol l^{-1}) did not affect membrane K^+ permeability in the rat submandibular gland, but this compound did, however, impair the regulation of membrane K^+ permeability in the human sweat gland. These data suggested that the ACh-evoked K^+ permeability coupling process in the human sweat gland is more sensitive to a fall in pH_i.

Another thesis put forward to explain this Na^+-dependency is that the transport of $[Ca^{2+}]_o$ into rodent submandibular acini occurs via 'reversed' Na^+-Ca^{2+} exchange i.e. $[Na^+]_i$ exchanged for $[Ca^{2+}]_o$, (Gallacher & Morris, 1987). I therefore used $^{45}Ca^{2+}$ to monitor the transport of Ca^{2+} in the rat submandibular gland. The data from these experiments demonstrated that the basal $^{45}Ca^{2+}$ efflux rate was unaffected when either Na^+ or Ca^{2+} was removed from the superfusing control solution. These results therefore suggest that Na^+-Ca^{2+} exchange does not play an important role in maintaining low internal Ca^{2+} in this tissue and it is therefore very unlikely that Ca^{2+}-influx occurs via 'reversed' Na^+-Ca^{2+} exchange.

Secretagogues that bind to muscarinic cholinoreceptors are functionally coupled to the enzyme phospholipase C which catalyses the hydrolysis of the membrane phospholipid phosphatidyl-inositol-bisphosphate (PIP_2) (Berridge & Irvine, 1984; 1989). The hydrophobic product of PIP_2 hydrolysis is a diacylglycerol (DAG) molecule that remains in the plasma membrane where it allosterically modulates the activity of the Ca^{2+} and phospholipid dependent enzyme, protein kinase C (PKC) (Nishizuka, 1984 & 1988). The physiological significance of this event is not fully understood. The phorbol ester 12-0 tetra-decanoylphorbol-13-acetate (TPA) is a potent exogenous activator of PKC (Nishizuka 1984, 1986) that can inhibit responses to Ca^{2+} mobilising agonists

(Ansah, Dho & Case, 1986; Llano & Marty, 1987; Maruyama, 1989). This result has led to the hypothesis that DAG may be able to regulate the stimulus-response coupling process by exerting a negative feedback mechanism. In order to test this hypothesis, I therefore explored the effects of TPA upon the ACh-evoked, biphasic increase in membrane K^+ permeability in the rat submandibular gland.

TPA only inhibited the transient component of the response to ACh. This result, therefore suggests that PKC may be capable of exerting a negative feedback mechanism on the mobilisation of Ca^{2+} from intracellular stores in the rat submandibular gland, but has no effect on Ca^{2+}-inflow.

CHAPTER 1 : GENERAL INTRODUCTION.

PHYSIOLOGICAL FEATURES OF SALIVARY GLANDS.

Structure and innervation.

In most mammalian species, the principle salivary glands communicating with the mouth and pouring their secretion into its cavity are the parotid, submandibular and sublingual. Mammalian salivary glands consist of parenchymal and stromal components. The parenchyma resembles a bunch of grapes in which the branching stems represent the ductal network and the grapes represent the acini, the secretory portion of the gland. The minimal physiological parenchymal unit of a salivary gland is sometimes referred to as a salivon (Johnson, 1981) and consists of an acinus and those duct segments that modify the acinar product (figure 1.1). The acinus is composed of pyramidal secretory cells (acinar cells) arranged about a central lumen and is surrounded by myoepithelial cells (Garrett, 1974). A basal lamina separates the secretory and myoepithelial cells from the adjacent connective tissue. The lumen of the acinus leads to a duct system that has three distinct segments (figure 1.1) : the intercalated duct, the striated duct and the excretory duct. The cells of the intercalated duct are also surrounded by myoepithelial cells.

Salivary glands are classified as serous or mucous depending on the composition of their secretions. The secretions from mucous glands (*e.g.* rat sublingual gland) are viscous. In contrast, the secretions released from serous glands (*e.g.* rat parotid gland) are more watery. A number of glands have been designated seromucous as their secretions are of an intermediate viscosity (e.g human and rat submandibular gland). Acini are thus composed of either serous or mucous cells. Both types of cell undergo cycles of activity. During part of the cycle the serous cells synthesise zymogen granules whereas the mucous cells form mucus which is stored as mucinogen granules.

The major salivary glands receive dual innervation from the autonomic nervous system (Garret, 1966). Parasympathetic innervation reaches the glands from the cranial outflow by way of the glossopharyngeal nerve (IX), the chorda tympani branch of the facial nerves (VII), and the hypoglossal nerve (XII), (Burgen, 1964; Garret & Holmberg, 1972; Greene, 1968). Parasympathetic ganglia lie close to the glands and their postganglionic fibres are distributed to all of the secretory cells. Activation of the parasympathetic pathway results in copious secretion. The final mediator of this pathway is acetycholine (ACh) and the injection of parasympathomimetric drugs can produce an equal but not a

greater response. The sympathetic innervation reaches the glands via the superior cervical ganglion (Yoshida, Sprecher, Schneyer & Schneyer, 1967; Garret & Thulin, 1975). The post ganglionic fibres are all adrenergic, but vasoactive intestinal polypeptide (VIP) and adenosine tri-phosphate (ATP) may also be released from these fibres (Burnstock, 1972, 1990a, b; Gabella, 1981).

Function.

Salivary secretions have a number of important biological functions : digestion, protection, maintenance of the teeth and growth development. The digestive function is accounted for by the presence in saliva of the enzyme alpha amylase which breaks down the 1,4-glycosidic bonds of carbohydrates. Saliva also serves to lubricate the epithelial surfaces of the buccal cavity, thereby aiding the chewing and swallowing of food.

The protective functions include general protection of the oral cavity. For example, the salivary glands secrete copiously just before one vomits and the saliva neutralises the regurgitated gastric acid juice. Saliva also plays several specific roles in maintaining the teeth : 1). calcium and phosphate of the saliva are instrumental in the post eruptive mineralization of newly erupted teeth and in the repair of white spot (precarious) lesions of the enamel, 2). proteins of the saliva cover the teeth with a protective coat called the acquired pellicle, 3). constituent antibodies and antibacterial agents serve to retard the activity of microbes in the oral cavity which would cause tooth decay (reviewed by Burgen & Emmelin, 1961).

It has been postulated that salivary secretions can aid tissue development, since hormones such as nerve growth factor (Goldstein and Burdman, 1965; Levi-Montalcini & Angeletti, 1961; Levi-Montalcini & Cohen, 1960), epidermal growth factor (Cohen, 1962; Cohen & Elliott, 1963) and mesodermal growth factors (Weimar & Haraguchi, 1975) have been isolated from salivary samples.

Formation of saliva.

It is now well established that saliva is formed by a process which is similar to the "two stage" hypothesis postulated by Thaysen (1960). This hypothesis proposes that the acinar cells secrete an isotonic "primary" fluid with an osmolarity and ionic composition similar to that of plasma. However, as this fluid passes along the ductal system its composition is modified by secondary processes which involve the active reabsorption of sodium (Na^+) and chloride (Cl^-) ions resulting in a hypotonic secretory product. In salivary glands the striated ducts play a major role in the reabsorption of Na^+, although some

reabsorption also takes place in the larger excretory ducts. At the same time that Na^+ and Cl^- are being absorbed, K^+ and HCO_3^- are secreted by the duct cells into the saliva, however, the amount of K^+ secreted does not match the amount of Na^+ being reabsorbed, so the saliva remains hypotonic (Young, 1979; Young & van Lennep, 1978, 1979; Young & Schneyer, 1981).

Direct evidence of an isotonic primary secretion and hypertonic reabsorption came from a number of different experimental techniques :

Martinez, Holzgreve & Frick (1966); Young & Schögel (1966) and Martin & Young (1971) successfully used the micropuncture method to collect samples of precursor saliva from the most distal regions of the ductal system (acinar-intercalated duct region, see figure 1.2). Analysis of these samples revealed that the ionic composition of the precursor saliva was always very similar to that of plasma.

If Thaysen's (1960) hypothesis is correct then at high flow rates you would expect the composition of the secretory product to differ from that produced during a slow flow rate since their would be less time for modification within the ductal epithelium. It is now well known that such a relationship does exist when salivary glands secrete in response to parasympathetic stimulation (e.g. Young & Martin, 1971; Martinez, Quissell, Wood & Giles, 1975a; Schneyer & Hall, 1965; Yoshidia, Sprecher, Schneyer & Schneyer, 1967; Smaje, 1973) and α-adrenoreceptor activation (Martinez *et al.*, 1975a; Young, Cook, Jones, McGirr & Thompson, 1979).

The enzyme Na^+, K^+ ATPase, which splits ATP and releases energy and so drives the Na^+/K^+ pump plays a central role in the active transport of ions within the ductal epithelium. Large quantities of this enzyme has been localised in the cat submandibular gland on the basal membrane of the striated ducts, whilst only small amounts of this enzyme are found in the cells of the intercalated duct and acini (Bungard, Møller & Poulsen, 1977). These data therefore provide the evidence that the striated duct cells are responsible for modifying the primary fluid, since the intracellular concentration of Na^+ in these cells will be kept low by active transport and will allow Na^+ to move from the lumen to the blood-side (see Young, 1979; Young & van Lennep, 1979; Young & Schneyer, 1981).

THE MECHANISM OF EXOCRINE FLUID SECRETION.

The secretory potential.

Up to the early 1950's the mechanisms responsible for the formation of primary saliva were not fully understood. However, two significant discoveries by Lundberg (1955; 1958) aided understanding of the response to the cholinergic agonist acetylcholine (ACh).

Lundberg (1955) using a double-barrelled microelectrode (Coombs, Eccles & Fatt, 1955) found that electrical stimulation of the parasympathetic nerves or intra-arterial injection of ACh, adrenaline or pilocarpine evoked a hyperpolarisation of the acinar cell membrane potential. This was referred to as the secretory potential and Lundberg (1955) postulated that it indicated the existence of an active electrogenic pump mechanism : *e. g.* either active cation extrusion or active anion uptake.

Subsequently, Lundberg (1958) found that stimulant-elicited hyperpolarisations of the salivary acinar membrane were dependent upon the presence of Cl^- ions in the extracellular fluid and concluded that ACh could activate electrogenic Cl^- ion uptake into salivary acini. This finding could not, however, be reproduced by Yoshimura & Imai (1967) or Petersen & Poulsen (1968b) who found that large secretory potentials could still be evoked when extracellular Cl^- was replaced with sulphate.

Studies of potassium transport.

Burgen (1956), investigated how K^+ ions were transported in mammalian salivary glands (dog submandibular and parotid gland and cat submandibular gland). He demonstrated two phases in the secretion of K^+ ions (figure 1.3). The first occurred at the onset of cholinergic activation was transient and very rapid. Most of this secreted K^+ was derived from the cytoplasm of the secretory cells. In the subsequent phase, the concentration of K^+ ions in the saliva was lower and most of the K^+ was derived from the plasma and transported across the glandular epithelium. Burgen (1956) also showed that during the initial part of this response, K^+ ions were lost to the blood-stream over the same time course. He therefore proposed that the mechanism underlying the loss of K^+ ions from the cytoplasm of the secretory cell to the saliva and to the blood were identical.

Petersen (1971) provided the first evidence that it was this efflux of K^+ ions rather than the active uptake of Cl^- ions which was responsible for the development of the secretory potential first described by Lundberg (1955). He demonstrated that the secretory potential was maintained when the cat submandibular gland was perfused with a Cl^--free solution (Petersen, 1971) and

showed that the metabolic inhibitor 2,4-dinitrophenol did not effect the amplitude of the secretory potential (Petersen, 1970b). Furthermore, he showed that the size of the secretory potential was dependent upon the concentration of extracellular K^+ in such a way that the amplitude was potentiated if extracellular K^+ was lowered and inhibited if the extracellular K^+ was raised (Petersen, 1970c). From these results, he proposed that the secretory potential was due to an increase in membrane K^+ permeability rather than active ion transport.

The role of extracellular calcium.

Douglas & Poisner (1963) demonstrated in the cat submandibular gland that fluid secretion in response to both ACh and adrenaline was dependent upon the presence of Ca^{2+} in the extracellular fluid (Douglas & Poisner, 1963). Schramm & Selinger (1974; 1975) similarly found that cholinergically or α–adrenergically-evoked K^+ loss from the rat parotid gland was sustained and required the presence of extracellular calcium ($[Ca^{2+}]_o$), (Schramm & Selinger, 1974; 1975). Contrary to these findings, electrophysiological experiments (Petersen & Pedersen, 1974) suggested that α-adrenergically-evoked K^+ efflux from the mouse parotid gland was transient and independent of $[Ca^{2+}]_o$. This discrepancy was resolved by Putney (1976a), who used the radiotracer $^{86}Rb^+$ to monitor K^+ transport in the rat parotid gland. He discovered that carbachol and phenylephrine-evoked K^+ efflux consisted of two phases : a transient phase that persisted when Ca^{2+} was removed from the perfusion solution and a sustained phase which was acutely dependent upon the presence of Ca^{2+} (Putney, 1976a). Similar results were also obtained from the rat lacrimal and sublingual gland in response to the cholineric secretagogue carbachol (Parod & Putney, 1978b; Putney, Leslie & Marier, 1978). Putney, therefore concluded that the loss of cellular K^+ from exocrine acini was mediated by an agonist-evoked increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), which was initiated by the mobilisation of Ca^{2+} from internal Ca^{2+} stores (transient phase) and subsequently sustained by an influx of Ca^{2+} from the extracellular fluid (sustained phase) (Putney, 1976a; Parod & Putney, 1978; Putney, Leslie & Marier, 1978).

Fluid and electrolyte secretion.

Petersen (1970a) examined the ACh-evoked release of K^+ from the cat submandibular gland into the perfusion medium and its subsequent reuptake into the gland. He demonstrated that K^+ reuptake, but not K^+ efflux, was abolished if Cl^- or Na^+ ions were removed from the perfusing medium (Petersen, 1970a). He also established that K^+ reuptake following ACh-elicited K^+ release was

inhibited by oubain, an inhibitor of the Na^+/K^+ pump (Petersen, 1970a). These results led Petersen to postulate that the reuptake of K^+ was a Cl^- and Na^+ dependent process and that this process was also dependent upon the operation of the Na^+/K^+ pump (Petersen, 1970a).

In 1983, it was found that the basolateral membrane of various acinar cells contained Ca^{2+}- and voltage-activated K^+ channels (Maruyama, Gallacher & Petersen, 1983a; Maruyama, Petersen, Flanagan & Pearson, 1983b). This provided the explanation, at the molecular level, for the stimulant-evoked acinar cell K^+ loss. At this point in time, however, it was not clear how an increase in basal membrane K^+ conductance could regulate fluid secretion. It was, however, known that the inward movement of Na^+, K^+ and Cl^- into red blood cells (Chipperfield, 1981) and rabbit kidney epithelial cells (Greger & Schlatter, 1981) were coupled and took place via a Na^+-K^+-Cl^- co-transport system. Petersen and Maruyama (1984) proposed that Na^+, K^+ and Cl^- may be transported into the cat submandibular gland in a similar manner and on this basis they proposed a cellular model (figure 1.4) to explain electrolyte transport in the basolateral membrane of fluid secreting salivary acini. In summary the model was based on the following criteria :

1) The three transport proteins (Na^+-K^+-$2Cl^-$ co-transporter, Na^+/K^+ pump and Ca^{2+}- and voltage-activated K^+ channel) in the basolateral membrane acted together as a Cl^- pump.

2) These three functionally linked transporters are controlled by minute changes in $[Ca^{2+}]_i$ that regulate the opening of basal K^+ channels and thereby the reuptake of K^+ into the cell via the Na^+-K^+-$2Cl^-$ co-transporter (stoichiometry proposed by Geck, Pietrzyk, Burckhardt, Pfeiffer & Heinz, 1980).

3) The Na^+/K^+ pump ensures that Na^+ entering into the cell via the co-transporter is extruded from the cytosol.

4) The Na^+/K^+ pump maintains an inwardly directed electrochemical gradient for Na^+ influx and it is this driving force which allows Cl^- and K^+ ions to accumulate above their equilibrium potential (e.g. secondary active transport).

5) The rate of Na^+ and Cl^- uptake is directly linked to the rate of transport in the cycle of K^+ outflow and uptake.

6) The main regulatory component of the model is the voltage- and Ca^{2+}-activated K^+ channel located on the basolateral membrane.

7) It was also proposed that Cl^- transport into the lumen could occur via luminally localised Ca^{2+}-activated Cl^- channels.

From this model, it was proposed that the cholinergic loss of cellular K^+ from secretory epithelia could be explained as follows : the binding of ACh to its target receptor in the basolateral membrane evokes an increase in the $[Ca^{2+}]_i$. This rise is initiated by the release of Ca^{2+} from internal stores and is subsequently sustained by an influx of Ca^{2+} from the external fluid (Putney, 1976a). The rise in $[Ca^{2+}]_i$ activates Cl^- channels in the apical membrane (Findlay & Peterson, 1985; Peterson, 1986) causing Cl^- to flow down its electrochemical gradient into the lumen. The shift in Cl^- increases the negativity of the luminal fluid above that of the interstitial fluid thereby generating a transepithelial potential difference. Charge balance on either side of the secretory cell is maintained by the diffusion of Na^+ from the blood side to the lumen via a transcellular or paracellular route (Ussing, Bindslev, Lassen & Sten-Knudsen, 1981). This process elevates the osmolarity of the luminal fluid and so creates a driving force for the passage of water across the epithelia cell into the lumen (Lundberg, 1958; Silva, Stoff, Field, Fine, Forrest & Epstein, 1977; Frizzell, Field & Schultz, 1979; Petersen & Maruyama, 1984).

Another consequence of the increase in luminal Cl^- conductance is a tendency for the secretory cell to become depolarised. Since the driving force for Cl^- secretion is dependant upon the maintenance of a negative intracellular potential, the rate of Cl^- secretion would therefore become diminished. However, this does not occur, because the rise in $[Ca^{2+}]_i$ and the depolarisation also activate Ca^{2+}- and voltage-dependent K^+ channels located on the basolateral membrane (Maruyama, Gallacher & Peterson, 1983a; Maruyama & Petersen, 1984a; Welsh, 1983; Findlay, 1984; Morris, Fuller,Gallacher & Scott, 1987) and this results in K^+ release into the interstitium. This event tends to repolarise the cell and the resultant increase in the membrane potential provides the driving force to sustain Cl^- secretion into the lumen (Welsh, 1983).

Experimental evidence for linked Na^+, K^+ and Cl^- transport in salivary acini.

Experimental data was subsequently produced to support linked Na^+, K^+ and Cl^- movement into salivary glands via a basolaterally localised Na^+-K^+-Cl^- co-transport carrier. Exley, Fuller & Gallacher (1986) demonstrated in salivary glands, that after cholinergically-evoked K^+ outflow, K^+ reuptake was inhibited by piretanide, an inhibitor of Na^+-K^+-Cl^- co-transport. They also showed that the

reuptake of K^+ was a Cl^--dependent process. This finding, however, was not absolute because Br^- could support K^+ transport.

The stoichiometry of the Na^+-K^+-ATPase pump has been extensively studied and if the coupling of Na^+ transported to Na^+ pumped by the ATPase is 1, then the amount of Na^+ transported per mole of O_2 consumed should be a maximum of 18:1. Such a figure has been measured in frog skin and toad bladder epithelia (Zerahn, 1956; Leaf & Renshaw, 1957), but, in kidney and shark rectal gland values closer to 30:1 have been measured (Lassen, Munck, Hess-Thaysen, 1961; Silva, Stoff, Solomon, Rosa, Stevens & Epstein, 1980). In the shark rectal gland the relatively low O_2 consumption is associated with Na^+-K^+-Cl^- co-transport. Since two Cl^- ions are transferred for every Na^+ pumped by the Na^+-K^+-ATPase it would seem that a maximum of 36 mol NaCl should be secreted per mol of O_2 consumed, sinced 2 mol Na^+ crossing the acinar epithelium via the paracellular route will accompany 2 mol of Cl^- and the K^+ will be recycled. Thus, if the metabolic rate of acinar secretion is measured, one can distinguish between Na^+-Cl^- co-transport or Na^+-K^+-Cl^- co-transport (i.e if O_2 consumption in the perfused gland is compared to the rate of Na^+ transport during activation). Based on this principle, Smaje, Poulsen & Ussing (1986) have produced strong evidence for the existence of a Na^+-K^+-Cl^- co-transport system in the perfused rabbit submandibular gland.

Ionic mechanisms regulating calcium influx.

In electrically excitable cells, it is known that $[Ca^{2+}]_o$ enters into the cytosol via voltage-activated Ca^{2+} channels (Reuter, 1983, 1985), the initial event being an agonist-evoked depolarisation which results in the activation of voltage-sensitive Ca^{2+} channels. However, electrophysiological experiments undertaken on rodent salivary acini indicate that these cells do not possess these channels and the mechanism by which the inflow of $[Ca^{2+}]_o$ occurs remains obscure (Berridge, 1980; Putney, 1986; Petersen & Gallacher, 1988; Gallacher, 1988).

OBJECTIVES OF PRESENT STUDY.

Experiments using fluorescent pH indicators have shown that removal of external HCO_3^- reduces cytosolic H^+-buffering capacity (Gillespie & Greenwell, 1988; Steward, Seo & Case, 1989). It has also been demonstrated that the activity of Ca^{2+}-activated K^+ channels in pancreatic ß cells, Necturus gall-bladder epithelial cells and cultured renal medullary thick ascending limb cells is reduced when pH_i falls (Cook, Ikeuchi, & Fujimoto, 1984; Cornejo, Guggino & Guggino, 1989; Copello, Segal & Reuss, 1991). Although, Petersen (1971) demonstrated

that cholinergically-evoked fluid secretion in the cat submandibular gland was not HCO_3^--dependent, it is still not known what effect the removal of HCO_3^- from the control solution will have upon the efflux of K^+ across the basolateral membrane of the rat submandibular gland. Therefore, one of my intentions was to assess the role played by HCO_3^- in regulating K^+ efflux.

The mechanisms regulating the inflow of extracellular Ca^{2+} into salivary acini are not yet known (Putney, 1986; Petersen & Gallacher, 1988; Gallacher, 1988). Patch-clamp experiments undertaken on mouse submandibular acini indicated that Ca^{2+}-activated K^+ channels become refractory to Ach if Na^+ is removed from the bathing solution and replaced with the impermeant cation N-Methyl-D-Glucammonium ($NMDG^+$) (Gallacher & Morris 1987; Morris, Fuller & Gallacher, 1987). This result led Gallacher & Morris to postulate that ACh-evoked Ca^{2+}-inflow into rodent salivary acini was Na^+-dependent and possibly occurred via reversed Na^+-Ca^{2+} exchange (*i.e.* internal Na^+ exchanged for $[Ca^{2+}]_o$). Contrary to the findings of Gallacher & Morris (1987), Petersen (1970) and Putney (1978) have demonstrated that ACh can evoke K^+ efflux from salivary glands if $[Na^+]_o$ is replaced with the permeant cation Li^+. There are thus conflicting views regarding the role played by $[Na^+]_o$ in controlling K^+ efflux. In an effort to resolve this, I have therefore compared the effects of replacing $[Na^+]_o$, with either $NMDG^+$ or Li^+, upon the cholinergic regulation of membrane K^+ ($^{86}Rb^+$) permeability in the rat submandibular gland. Furthermore, to explore the possibility that the Na^+-Ca^{2+} exchanger may mediate Ca^{2+}-influx into the acini of the rat submandibular gland, radiotracer experiments were also undertaken using $^{45}Ca^{2+}$ as an isotopic marker for the transport of cellular Ca^{2+}.

An alternative hypothesis proposed by Gallacher & Morris (1987) was that the influx of $[Ca^{2+}]_o$ into rodent acini may not be directly dependent upon sodium, but that the removal of $[Na^+]_o$ compromises Na^+-H^+ exchange and so evokes a fall in pH in the cytosol of the acini. Arkle, Gillespie and Greenwell (1988) provided the evidence to support this proposal, as they showed that $[Ca^{2+}]_i$ is elevated and pH_i lowered when unstimulated rodent salivary acini are exposed to $NMDG^+$-containing solutions. These findings led Arkle *et al.*, (1988) to propose that $NMDG^+$ did not act as a simple substitute for $[Na^+]_o$ and that its effects may be secondary to a fall in pH_i. It is thus possible that the Ca^{2+}-influx pathway in rodent salivary acini and other fluid secreting epithelia is regulated via a pH_i sensitive mechanism (e.g. Na^+-H^+ exchange). In order to examine this posssibility, I therefore examined the effects of amiloride, an inhibitor of Na^+-H^+ exchange, upon the cholinergic regulation of membrane K^+ permeability in exocrine glands (*e.g.* the rat submandibular and human sweat gland).

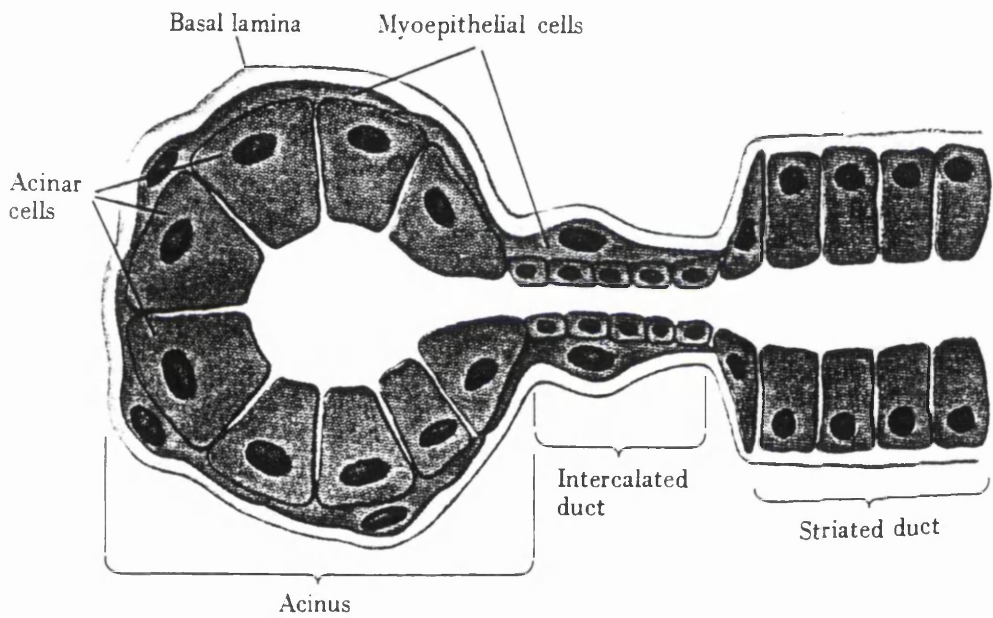

FIGURE 1.1. Diagram of the basic physiological parenchymal unit of a salivary gland sometimes referred to as a 'salivon'. This consists of the acinar cells, the surrounding myoepithelial cells, the intercalated duct and the striated duct. (Johnson, 1981).

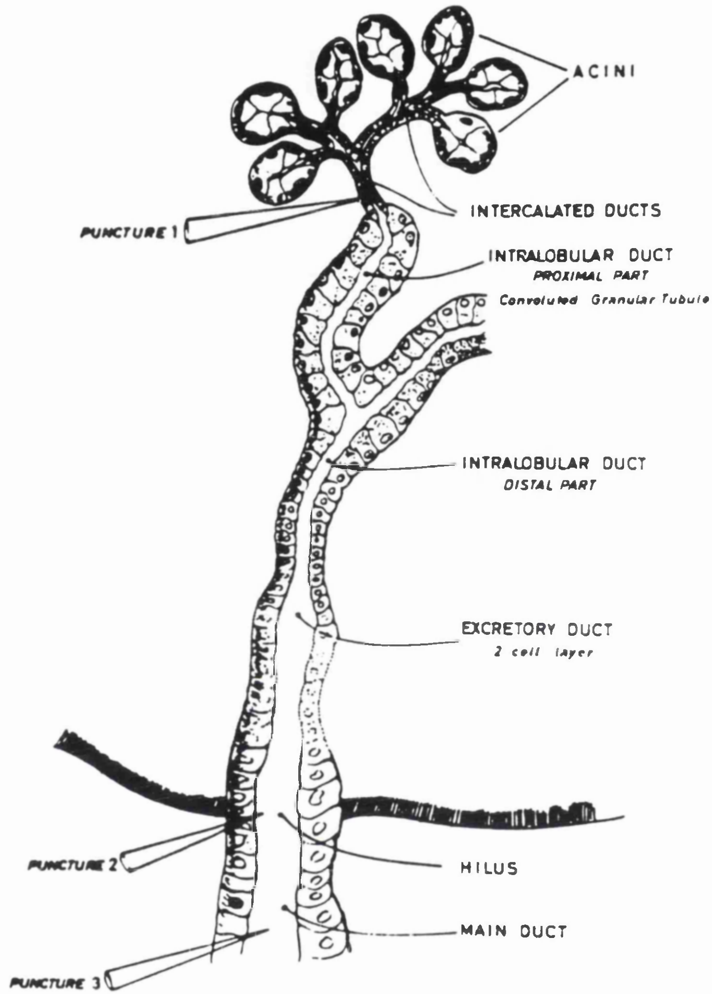

FIGURE 1.2. Puncture sites at the different levels of the salivary ductal system. (taken from Martinez, (1968); fig 16).

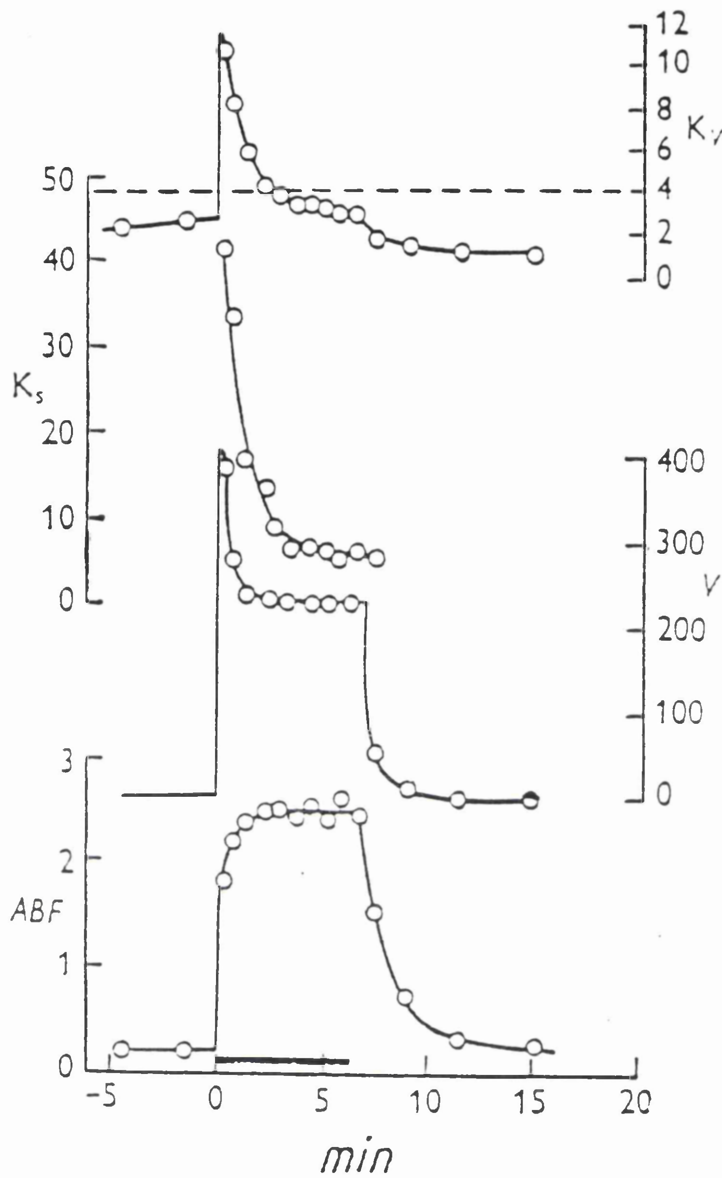

FIGURE 1.3. Potassium balance in the perfused dog submaxillary gland during stimulation of the chorda tympani at 20 Hz for 6.6 min. Ordinates : from below to top : ABF, arterial blood flow in ml./g/min; V, rate of saliva secretion mg/g/min; K_S, saliva potassium m-equiv/l.; K_V = venous plasma potassium in mEq/l and K_A = arterial plasma potassium in mEq/l. K_V is indicated by the solid line K_A by the dotted line. At the beginning of stimulation the concentration of potassium in the venous plasma reached over 12 mEq/l and later settled down to 2.6 mEq/l (taken from Burgen, (1956); fig. 9).

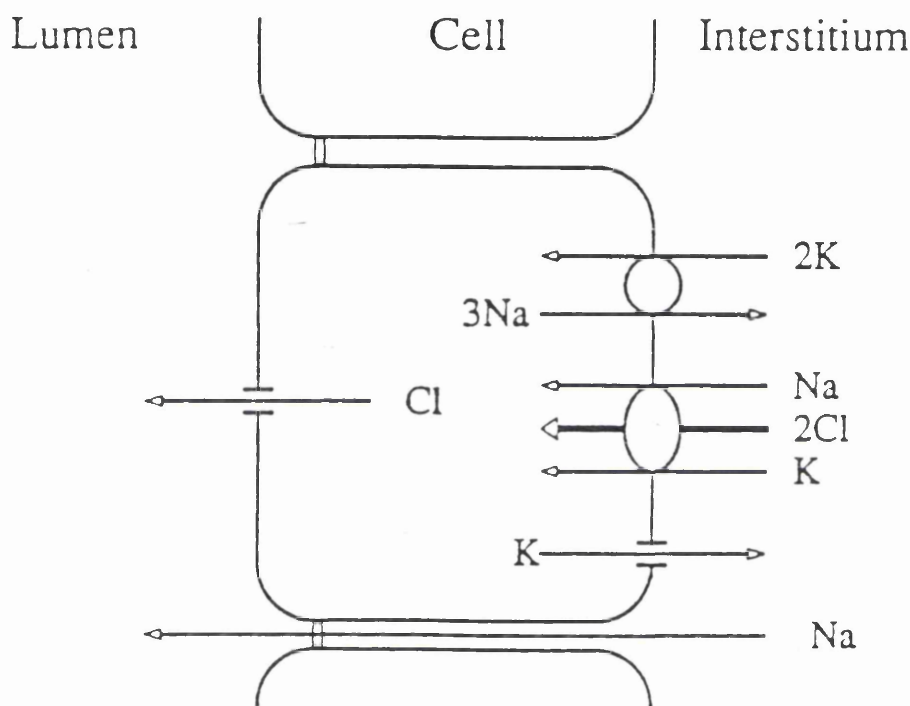

FIGURE 1.4. The cellular model proposed by Petersen & Maruyama, (1984) to account for the control of electrolyte transport in the basolateral membrane of fluid secreting salivary acini. The figure shows an electroneutral Na^+, K^+, $2Cl^-$ co-transporter, a voltage- Ca^{2+}-activated K^+ channel and a Na^+, K^+-ATPase located in the basolateral membrane. These provide the mechanism that accumulates Cl^- within the cell and Cl^- exits from the cell via Cl^- channels located in the apical membrane. Na^+ enters secretion passively via tight junctions.

CHAPTER 2 : MATERIALS AND METHODS.

BASIS OF THE $^{86}Rb^+$-EFFLUX METHOD.

The radioactive isotopes rubidium-86 ($^{86}Rb^+$), potassium-42 ($^{42}K^+$) and potassium-43 ($^{43}K^+$) have been frequently used as tracers to monitor K^+ transport from secretory epithelia upon receptor activation (Sehlin & Taljedal, 1975, Putney, 1976a, Parod & Putney, 1978b, Weiss & Putney, 1978, Henquin 1979, Henquin & Meissner, 1981, Danielsson & Sehlin, 1983; Gallacher 1982, 1983). Although all of these nuclides have been successfully used to mimic cellular K^+ transport, there are several disadvantages associated with the use of $^{42}K^+$ and $^{43}K^+$. Firstly, they are more radiotoxic than $^{86}Rb^+$ and hence are less safe to handle. Secondly, they have shorter half lives ($^{42}K^+$, 12.4 hrs; $^{43}K^+$, 22.4 hrs) than $^{86}Rb^+$ (18.7 days) and this significantly complicates their routine use.

The $^{86}Rb^+$ efflux technique was first used in mammalian exocrine glands by Putney in 1976 and the main aim of the isotopic experiments presented in this thesis was to assess the role played by Ca^{2+}-activated K^+ channels in regulating fluid secretion in the rat submandibular gland and human sweat gland. This was achieved by incubating intact fragments of glandular tissue in a physiological saline solution containing radioactive $^{86}Rb^+$ prior to cholinergic stimulation. This process allowed the isotope to equilibrate across the cell membrane and the cells to become loaded with $^{86}Rb^+$. The release of $^{86}Rb^+$ from the glandular tissue was then measured during resting and stimulated conditions.

Rb^+ and K^+ are closely related alkali metals and, initially, it was thought that their physiochemical properties were sufficiently similar to prevent membrane transport proteins from discriminating between them (Ussing, 1960). However, patch-clamp single-channel current recordings (excised inside-out patches of basolateral membrane) from exocrine acinar cells (Gallacher, Maruyama & Peterson, 1984) showed : 1). when all the K^+ (4.7 mmol l^{-1}) and Na^+ (140 mmol l^{-1}) in the patch pipette (external environment) was replaced with $^{86}Rb^+$, then K^+ current flowed easily from the inside to the outside, but no inward $^{86}Rb^+$ current was elicited, 2). if all the K^+ (145 mmol l^{-1}) in the bath solution (intracellular environment) was replaced with $^{86}Rb^+$, then inward K^+ current but no outward $^{86}Rb^+$ current was observed, 3). if the K^+ in the bathing solution was only partially replaced (50%) with $^{86}Rb^+$, then significantly reduced outward current steps could be recorded. These three findings led Gallacher, Maruyama & Peterson (1984), to postulate that the high-conductance K^+ channel was extremely selective and that it could discriminate between $^{86}Rb^+$ and K^+. $^{86}Rb^+$ did not, however, cause any shift in reversal potential demonstrating that it

can pass through these channels albeit at a very low rate. It thus appears that $^{86}Rb^+$ can act as a 'permeant blocker' of this channel. These authors therefore proposed that $^{86}Rb^+$ could serve as a useful marker for K^+ transport provided the cytosolic $^{86}Rb^+$ to K^+ ratio was low. A measure of the cytosolic $^{86}Rb^+$ to K^+ ratio is therefore essential when undertaking $^{86}Rb^+$ efflux experiments to ensure that it is low enough to give meaningful results.

ISOTOPE EFFLUX EXPERIMENTS.

Isolation of rat submandibular glands and loading of $^{86}Rb^+$ or $^{45}Ca^{2+}$.

Male Wistar rats, weighing approximately 300g were sacrificed by cervical dislocation. Their submandibular glands were removed and placed in ice-cold control solution (appendix 1). The glandular tissue was chopped into small fragments (<0.5mm) using a razor blade and loaded with radiotracer ($^{86}Rb^+$ or $^{45}Ca^{2+}$) by placing the pieces of glandular tissue in a glass vial containing 2ml of the control solution plus ~0.2-0.8 µCi ml^{-1} of the appropriate isotope (Amersham International plc). The vial and its contents were then partially immersed in a temperature-controlled water bath and incubated at 37°C for 30 minutes. Throughout the loading process the incubation medium was continually gassed with $95\%O_2/5\%CO_2$.

Superfusion.

After loading, 40 to 50 mg of the radioactive tissue was transferred to a flow chamber (volume 120 µl, Figs 2.1 & 2.2) the outlet of which was protected by nylon mesh to prevent any tissue fragments from passing through. The tissue was washed free from any extracellular nuclide prior to sample collection by superfusing (2ml min^{-1}) at 37°C with the unlabelled experimental superfusing solution ($^{86}Rb^+$-loaded tissue washed for 2 min, $^{45}Ca^{2+}$-loaded tissue washed for 5 min).

Sample collection.

During each experiment, a sequential series effluent samples were collected, at 1 minute intervals, directly into scintillation vials using a Redirac fraction collector (LKB Produkter, Sweden, figure 2.2). At the end of each experiment the tissue was recovered from the flow cell and dissolved in 2 ml of concentrated nitric acid. 8 mls of Scintillation fluid (National Diagnostics 'Ecoscint') was added to each effluent sample and to aliquots (50µl) of the dissolved glandular tissue and their radioactive contents were determined by liquid scintillation counting (Packard Tri Carb 300C counter). Vials (two)

containing aliquots (2ml) of the non-radioactive experimental saline solution plus scintillant were also counted to evaluate the background counts per minute which were automatically subtracted from the sample counts per minute. All samples counted with similar efficiency (~80%), so individual quench corrections were not applied.

Data analysis.

The total amount of radioactivity (counts per minute) present in the glandular tissue at the onset of sample collection was defined as the amount of radioactivity retained by the tissue after perfusion and the sum of the radioactivity present in each effluent sample. Rate constants describing the rate of isotope efflux were calculated using the following equation (Putney, 1976a) :

$$K_n = \frac{\Delta \ln \%_n}{\Delta t_n} \times 100\%$$

Where K_n = the rate constant over the nth interval, $\Delta \ln \%_n$ is the decrease in the natural logarithm of the percent isotope remaining in the tissue and Δt_n is the duration of the interval. A computer program (written by Dr S. M. Wilson) based on the above formula was used to calculate rate constants.

Isolation of human sweat glands and loading of $^{86}Rb^+$.

Biopsies of human skin were obtained from patients undergoing general surgery. This process had the approval of the local Medical Ethics Committee and the informed consent of the patient. The skin samples were placed in isolation medium (appendix 1) and repeatedly sheared using sharp scissors (Lee, Jones & Kealey, 1984). Sweat glands were isolated from the resultant slurry with the aid of a stereo dissection microscope. Although some glands floated freely, most were bound to surrounding collagen (figure 2.3) and were gently teased free using mounted entomological pins. Glands were transferred directly into a flow chamber containing isolation medium. Batches of between 40 to 50 glands were collected for each experiment.

Due to their small size, the glands were loaded with $^{86}Rb^+$ by continuos loop-superfusion (1 h, 2ml min^{-1}, 37°C). This was achieved by pumping the $^{86}Rb^+$-containing control solution through the flow chamber using a peristaltic pump (LKB, Produkter, Sweden). The sweat glands were washed free from any extraneous $^{86}Rb^+$ by superfusing the chamber for 2 minutes with the unlabelled

superfusing solution. Thereafter, sequential effluent fractions (30 second intervals) were collected. These samples were analysed as described above.

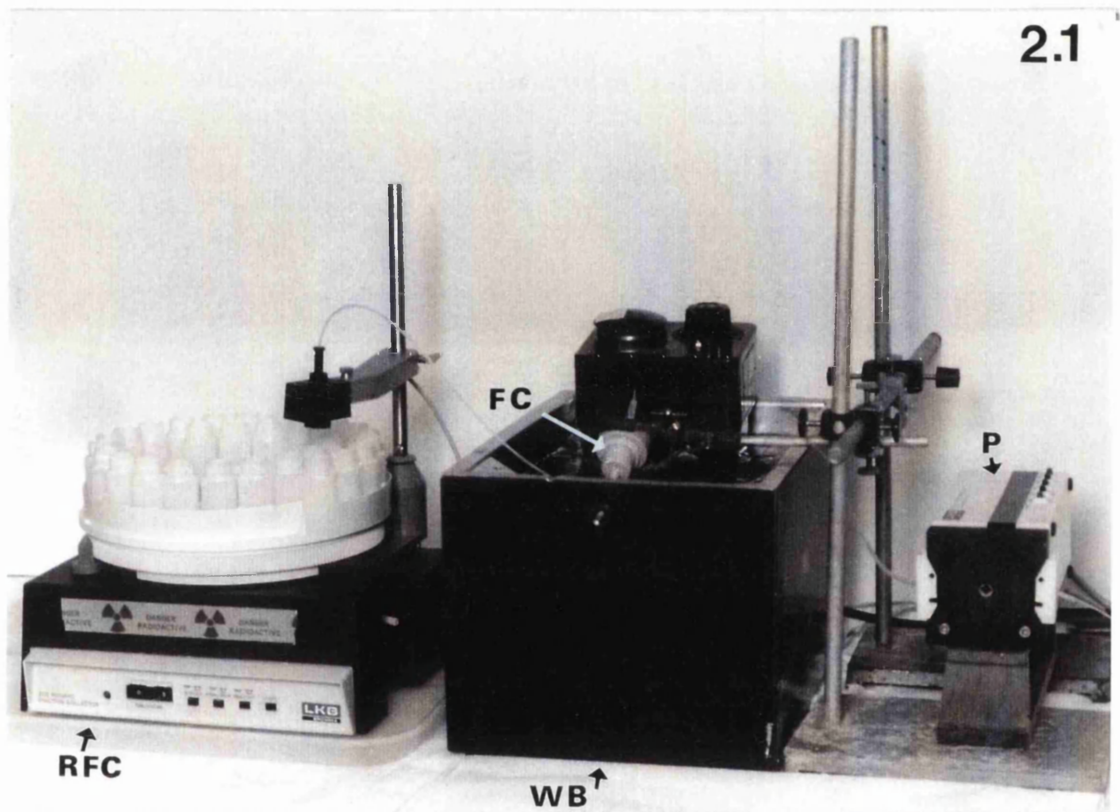

FIGURE 2.1. The experimental equipment used for isotopic flux experiments. Isolated sweat glands or salivary fragments were transferred into a flow chamber (FC) which was immersed into a water bath (W B) set at 37°C. Non-radioactive physiological saline solution was then pumped through the flow chamber using a peristaltic pump (P). Superfusion was maintained at a constant rate and sequential effluent fractions were collected directly into scintillation vials using a Redirac fraction collector (RFC).

2.2

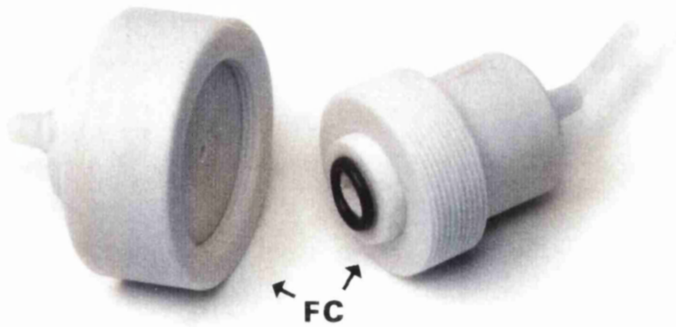

FIGURE 2.2. The internal compartment of the flow chamber (F C).

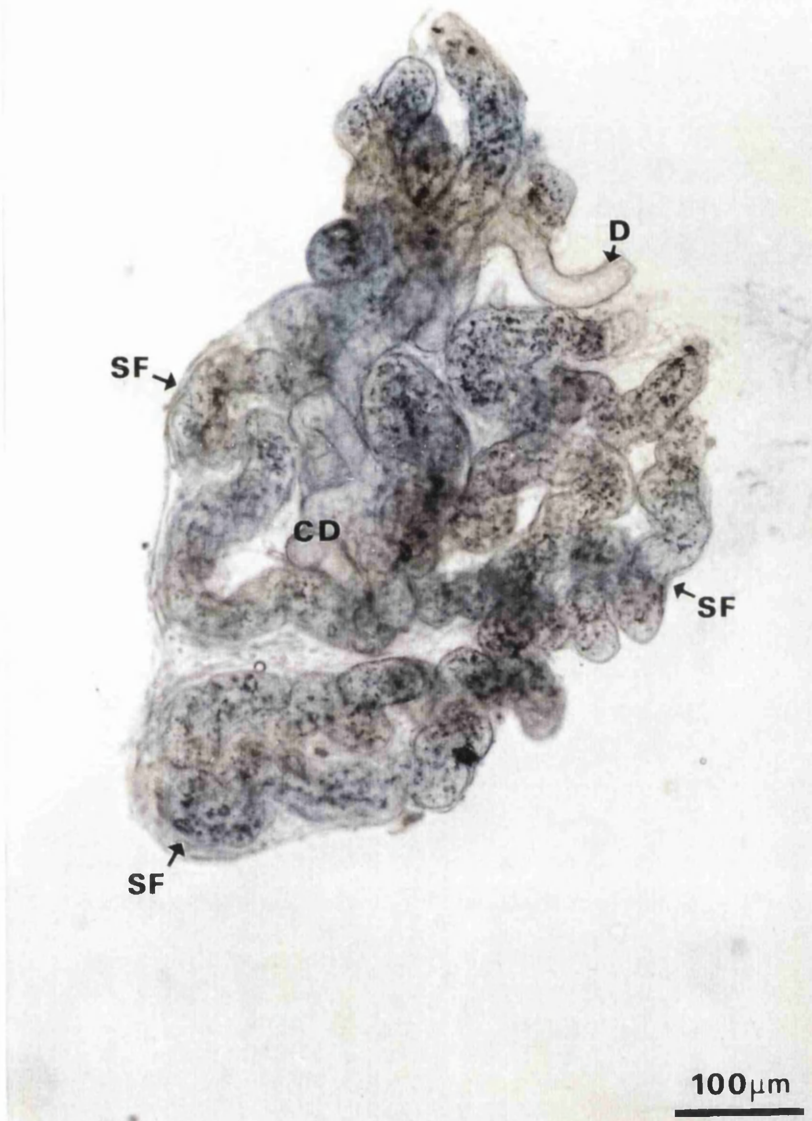

FIGURE 2.3. Light micrograph taken using differential interference contrast of an isolated human sweat gland. The cells of the secretory fundus (S F) have a more granular appearance than those of the coiled duct (C D) and duct (D).

CHAPTER 3 : THE EFFLUX OF POTASSIUM FROM THE RAT SUBMANDIBULAR GLAND UNDER SODIUM-FREE CONDITIONS IN VITRO.

INTRODUCTION.

It is now well established that rodent salivary acini possess large conductance Ca^{2+}-activated K^+ channels (Maruyama, Gallacher & Petersen, 1983; Petersen & Maruyama, 1984) whose activity increases during stimulation with cholinergic agonists (Gallacher & Morris, 1986; 1987). The resultant rise in membrane K^+ permeability (Burgen, 1956; Petersen, 1970; Putney, 1976a) is an important component of the mechanism of fluid secretion (Petersen & Maruyama, 1984; Petersen & Gallacher, 1988) and can be resolved into two distinct phases. An early, transient increase in membrane K^+ permeability which is independent of extracellular Ca^{2+} ($[Ca^{2+}]_o$) and a second, sustained phase which is dependent upon $[Ca^{2+}]_o$ (Putney, 1976a). It has been concluded that the transient increase is due to the mobilisation of Ca^{2+} from internal stores and the sustained increase is attributed to an inflow of Ca^{2+} from the external medium (Putney, 1976b).

The dependence of ACh-evoked changes in membrane K^+ permeability upon extracellular sodium ($[Na^+]_o$) have already been investigated (Petersen,1970; Putney, 1978). Experiments using the permeant cation Li^+ as a substitute for $[Na^+]_o$ have demonstrated that the cholinergically-induced loss of K^+ from mammalian salivary glands is normal (Petersen, 1970) or potentiated (Putney, 1978). Contrary to these findings, patch-clamp experiments undertaken on mouse submandibular acini have suggested that Ca^{2+}-activated K^+ channels become refractory to ACh when $[Na^+]_o$ is substituted with the impermeant cation N-Methyl-D-Glucammonium ($NMDG^+$), (Gallacher & Morris, 1987). This effect has been attributed to an inhibition of ACh-evoked Ca^{2+}-influx, which therefore suggests that the sustained increase in membrane K^+ permeability is Na^+ dependent. There thus appear to be conflicting views regarding the role played by $[Na^+]_o$ in regulating K^+ efflux from the acini of salivary glands.

The radioisotope $^{86}Rb^+$ is an effective tracer for studying increases in membrane K^+ permeability in the acini of rodent salivary glands (Putney, 1976a, 1978; Putney, Leslie & Marier, 1978). Therefore the main objective of the present experiments was to examine the effects of replacing $[Na^+]_o$ with either $NMDG^+$ or Li^+ upon the cholinergic regulation of membrane K^+ permeability in the rat submandibular gland using $^{86}Rb^+$ as a marker of K^+ permeability.

METHODS.

The methods used for preloading the salivary fragments with $^{86}Rb^+$, superfusion and calculating $^{86}Rb^+$-efflux rate constants are described in detail in the methods section (chapter 2).

Data Analysis.

The basal (unstimulated) efflux rate was defined, for each experiment, as the mean efflux rate measured over a 3 min period prior to the addition of ACh (10^{-5} mol l^{-1}). Responses to ACh were quantified by subtracting this basal efflux rate from the peak response. In experiments where ACh (10^{-5} mol l^{-1}) and $CaCl_2$ (2.56 mmol l^{-1}) were added sequentially, the rate constant measured immediately prior to the addition of $CaCl_2$ was similarly used to quantify the response to calcium. The 'combined response' in such experiments was defined as the sum of the individual responses to ACh and $CaCl_2$. The percentage inhibition of the combined response was evaluated as :

$$\{(\text{control response - combined response}) / \text{control response}\} \times 100,$$

where the control response is the response elicited by ACh (10^{-5} mol l^{-1}) in the appropriate Ca^{2+}-containing solution. The results are given as the mean ± standard error and differences between the means were statistically analysed using a Student's unpaired t-test. The value n refers to the number of individual animals.

Solutions.

The composition of the HCO_3^--buffered control solution was (in mmol l^{-1}) : NaCl, 103; KCl, 4.7; $CaCl_2$, 2.56; $MgCl_2$, 1.13; $NaHCO_3$, 25; NaH_2PO_4, 1.15; D-glucose, 2.8; sodium fumarate, 2.7; sodium glutamate, 4.9; sodium pyruvate, 4.9. Its pH was 7.3-7.4 when gassed with 95% O_2/5% CO_2.

The Na^+-HEPES solution was similar in composition to the control solution except that all the $NaHCO_3$ was iso-osmotically replaced with 4-(2-hydroxyethyl)-1- piperazine sulphonic acid (HEPES). The pH of this solution was adjusted to 7.4 using NaOH and it was gassed with 100% O_2.

Na^+-free solutions were HEPES-buffered and prepared by iso-osmotically replacing Na^+ with either $NMDG^+$ or Li^+. Metabolic substrates were added to these solutions as free acids and the pH was adjusted to 7.4 with lithium hydroxide or hydrochloric acid. All HEPES-buffered-solutions were gassed with 100% O_2.

Low Ca^{2+}-solutions were prepared simply by omitting $CaCl_2$ (2.56 mmol l^{-1}) from the above solutions ($[Ca^{2+}]_o$ ~20 µmol l^{-1}; Miller & Smith, 1984). Ca^{2+}-free solutions were prepared by also adding the Ca^{2+} chelating agent ethyleneglycol-bis-(aminoethylether) tetra acetic acid (EGTA). The $[Ca^{2+}]_o$ of these solutions was estimated to be ~0.02 µmol l^{-1} (Miller & Smith, 1984).

ACh was prepared as a 10 mmol l^{-1} stock in distilled water, aliquoted and stored at -20°C for up to 1 week. Each aliquot was thawed only once and kept on ice just before use.

RESULTS.

Efflux in sodium–containing solutions.

Basal Efflux (unstimulated).

Figure 3.1A shows the decline in the glandular $^{86}Rb^+$ content as $^{86}Rb^+$ is washed from the salivary fragments during superfusion with the HCO_3^--buffered control solution (n=4). The amount of $^{86}Rb^+$ retained in the salivary tissue after 30 minutes superfusion was 25 ± 3%. Rate constants describing the efflux of $^{86}Rb^+$ under these conditions are presented in figure 3.1B (circles, n=9). There is some tendency for the rate constant to decline, but these data are essentially consistent with the washout of a single compartment. Figure 3.1B also shows equivalent data obtained during superfusion with the HCO_3^--free, Na^+-HEPES solution (squares; n=3). The basal efflux rate measured under these conditions did not differ significantly from the control values. Removal of HCO_3^- does not, therefore, effect the basal efflux of $^{86}Rb^+$.

Responses to acetylcholine in the presence of Ca^{2+}.

The increase in $^{86}Rb^+$-efflux evoked by ACh (10^{-5} mol l^{-1}) (Δmin^{-1}, 0.068 ± 0.004, n=17) during superfusion with the HCO_3^--buffered control solution is illustrated in figure 3.2A. This response consists of an immediate and rapid increase in the $^{86}Rb^+$ efflux rate which reached a peak after 2 min. Thereafter, there was initially a rapid decline to a much more slowly declining phase. Equivalent data obtained during superfusion with the HCO_3^--free, Na^+-HEPES solution are presented in figure 3.2B. Under these experimental conditions a similar, but significantly smaller increase in the efflux rate was elicited by ACh (Δmin^{-1}, 0.049 ± 0.005, n=7, $p<0.02$). Removal of HCO_3^- therefore impairs the response to ACh.

Biphasic responses to acetylcholine.

In the experiments presented in figure 3.3A, the salivary fragments were initially perfused with the Ca^{2+}-free, HCO_3^--buffered control solution. After 12 min, ACh (10^{-5} mol l^{-1}) was added to the superfusing solution and, 5 min later $CaCl_2$ (2.56 mmol l^{-1}) was introduced. The initial response to ACh was entirely transient (Δmin^{-1}, 0.019 ± 0.003, figure 3.3A, n=9), but, when $CaCl_2$ (2.56 mmol l^{-1}) was introduced to the superfusing solution, in the continued presence of ACh (second arrow) a second response was evoked by ACh. This secondary response consisted of an immediate increase in the efflux rate to a peak, from which there is an initial rapid decline to a much more slowly declining phase (Δmin^{-1}, 0.032 ± 0.004, figure 3.3A, n=9).

An essentially similar biphasic response was obtained during superfusion with the Na^+-HEPES solution (transient Δmin^{-1}, 0.017 ± 0.004; secondary response Δmin^{-1}, 0.028 ± 0.005, n=10, figure 3.3B).

Efflux in sodium-free solutions.

Basal Efflux (unstimulated).

Table 3.1 shows the basal efflux rate measured during superfusion with the HEPES-buffered Na^+, $NMDG^+$ or Li^+-containing solutions in the presence and absence of $CaCl_2$. The basal efflux rate measured during superfusion with the $NMDG^+$-solution was greater than normal and this effect was not Ca^{2+} dependent (table 3.1).

The data presented in figure 3.4 confirmed that the impermeant cation $NMDG^+$ causes a rise in the basal rate of $^{86}Rb^+$-efflux. This developed slowly and reaches a sustained level after ~5-6 minutes. This increase could be reversed rapidly on return to the control solution. Similar results were also recorded under Ca^{2+}-free conditions (n=7), and the addition of calcium to Ca^{2+}-free, $NMDG^+$-solution did not alter the efflux rate (n=3).

Higher basal efflux rates were also obtained during perfusion with the Ca^{2+}-containing Li^+-solution, and this increase in efflux was higher than that evoked in the presence of $NMDG^+$ (table 3.1). In the absence of extracellular Ca^{2+}, however, both Na^+ substitutes elicited an equal effect and the additional effect of Li^+ elicited in the presence of extracellular Ca^{2+} disappeared (table 3.1). In spite of this finding, the addition of Ca^{2+} to Ca^{2+}-free, Li^+-solutions did not change the efflux rate (n=3).

Responses to acetylcholine in the presence of Ca^{2+}.

The response to ACh (10^{-5} mol l^{-1}) evoked during superfusion with the HEPES-buffered Li^+ or $NMDG^+$-solution containing 2.56 mmol l^{-1} $CaCl_2$ is illustrated in figure 3.5. The increase in the efflux rate evoked by ACh in the presence of Li^+ (figure 3.5A, Δmin^{-1}, 0.087 ± 0.012, n=4) was larger ($P<0.01$) than that elicited in the presence of Na^+ (see figure 3.2B). However, when $NMDG^+$ was used as the Na^+ substituent, the ACh-evoked increase in the $^{86}Rb^+$ efflux rate (Δmin^{-1}, 0.031 ± 0.004, n=7, (figure 3.5B) was smaller ($P<0.02$) than that measured in the Na^+-HEPES solution (figure 3.2B).

Biphasic responses to acetylcholine.

In the presence of the Ca^{2+}-free, Li^+-solution, ACh again elicited a transient increase in the efflux rate (Δmin^{-1}, 0.028 ± 0.005, n=6, figure 3.6). On the subsequent addition of $CaCl_2$ (2.56 mmol l^{-1}), (second arrow) to the Li^+-solution, ACh again evoked a further increase in the efflux rate (Δmin^{-1}, 0.012 ± 0.004, n=6, figure 3.6). However, the magnitude of this secondary response was significantly smaller than that evoked in the presence of Na^+ ($p<0.05$; table 3.2). The combined response evoked by the sequential addition of ACh (10^{-5} mol l^{-1}) and $CaCl_2$ (2.56 mmol l^{-1}) to the initially Ca^{2+}-free, Li^+-solution, was significantly ($p<0.01$) smaller than that elicited by an equimolar dose of ACh in the Ca^{2+}-containing, Na^+-solution (table 3.2). This inhibition of the combined response was quantified and is presented in table 3.2.

The transient increase in the efflux rate (Δmin^{-1}, 0.009 ± 0.007, n=12) evoked by ACh during superfusion with the nominally Ca^{2+}-free $NMDG^+$-solution ($[Ca^{2+}]_o \sim 0.02 \mu mol\ l^{-1}$ did not significantly differ from the equivalent response elicited in the presence of $[Na^+]_o$ (table 3.2). When the $[Ca^{2+}]_o$ of this solution was raised to 2.56 mmol l^{-1}, ACh could only evoke a small increase in the efflux rate (Δmin^{-1}, 0.006 ± 0.001, n=12, figure 3.7A) that was poorly sustained and significantly smaller than that evoked in the presence of Na^+ ($p<0.05$, table 3.2). The combined response is therefore inhibited and this inhibitory effect was similar to that evoked in the presence of Li^+ (table 3.2).

In a further series of experiments the salivary tissue was initially perfused with a low Ca^{2+}-containing $NMDG^+$-solution ($[Ca^{2+}]_o \sim 20\ \mu mol\ l^{-1}$; figure 3.7B). Under these experimental conditions there was no significant inhibition of the combined response, but the sustained component of the response to ACh was still significantly smaller in magnitude ($P<0.05$) when compared to the equivalent response evoked in $[Na^+]_o$ (figure 3.7B; table 3.2).

DISCUSSION.

The present results confirmed that on superfusion with the Na^+-containing solutions, ACh could evoke an increase in membrane K^+ permeability in the rat submandibular gland. This increase could be resolved into two distinct phases by manipulation of the $CaCl_2$ present in these solutions. In the absence of $CaCl_2$, ACh-elicited a transient response that could be attributed to the mobilisation of Ca^{2+} from intracellular stores (Putney, 1976a; Aub, McKinney & Putney, 1982; Aub & Putney, 1987). When $CaCl_2$ (2.56 mmol l^{-1}) was restored to these solutions, ACh evoked a second response which was more sustained. This latter phase was attributed to an inflow of Ca^{2+} from the external fluid (Putney, 1976a, 1977; Marier, Putney & van De Walle, 1978).

In mammalian salivary acini, removal of HCO_3^- reduces the pH buffering capacity of the cytoplasm and can therefore disturb pH_i (Steward, Seo & Case, 1989). It has been demonstrated in various cell types that the activity of Ca^{2+}-activated K^+ channels is reduced if pH_i is lowered (Cook, Ikeuchi & Fujimoto, 1984; Cornejo, Guggino & Guggino, 1989; Copello, Segal & Reuss, 1991). In the present study, the basal efflux rate and biphasic response to ACh was essentially normal under HCO_3^--free conditions. However, the response to ACh during superfusion with the control solution was significantly inhibited.

It has been recently reported that exposure to $NMDG^+$-containing solutions reduces the activity of Ca^{2+}-activated K^+ channels in unstimulated mouse submandibular acini, (Gallacher & Morris, 1987). This result suggests that a reduction in the basal efflux of $^{86}Rb^+$ rate would occur in the presence of $NMDG^+$. However, the present results demonstrated that the basal efflux rate was significantly elevated on exposure to the $NMDG^+$-solution. A similar effect was also evoked when $[Na^+]_0$ was replaced with Li^+. Putney (1978) has shown that a similar effect occurs in the rat parotid gland when extracellular Na^+ is partially replaced with either Li^+ or choline. For $NMDG^+$, I was able to demonstrate that this rise in the basal efflux was reversible and occurred after a latency period of 5 to 6 minutes. Although Ca^{2+} efflux via Na^+-Ca^{2+} exchange would be prevented under Na^+-free conditions, Ca^{2+}-influx via this transporter could still occur provided sufficient internal Na^+ ($[Na^+]_i$) is present. If the measured rate constant for Na^+ efflux across the basolateral membrane was approximately 0.1 min^{-1}, then 6 minutes exposure to a Na^+-free solution would reduce $[Na^+]_i$ by about 60% (Katoh, Nakasato, Nishiyama & Sakai, 1983). It is thus possible that the increase in the basal efflux could be due to an increase in the $[Ca^{2+}]_i$ via Na^+-Ca^{2+} exchange.

There is evidence that part of the increased basal efflux caused by Li^+, but not $NMDG^+$, is due to Ca^{2+}-influx. However, not all of our data supports this since the addition of Ca^{2+} to the Ca^{2+}-free, Li^+ -solution did not alter the efflux rate.

In the presence of external Ca^{2+}, the peak response to ACh was inhibited in the presence of $NMDG^+$ and potentiated on exposure to Li^+. The effects of Li^+-containing solutions during cholinergic stimulation in other exocrine organs have been variable. In cat salivary acini cells, ACh can still elicit increased K^+-efflux, however, desensitisation to repeated doses of ACh occurs very quickly in the presence of Li^+ (Petersen, 1970). In common with the present findings cholinergically-evoked amylase secretion (Putney, 1976a) and K^+-efflux (Putney, 1978) from the rat parotid gland are enhanced when external Na^+ is replaced with either choline or Li^+ and is thought to be due to an effect on the Ca^{2+}-dependent component of the response.

A complex picture emerged from the Na^+-free experiments which were designed to assess the effects of $NMDG^+$ upon the transient and sustained increases in the efflux rate. In preliminary experiments where the initial $[Ca^{2+}]_o$ of the nominally Ca^{2+}-free $NMDG^+$-solution was ~0.02 $\mu mol\ l^{-1}$, the transient efflux rate was not significantly inhibited, whereas the sustained phase was. However, when these experiments were repeated using nominally Ca^{2+}-free $NMDG^+$-solutions containing an initial $[Ca^{2+}]_o$ of ~20 $\mu mol\ l^{-1}$, an intermediate stage was reached, in that the magnitude of the transient increase in the efflux rate returned to normal levels, but the sustained phase was still significantly inhibited. These latter findings therefore support Gallacher & Morris's hypothesis that Ca^{2+}-inflow, which supports the sustained increase in the efflux rate from rodent salivary acini, is Na^+-dependent and suggests there could be some interaction between external Na^+ and the mobilisation of Ca^{2+}. The physiological basis of this relationship is not known yet and therefore requires further investigation and will be examined in the succeeding chapter (chapter 4).

The data obtained from the Na^+-free experiments also demonstrated that in the presence of either Li^+ or $NMDG^+$, the combined response evoked by the sequential addition of ACh and $CaCl_2$ was smaller than the response evoked by an equimolar dose of ACh in the presence of $CaCl_2$. It would thus appear that the simultaneous removal of extracellular Na^+ and Ca^{2+} reduces the responsiveness to ACh and this effect is not reversed when the external Ca^{2+} concentration of the superfusing Na^+-free solution is elevated.

TABLE 3.1. Basal efflux rate constant values measured from the rat submandibular gland during superfusion with the HEPES-buffered Na^+, Li^+ or $NMDG^+$ solution. Values represent the mean ± standard error and differences between the means were analysed using an unpaired Student's t-test.

Principal cation	Basal $^{86}Rb^+$ efflux rate constant, min^{-1}. Ca^{2+}-containing	Ca^{2+}-free
Na^+	0.024 ± 0.002 (7)	0.026 ± 0.002 (10)
Li^+	0.059 ± 0.002*** (4)	0.041 ± 0.006 (6)**, †
$NMDG^+$	0.039 ± 0.001*** (5)	0.041 ± 0.003 (12)***

**P<0.01, ***P<0.001 when compared to the appropriate Na^+-HEPES solution. †P<0.05 with respect to the Ca^{2+}-containing Li^+-solution. Number of individual experiments indicated in brackets.

TABLE 3.2. ACh-evoked transient and sustained increases in $^{86}Rb^{+}$-efflux obtained during superfusion with the HEPES-buffered solutions containing Na^{+}, Li^{+} or $NMDG^{+}$ as the principal cation. The values are given as the mean ± standard error. Differences between mean values were compared using an unpaired Student's t-test and n refers to the number of individual experiments.

Principal cation	n	Initial $[Ca^{2+}]_o$ ($\mu mol\ l^{-1}$)	Responses to ACh (Δmin^{-1}) Ca^{2+} Independent	Ca^{2+} Dependent	Inhibition (%)
Na^{+}	10	0.02	0.017 ± 0.004	0.028 ± 0.005	4 ± 11
Li^{+}	6	0.02	0.028 ± 0.005	0.012 ± 0.004 *	55 ± 10 **
$NMDG^{+}$	12	0.02	0.009 ± 0.007	0.006 ± 0.001 *	54 ± 8 ***
$NMDG^{+}$	6	20	0.017 ± 0.003	0.012 ± 0.003 *	24 ± 15

*$P<0.05$, **$P<0.01$ and ***$P<0.001$ with respect to equivalent data obtained in the presence of the Na^{+}-HEPES solution.

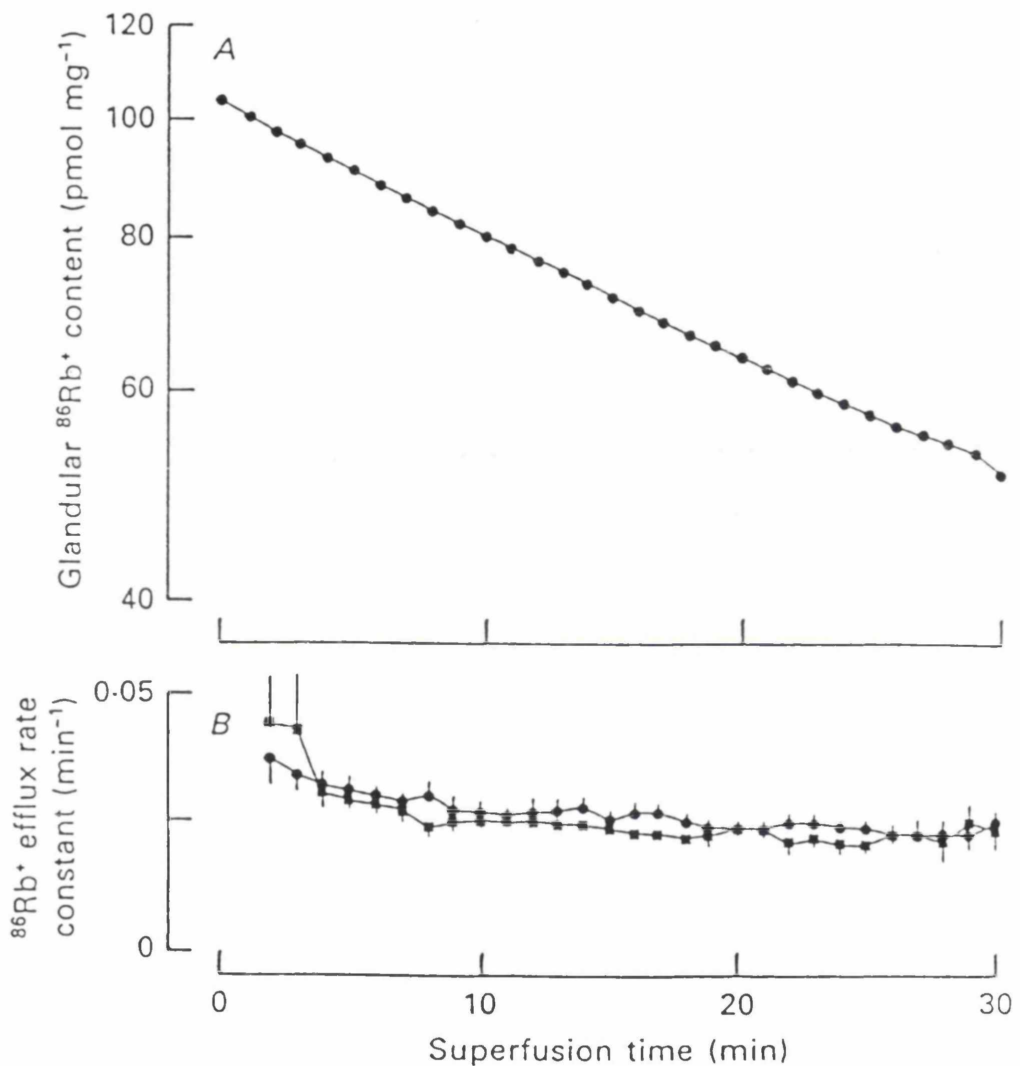

FIGURE 3.1. Figure 3.1A. This figure shows the decline in the glandular $^{86}Rb^{+}$ content as $^{86}Rb^{+}$ is washed out from the salivary fragments during superfusion with the HCO_3-buffered control solution (n=4). First order rate constants for $^{86}Rb^{+}$ efflux expressed as a function of the superfusion time are presented in figure 3.1B. Data obtained during superfusion with the control solution (circles, n=9) or the Ca^{2+}-containing Na^{+}-HEPES solution (squares, n=3) are presented.

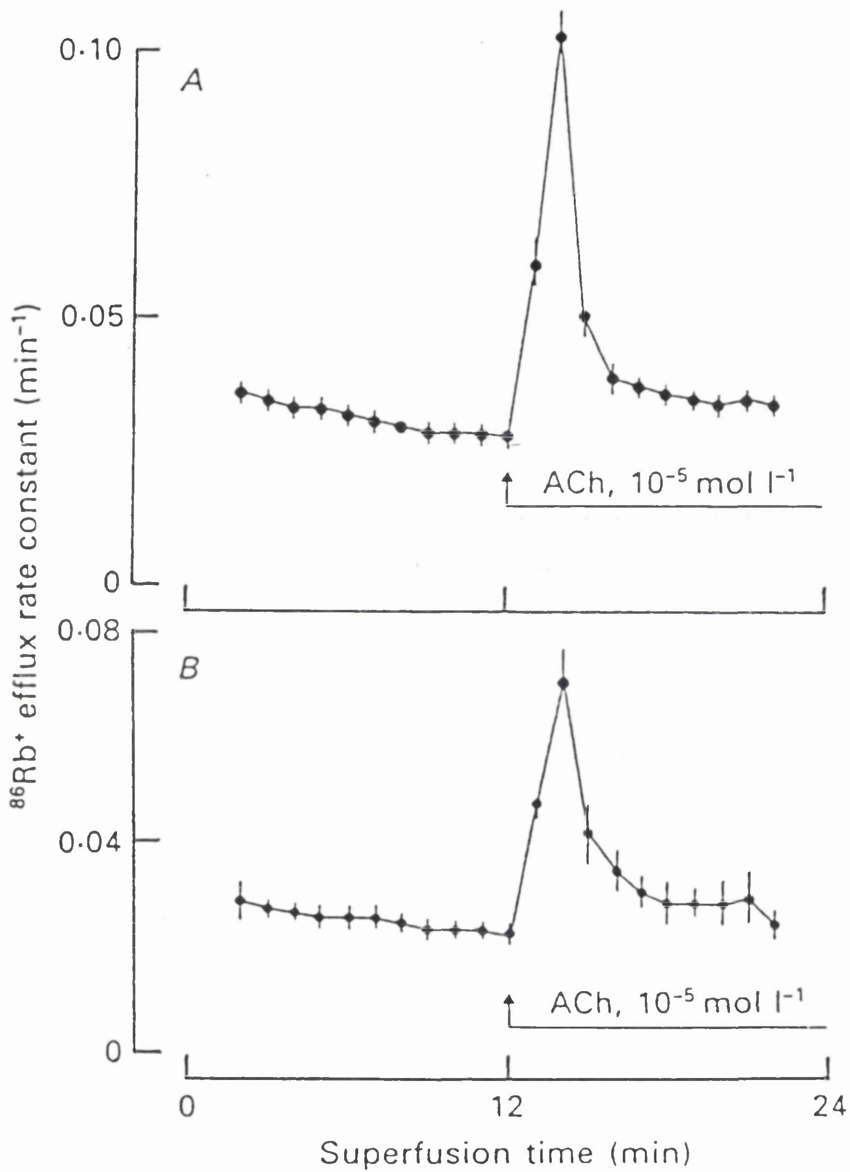

FIGURE 3.2. (A) shows the increase in the $^{86}Rb^{+}$ efflux rate elicited by ACh (10^{-5} mmol l^{-1}) during superfusion with the control solution (n=17). The increase in the efflux rate evoked by ACh during superfusion with the Ca^{2+}-containing Na^{+}-HEPES solution is illustrated in (B).

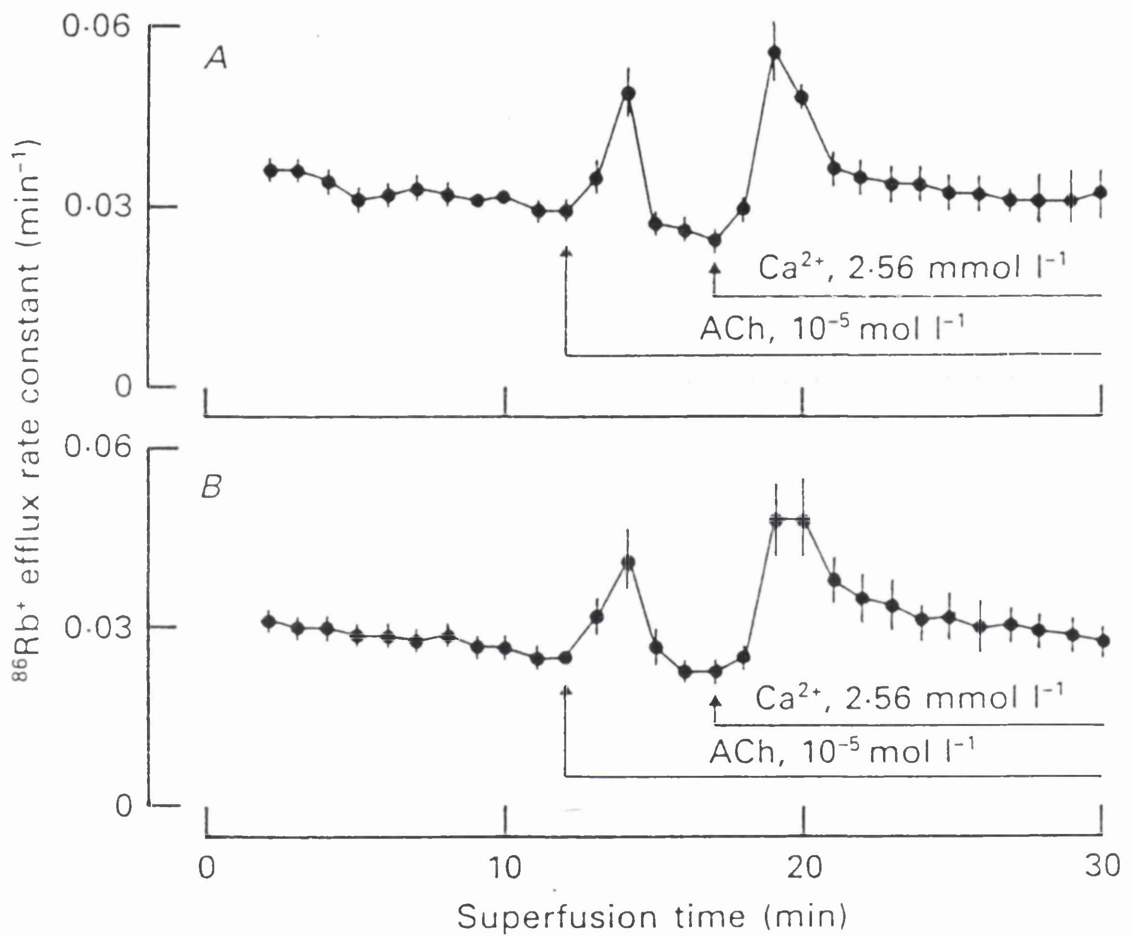

FIGURE 3.3. Transient and sustained increases in the efflux rate evoked by ACh (10^{-5} mmol l^{-1}) during superfusion with the Na^+-containing solutions are presented. The biphasic response to ACh during superfusion with the Na^+-HCO_3^- solution is illustrated in (A), (n=9). (B) shows the equivalent biphasic response evoked in the Na^+-HEPES solution (n=10).

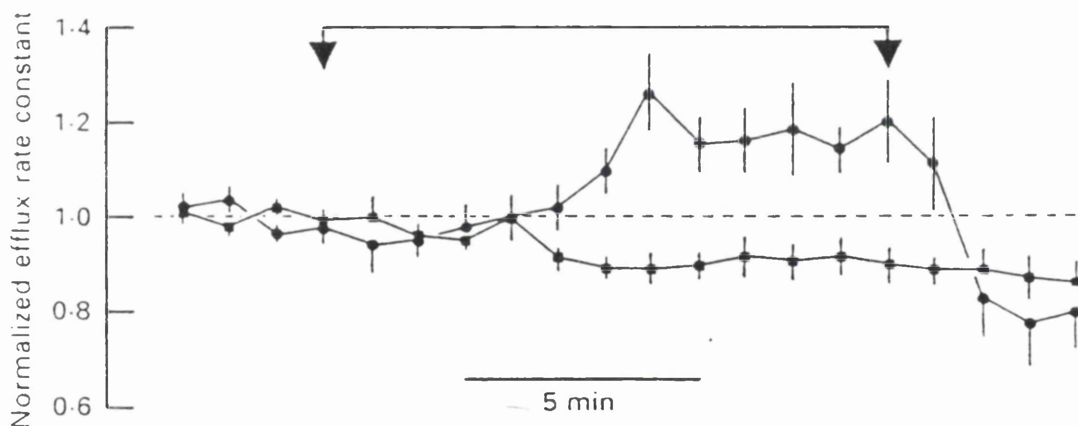

FIGURE 3.4. The effects of superfusion with the $NMDG^+$-solution upon the basal rate of $^{86}Rb^+$-efflux. The y-axis represents the basal efflux rate normalised to the mean $^{86}Rb^+$ efflux rate over the first 4 minutes of the experimental period. Circular points show data derived from salivary fragments superfused successively with the Na^+-HEPES solution; $NMDG^+$-HEPES solution for the period indicated by the arrows on the diagram, and then the Na^+-HEPES solution (n=6). The squares represent data from the Na^+-HEPES solution only (n=4).

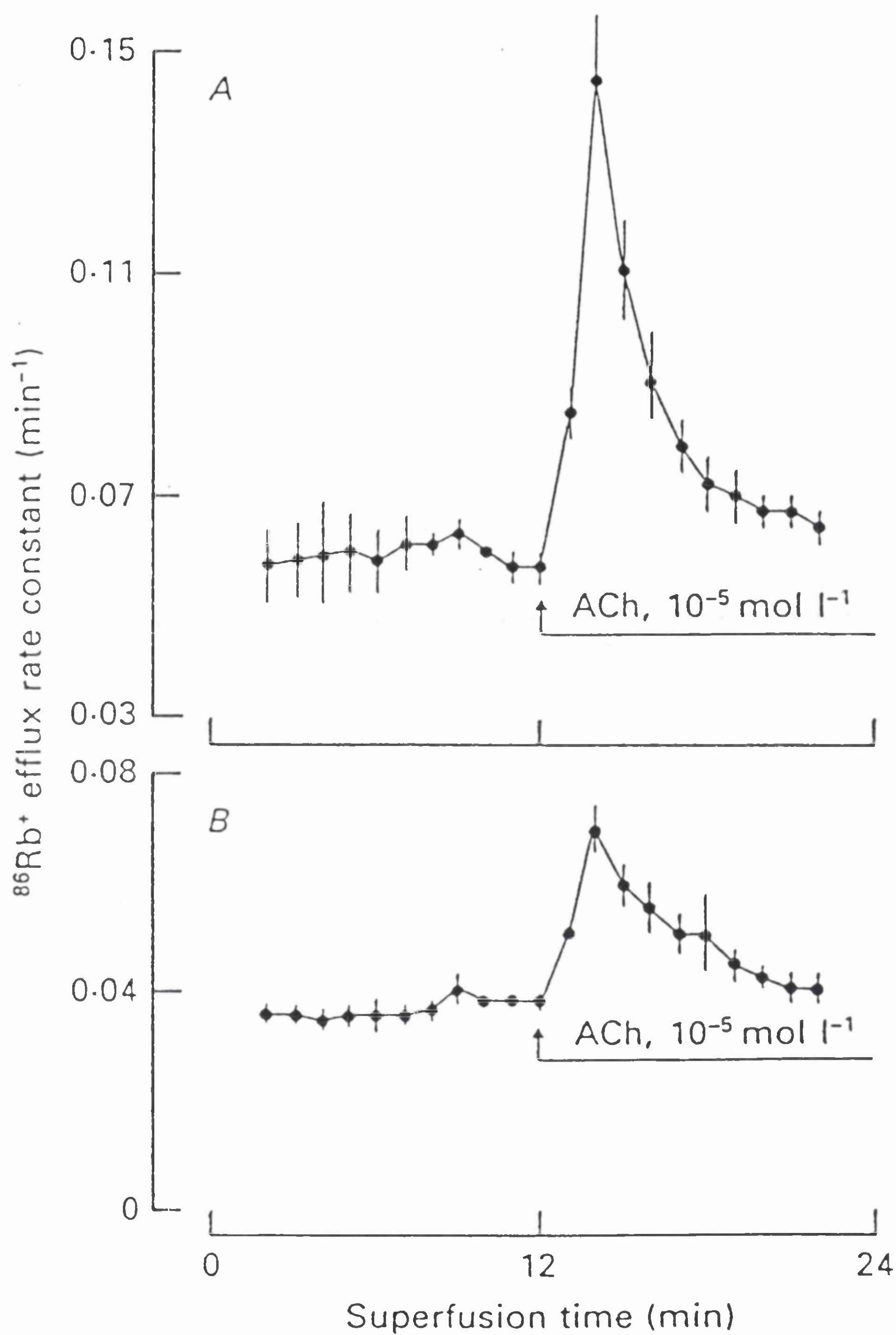

FIGURE 3.5. The response to ACh (10^{-5} mmol l^{-1}) obtained during superfusion with the Ca^{2+}-containing HEPES-buffered Li^{+} (figure 3.5A, n=4) or $NMDG^{+}$ (figure 3.5B, n=5) solution is presented.

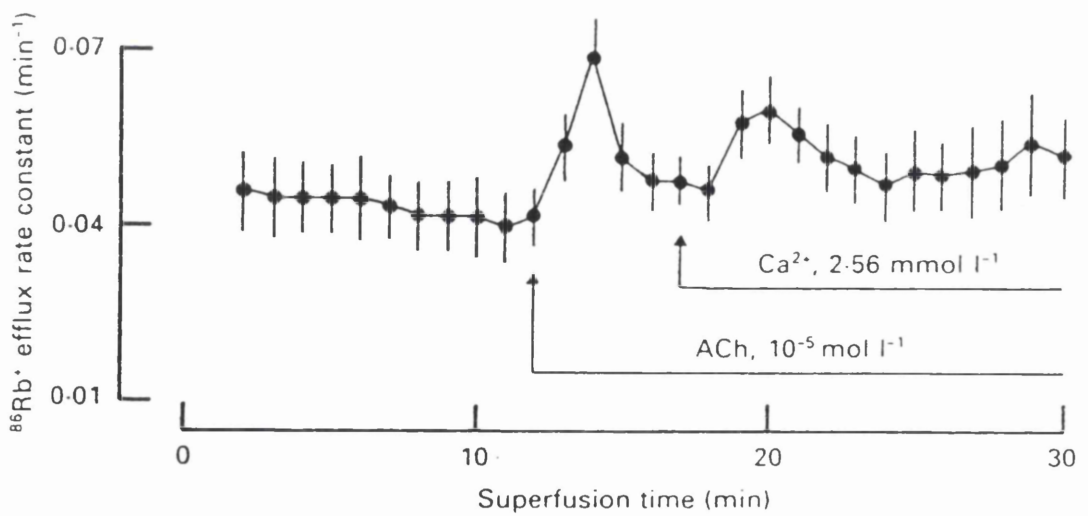

FIGURE 3.6. The biphasic increase in the efflux rate elicited by ACh (10^{-5} mmol l^{-1}) during superfusion with the Li^{+}-HEPES solution (n=6) is depicted.

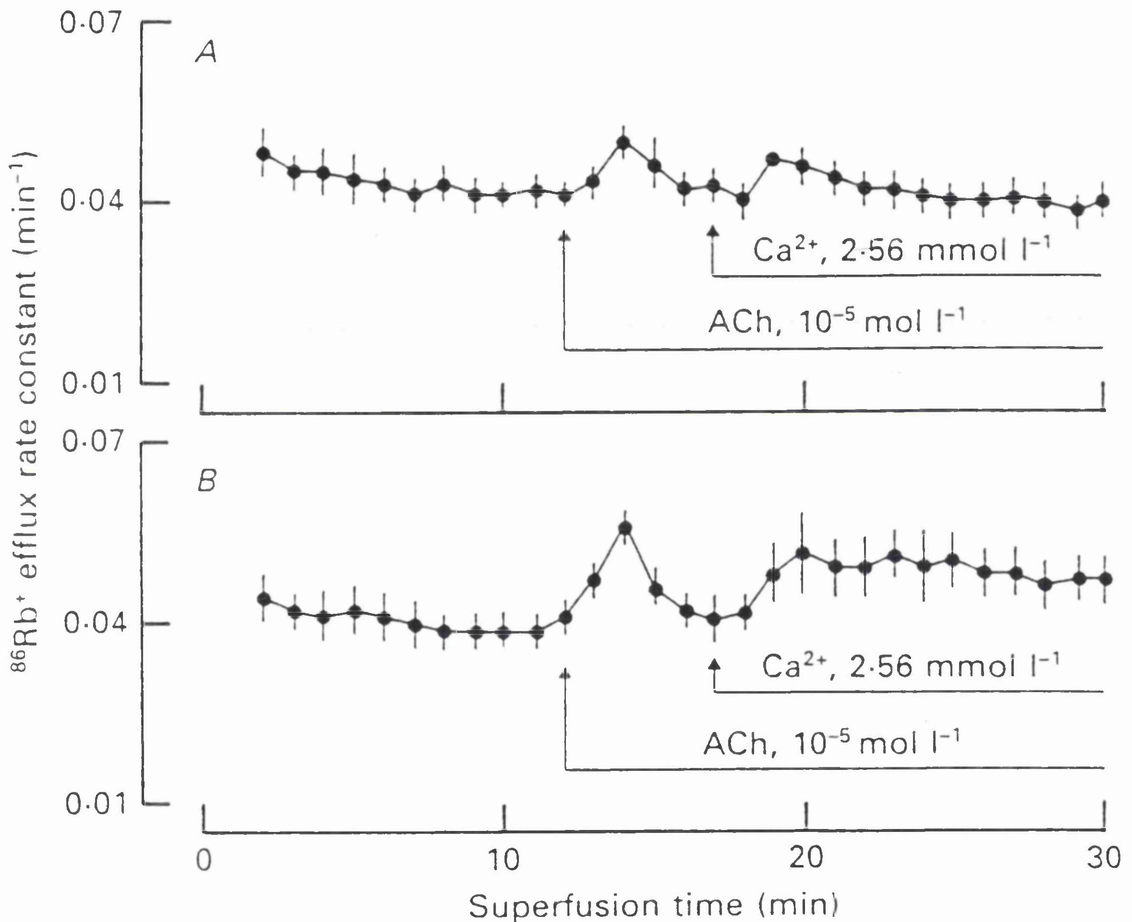

FIGURE 3.7. Transient and sustained increases in the efflux rate evoked by ACh during exposure to the impermeant cation $NMDG^+$. The biphasic response obtained when the salivary fragments were initially perfused with a $NMDG^+$-solution containing a $[Ca^{2+}]_o$ of ~0.02μmol l^{-1} is shown in figure 3.7A (n=12). Figure 3.7B shows the equivalent biphasic response evoked by ACh when the initial $[Ca^{2+}]_o$ of the $NMDG^+$-solution was raised to ~20μmol l^{-1} prior to the addition of ACh and $CaCl_2$ (n=6).

CHAPTER 4 : Na^+-DEPENDENT K^+ EFFLUX FROM THE RAT SUBMANDIBULAR GLAND IN VITRO : STUDIES IN HCO_3^--CONTAINING SOLUTIONS.

INTRODUCTION.

The results presented in chapter 3 confirmed that ACh evokes a biphasic increase in membrane K^+ permeability in the rat submandibular gland and demonstrated that this response was slightly impaired under HCO_3^--free conditions

Patch-clamp experiments undertaken on rodent submandibular acini have suggested that Ca^{2+}-influx, which supports the sustained increase in membrane K^+ permeability, is inhibited if external Na^+ ($[Na^+]_o$) is replaced with $NMDG^+$ (Gallacher & Morris 1987). The results from the Na^+-free experiments presented in the preceding chapter indicated that the removal of $[Na^+]_o$ inhibits only the Ca^{2+}-dependent component of the cholinergic response. However, these experiments also suggested that Na^+ removal could compromise cholinergic responses by eliciting complex effects if the external free Ca^{2+} concentration was also reduced to very low levels (~0.02μmoll^{-1}) under HCO_3^--free conditions. The possibility that receptor regulated Ca^{2+}-influx in exocrine cells occurs via a Na^+-dependent mechanism (Gallacher & Morris, 1987) thus remains equivocal.

Purpose of the experiments presented in this chapter was to re-examine the effects of removing $[Na^+]_o$ upon the cholinergic regulation of K^+ permeability in the rat submandibular gland using HCO_3^--buffered-solutions which contained sufficient Ca^{2+} to ensure that the unstimulated tissues were never exposed to an outwardly directed Ca^{2+} gradient.

METHODS.

The methods used for loading the salivary tissue with $^{86}Rb^+$, superfusion, calculating efflux rate constants were as described in the methods section (chapter 2).

Solutions.

Na^+-containing solution (control) : The composition of this solution (in mmol l^{-1}) was : NaCl, 103; KCl, 4.7; $CaCl_2$, 2.56; $MgCl_2$, 1.13; $NaHCO_3$, 25; NaH_2PO_4, 1.15; D-glucose, 2.8; sodium fumarate, 2.7; sodium glutamate, 4.9; sodium pyruvate, 4.9. The pH of this solution was adjusted to 7.3-7.4.

Na^+-free solution : $NMDG^+$ was used as the Na^+ substituent in experiments where the Na^+ content of the control solution was lowered. The metabolic substrates were added to the $NMDG^+$-solution in their free acid form. NaCl was iso–osmotically replaced with $NMDG^+$-OH^- (119 mmol l^{-1}) and the pH was adjusted to 7.0 with 1N HCl. $NaHCO_3^-$ was replaced with $NMDG^+$-OH^- (25 mmol l^{-1}) and the solution then gassed with 95% O_2/ 5% CO_2 to produce $NMDG^+$-HCO_3^-. After 1-2 hours gassing the pH of the solution was 7.4.

Ca^{2+}-free solutions : Great care was taken to ensure that the salivary tissue was not exposed to an outwardly directed Ca^{2+} gradient. In preliminary experiments, this was achieved by reducing the $CaCl_2$ of the above solutions to 45 μmol l^{-1} and adding 0.1 mmol l^{-1} EGTA. However, in later experiments, low Ca^{2+}-containing solutions were prepared simply by omitting $CaCl_2$ and EGTA. The amount of free ionised Ca^{2+} present in these solutions was assumed (Miller & Smith, 1984) to be ~0.2 μmol l^{-1} and ~20 μmol l^{-1} respectively. These solutions provided similar results, so the data was pooled.

Experimental procedures.

Transient and sustained increases in the $^{86}Rb^+$-efflux rate were evoked using the following protocol : salivary fragments preloaded with $^{86}Rb^+$ were superfused with the appropriate, nominally Ca^{2+}-free solution to which ACh (10^{-5} mol l^{-1}) was added directly to elicit the transient response. The $[Ca^{2+}]_o$ of the superfusing solution was elevated to (0.25-3.0 mmol l^{-1}) in the continued presence of ACh to evoke the sustained response. This was achieved either by directly adding the appropriate amount of $CaCl_2$ to the superfusing solution or by switching to an alternative reservoir using a small tap.

Data analysis.

The basal (unstimulated) rate of $^{86}Rb^+$ efflux was defined as the mean rate measured 3 min prior to the addition of ACh (10^{-5} mol l^{-1}). Responses to ACh were calculated by subtracting this basal efflux from the efflux rate measured at the peak of the response. Increases in efflux rate evoked by raising $[Ca^{2+}]_o$ were similarly quantified using the rate constant measured immediately prior to the elevation of Ca^{2+}. The results are expressed as the mean ± standard errors and the significance of any differences between mean values were assessed using a Student's unpaired t-test. The value n refers to the number of individual experiments. Analysis of the $[Ca^{2+}]_o$-dependent increase in the efflux rate was determined from a single rectangular hyperbolae fitted to the experimental data using a least squares regression technique in which each mean value was

weighted to the reciprocal of its standard deviation. This technique calculated the "best fit" curve through the experimental data.

RESULTS.

Basal efflux rate.

The basal (unstimulated) efflux rate constant measured under control conditions (0.032 ± 0.001, n=65, table 4.1) did not differ significantly from the value previously measured during superfusion with the control solution (chapter 3). In the complete absence of $[Na^+]_o$, the basal efflux rate was significantly ($p<0.001$) elevated (table 4.1). This effect was reversible when the $[Na^+]_o$ of the $NMDG^+$-solution was raised to 4.5 mmol l^{-1} (table 4.1).

Transient increases in the efflux rate.

In the presence of $[Na^+]_o$ and under nominally Ca^{2+}-free conditions, ACh elicited a transient increase in the $^{86}Rb^+$-efflux rate (Δ min^{-1} 0.023 ± 0.001, n=65; figure 4.1 & table 4.1). This value was ~30% greater than the equivalent response measured from initial experiments presented in chapter 3 (Δ min^{-1} 0.017 ± 0.004, n=10; figure 3.3A).

On complete removal of $[Na^+]_o$, ACh could still elicit a transient increase in the efflux rate (Δ min^{-1} 0.016 ± 0.002, n=29, figure 4.3A; table 4.1). However, the magnitude of this response was significantly ($P<0.05$) smaller than that evoked under control conditions (table 4.1). This inhibitory effect still persisted when $[Na^+]_o$ was raised to 4.5 mmol l^{-1}, (figure 4.3B; table 4.1). However, normal transient responses could be evoked when $[Na^+]_o$ was elevated to 9.0 mmol l^{-1} or greater (figure 4.3C; table 4.1). Therefore, in contrast to earlier results, these findings suggest that the transient component of the response to ACh displays some dependence for $[Na^+]_o$.

Ca^{2+}-dependent increases in the efflux rate.

A sustained increase in the efflux rate could be evoked by ACh when the $[Ca^{2+}]_o$ of the control solution was subsequently elevated (0.25-1.5 mmol l^{-1}; figure 4.1) and it was found that this response was Ca^{2+}-dependent. The maximal increase in the efflux rate was 0.038 ± 0.004 min^{-1} and $[Ca^{2+}]_{50}$ was estimated to be ~1.0 ± 0.2 mmol l^{-1} (figure 4.2, circles).

Raising $[Ca^{2+}]_o$, (2 mmol l^{-1}), in the complete absence of $[Na^+]_o$ also evoked an increase in the efflux rate (figure 4.3A), but the maximal response (0.015 ± 0.003 min^{-1}; n=6) was significantly ($P<0.02$) smaller than the control value, could not be sustained and did not appear to be Ca^{2+}-dependent (figure

4.2, squares). The Ca^{2+}-dependent response to ACh is again Na^{+}-dependent and this result therefore supports earlier observations (chapter 3).

Na^{+} dependence of the biphasic response to ACh.

This dependence for Na^{+} was assessed in a further series of experiments in which biphasic responses were elicited at different concentrations of Na^{+} (figure 4.3). The inhibition of the transient component persisted when $[Na^{+}]_o$ was 4.5 mmol l^{-1}, but both the basal and the sustained response were normal under such conditions (table 4.1). The two phases of the response could therefore be further resolved by their requirement for $[Na^{+}]_o$. Essentially normal responses were obtained when $[Na^{+}]_o$ was raised to 9 mmol l^{-1} or above (figure 4.3; table4.1).

The extent to which raising $[Na^{+}]_o$ could reverse the inhibition exerted by $NMDG^{+}$ on the sustained phase was examined (figure 4.4). Salivary fragments were initially perfused with a Na^{+}-free, low Ca^{2+} solution and under these conditions ACh evoked a small transient increase in the efflux rate (figure 4.4). A further increase in the efflux rate was obtained when the $[Ca^{2+}]_o$ of this superfusing solution was raised to 2 mmol l^{-1}. When the $[Na^{+}]_o$ of this superfusate was raised to 36.1 mmol l^{-1} in the continued presence of both Ca^{2+} (2 mmol l^{-1}) and ACh (10^{-5} mol l^{-1}) the inhibitory effect exerted on the sustained response was not reversed (figure 4.4).

DISCUSSION.

In accordance with earlier experiments (chapter 3), the basal $^{86}Rb^{+}$ efflux rate was significantly elevated during superfusion with the Na^{+}-free, $NMDG^{+}$-solution. However, this effect was abolished when the $[Na^{+}]_o$ of the above solution was raised to 4.5 mmol l^{-1} or more. These findings confirm that the basal outflow of $^{86}Rb^{+}$ is dependent upon $[Na^{+}]_o$.

The transient increase in the efflux rate obtained in the presence of 144.4 mmol l^{-1} $[Na^{+}]_o$, was ~30% larger than that evoked in initial analagous experiments (table 3.2, chapter 3). This result could well be due to more efficient retention of Ca^{2+} within internal stores (endoplasmic reticulum) in the rat submandibular acini and provides further support for the proposal that the salivary fragments may have been exposed to an outwardly directed Ca^{2+} gradient when the initial $[Ca^{2+}]_o$ of the superfusing solution is reduced to ~0.02 μmol l^{-1}.

The present results indicated that the transient and sustained increase in the efflux rate were significantly inhibited in the complete absence of $[Na^{+}]_o$,

(table 4.1). However, the inhibitory effect exerted on the sustained phase (~60% inhibition) was much greater than that exerted on the transient phase (~30% inhibition). The inhibition of the transient component persisted until $[Na^+]_o$ was raised to 9.0 mmol l^{-1}. The inhibition of the sustained phase was removed when $[Na^+]_o$ was raised to 4.5 mmol l^{-1}. It would thus appear in the rat submandibular gland that the mobilisation of Ca^{2+} from internal and external pools is Na^+-dependent. Furthermore, these results also indicate that the transient and sustained increases in membrane K^+ permeability have differential sensitivity for $[Na^+]_o$. This finding therefore does not support the view that Na^+ removal specifically inhibits Ca^{2+}-influx (Gallacher & Morris, 1987).

The results from the Na^+-free experiments also demonstrated that the inhibitory effect exerted on the sustained phase was not readily reversible when the $[Na^+]_o$ of the Na^+-free $NMDG^+$-solution was raised from 0 to 36.1 mmol l^{-1}. This latter result is contrary to electrophysiological data obtained from isolated mouse submandibular acini. These experiments showed that the Ca^{2+}-activated K^+ channels which have become refractory to ACh in the presence of $NMDG^+$, can become re-activated (~3 to 4 min) when 20mmol l^{-1} $[Na^+]_o$ is introduced to the bath solution (Gallacher & Morris, 1987). Gallacher & Morris (1987) suggested that the inhibitory effect exerted by $NMDG^+$ on these Ca^{2+}-activated K^+ channels may be due to a depletion of intracellular Na^+ which would occur under Na^+-free conditions. It is thus possible that elevations in $[Na^+]_o$ can raise $[Na^+]_i$ rapidly in isolated rodent salivary acini, but not in pieces of rodent salivary tissue.

The present results suggest that both Ca^{2+} mobilisation and Ca^{2+}-influx are inhibited under Na^+-free conditions which therefore suggests that Na^+-dependent processes may contribute to these events. The physiological basis for this Na^+-dependency is not yet known. However, one hypothesis proposed is that if proton (H^+) extrusion via the Na^+-H^+ exchanger is blocked in the presence of $NMDG^+$, then the resultant fall in pH_i could inhibit the mobilisation of Ca^{2+} from internal and external pools (Grinstein & Goetz, 1985; Siffert & Akkerman, 1987; Gallacher & Morris, 1987). Another thesis put forward is that Ca^{2+}-inflow into rodent submandibular acini occurs via reversed Na^+-Ca^{2+} exchange ($[Na^+]_i$ would be exchanged for $[Ca^{2+}]_o$), (Gallacher & Morris, 1987). These theories thus require investigation and are therefore tested in the next chapter.

TABLE 4.1. Basal and biphasic responses to ACh at different $[Na^+]_o$. The values represent the mean ± S. E. and the number of individual experiments is indicated in parenthesis. Differences between the mean values were evaluated using an unpaired Student's t-test. *P<0.05, **P<0.01, ***P<0.001.

$[Na^+]_o$ (mmol)	Basal efflux rate constant (min^{-1})	ACh-evoked increase in $^{86}Rb^+$ efflux (Δ min^{-1}). Ca^{2+}-independent	Ca^{2+}-dependent
144.4	0.032 ± 0.001 (65)	0.023 ± 0.001 (65)	0.025 ± 0.003 (20)
36.1	0.032 ± 0.003 (6)	0.022 ± 0.006 (6)	0.023 ± 0.004 (6)
18	0.030 ± 0.001 (8)	0.023 ± 0.004 (8)	0.026 ± 0.005 (8)
9	0.031 ± 0.004 (9)	0.027 ± 0.007 (9)	0.021 ± 0.005 (9)
4.5	0.033 ± 0.002 (10)	0.014 ± 0.002 (10)**	0.018 ± 0.002 (10)
0	0.039 ± 0.002 (29)***	0.016 ± 0.002 (29)*	0.010 ± 0.001 (12)***

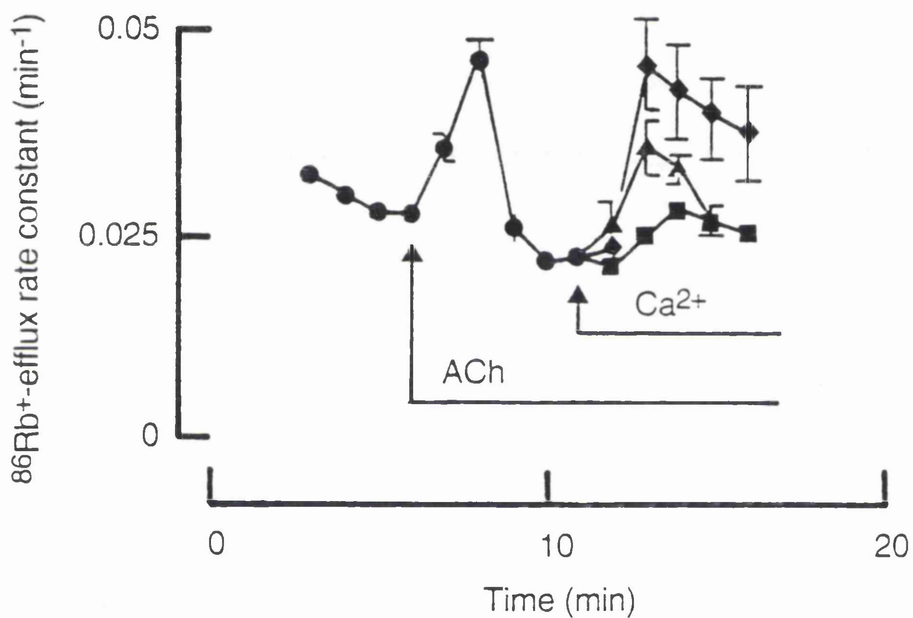

FIGURE 4.1. Dependence of the sustained increase in the $^{86}Rb^{+}$-efflux rate upon $[Ca^{2+}]_o$. The salivary fragments were initially superfused with the Ca^{2+}-free control-solution ($[Ca^{2+}]_o$~0.2 μmol l^{-1}), to which ACh (10^{-5} mmol l^{-1}) was added as shown. $[Ca^{2+}]_o$ was subsequently raised to 0.25 mmol l^{-1} (squares, n=12), 1.0 mmol l^{-1} (triangles, n=4) or 1.5 mmol l^{-1} (diamonds, n=8).

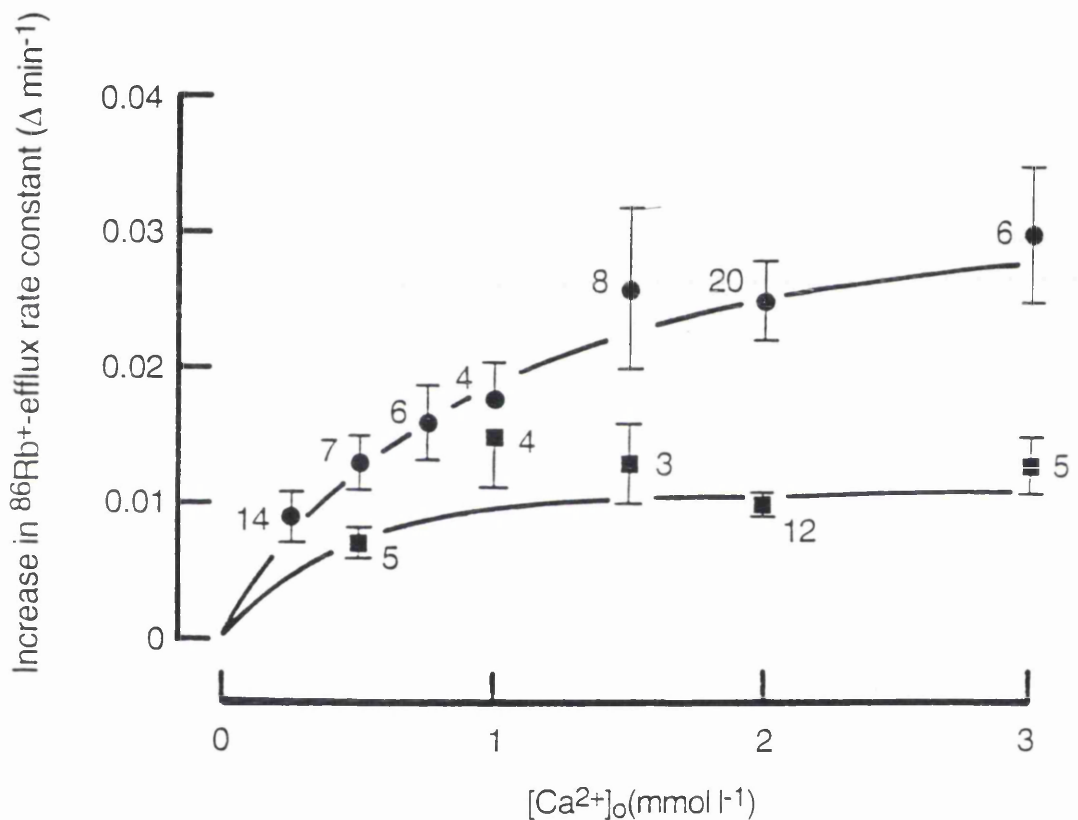

FIGURE 4.2. Ca^{2+}-dependent increases in the efflux rate evoked by ACh in figure 4.1 and other experiments (0.25-3.0 mmol l^{-1} $[Ca^{2+}]_o$) were quantified and then plotted against the appropriate $[Ca^{2+}]_o$ (figure 4.2, circles). The solid lines are single rectangular hyperbolae which were fitted to the experimental data and weighted to the standard deviation of each point using a least squares regression technique. Also presented in this figure are data obtained from a series of analogous experiments undertaken in the complete absence of $[Na^+]_o$ (0 mmol l^{-1} $[Na^+]_o$, squares).

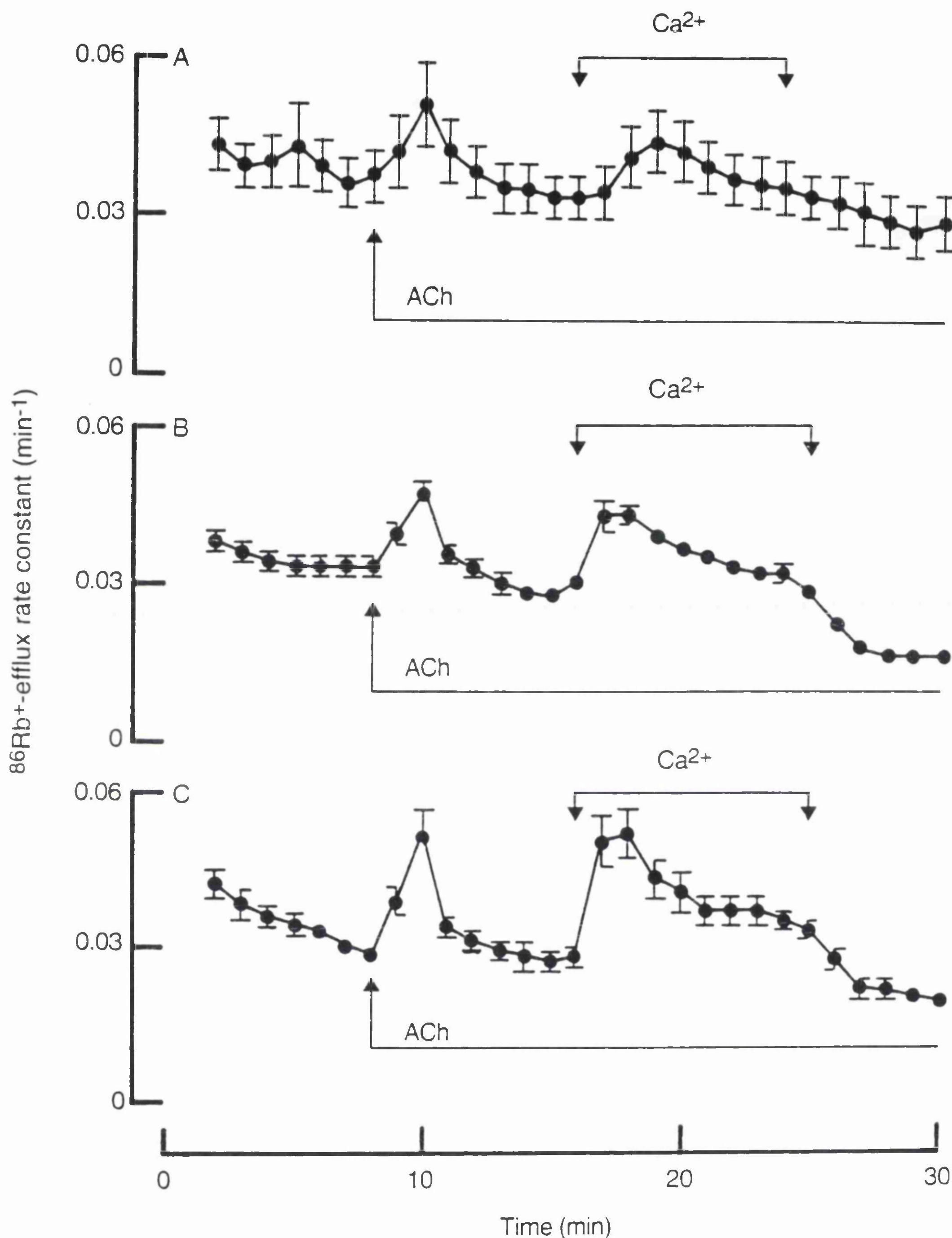

FIGURE 4.3. Biphasic responses to ACh evoked when the $[Na^+]_o$ of the $NMDG^+$-solution was 0 mmol l^{-1} (A, n=6); 9.0 mmol l^{-1} (B, n=9) and 18 mmol l^{-1} (C, n=8). In all experiments the salivary fragments were initially superfused with a Ca^{2+}-free solution ($[Ca^{2+}]_o$~20 µmol l^{-1}), to which ACh (10^{-5} mmol l^{-1}) and $CaCl_2$ (2 mmol l^{-1}) were added as indicated.

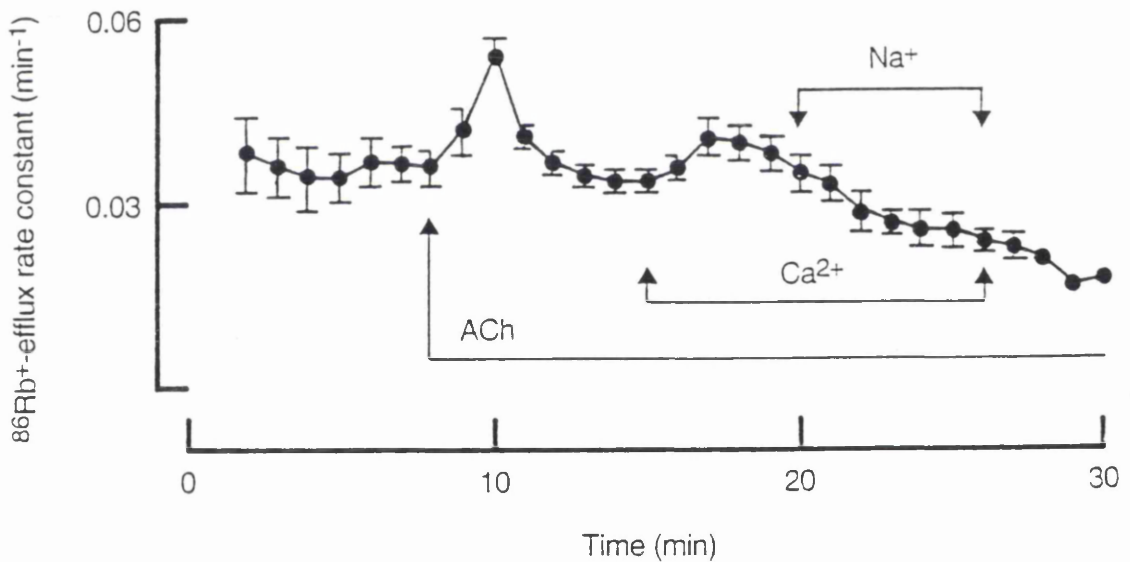

FIGURE 4.4. Effects of elevating $[Na^+]_o$ upon the Ca^{2+}-dependent component of the response to ACh. Salivary fragments were initially superfused with a Na^+ and Ca^{2+}-free solution ($[Na^+]_o$ = 0 mmol l^{-1}; $[Ca^{2+}]_o$~0.2 μmol l^{-1}) to which ACh (10^{-5} mmol l^{-1}) was initially added as indicated. The $[Ca^{2+}]_o$ of the superfusing solution was then raised to 2 mmol l^{-1} in the continued presence of ACh and 4 min later $[Na^+]_o$ was raised to 36.1 mmol l^{-1} again in the presence of ACh (n=6).

CHAPTER 5 : Na^+-DEPENDENT MECHANISMS REGULATING K^+ ($^{86}Rb^+$) and Ca^{2+} ($^{45}Ca^{2+}$) PERMEABILITY IN EXOCRINE GLANDS *IN VITRO*.

INTRODUCTION.

The results from the previous chapter suggested that ACh-evoked Ca^{2+} mobilisation and Ca^{2+}-influx involve Na^+-dependent processes. These findings are in contrast to analogous experiments undertaken in the human sweat gland where only Ca^{2+} mobilisation appears to be Na^+-dependent (Wilson, Bovell, Elder, Jenkinson & Pediani, 1990).

The physiological basis for this dependence upon $[Na^+]_0$ has not been established. However, fluorescent experiments performed on thrombin-stimulated human platelets have indicated that if proton (H^+) extrusion via the Na^+-H^+ exchanger is impaired, either by the removal of $[Na^+]_0$ or pharmacological blockade, then Ca^{2+} mobilisation and to a lesser extent Ca^{2+}-influx may be inhibited (Siffert & Akkerman, 1987).

It is thus possible that the inhibitory effects observed in the rat submandibular gland and human sweat gland may also be due to such an effect. This possibility was therefore investigated by examining the effects of amiloride, a potent inhibitor of Na^+-H^+ exchange, upon the cholinergic regulation of membrane K^+ ($^{86}Rb^+$) permeability in the rat submandibular gland and human sweat gland.

Contrary to the data obtained from the $NMDG^+$-experiments presented in chapter 4, patch-clamp experiments have indicated that only Ca^{2+} influx into rodent submandibular acini is inhibited in the presence of $NMDG^+$ (Gallacher & Morris, 1987). This result has led these scientists to postulate that Ca^{2+} inflow into rodent submandibular acini is Na^+-dependent and possibly occurs via reversed Na^+-Ca^{2+} exchange (intracellular Na^+ exchanged for extracellular Ca^{2+}). Therefore, another purpose of the present experiments was to test this thesis by using the radioactive isotope $^{45}Ca^{2+}$ to monitor the movement of cellular Ca^{2+} in the rat submandibular gland.

METHODS.

Isolation and superfusion of human sweat glands.

Sweat glands were isolated (Lee, Jones & Kealey, 1984) from samples of human skin which were obtained during general surgery with the informed consent of the patient and the approval of the local medical ethics committee.

Batches of 30-50 glands were transferred to a flow cell and loaded with $^{86}Rb^+$ (1 hour, 37°C) by superfusion with the control solution containing $^{86}RbCl$ (1-10 μCi l^{-1}). The glands were then washed for 2 min by superfusion (2 ml min^{-1}, 37°C) with unlabelled saline solution.

Submandibular glands were removed from freshly killed rats, chopped into pieces (<0.5 mm) and preloaded with $^{86}Rb^+$ or $^{45}Ca^{2+}$ in the usual way.

Solutions.

All experimental solutions were HCO_3^--buffered and low Ca^{2+}-solutions were prepared as described in chapter 4 to ensure that the glandular tissue was not exposed to an outwardly directed Ca^{2+} gradient.

Experimental procedures.

Glandular tissues were initially superfused with a nominally Ca^{2+}-free control solution to which ACh (10^{-5} mol l^{-1}) and $CaCl_2$ (sweat glands, 2.56 mmol l^{-1}; submandibular glands, 2 mmol l^{-1}) were added sequentially. The effects of amiloride (1 mmol l^{-1}) were assessed by including this drug in the superfusing solution throughout the duration of the experiment.

Data analysis.

Basal, transient and sustained increases in the $^{86}Rb^+$-efflux rate were evaluated as described in the preceding chapters. Data are expressed as the mean ± standard error. Differences between mean values were statistically analysed using a Student's unpaired t-test and n refers to the number of individual animals. Initial $^{45}Ca^{2+}$ washout experiments indicated that the basal outflow of $^{45}Ca^{2+}$ was not monoexponential (figure 5.3A). Using the linear regression technique, the slower exponential component (15-45 min period) was extrapolated from earlier time points (0-14 min) and replotted (figure 5.3B). The $t_{1/2}$ value (time required for the intracellular $^{45}Ca^{2+}$ content to be reduced by 50%) of the slow and fast exponential component of the basal $^{45}Ca^{2+}$ efflux rate was calculated by dividing the log of 0.5 by the slope value of each line.

RESULTS.

$^{86}Rb^+$-efflux experiments.

Human sweat gland.

Under Ca^{2+}-free conditions, ACh normally evoked a transient increase in the efflux rate (Δ min^{-1}, 0.077 ± 0.015, n=8, figure 5.1, circles). On the subsequent elevation of the $[Ca^{2+}]_o$ of the superfusing solution to 2.56 mmol l^{-1},

ACh evoked a further increase in the efflux rate which was sustained (Δ min^{-1}, 0.125 ± 0.015, n=8, figure 5.1, circles).

In the presence of amiloride, the basal efflux rate (0.065 ± 0.004 min^{-1}, n=7; figure 5.1, diamonds) was significantly inhibited (P<0.01) when compared to the control data (table 5.1). Under these experimental conditions ACh (10^{-5} mol l^{-1}) and Ca^{2+} (2.56 mmol l^{-1}) could still elicit an increase in the efflux rate. However, the peak increase of each these responses (transient; Δ min^{-1}, 0.030 ± 0.009; P<0.02; sustained; Δ min^{-1}, 0.039 ± 0.007; n=7; figure 5.1, diamonds) was significantly inhibited (transient, P<0.02; sustained, P<0.01; table 5.1).

Rat submandibular gland.

On superfusion with the Ca^{2+}-free control solution, ACh evoked a transient increase in the efflux rate (Δ min^{-1} 0.025 ± 0.002, n=14; figure 5.2; circles). When $[Ca^{2+}]_o$ was subsequently raised to 2 mmol l^{-1}, a further sustained increase in the efflux rate was evoked by ACh, (Δ min^{-1} 0.026 ± 0.004, n=14; figure 5.2; circles).

Contrary to the effects evoked by amiloride in the human sweat gland, the basal efflux rate (0.025 ± 0.001 min^{-1}, n=12) and the magnitude of the transient (Δ min^{-1} 0.019 ± 0.003) and sustained (Δ min^{-1} 0.028 ± 0.003) increases in the efflux rate were essentially normal (figure 5.2, diamonds; table 5.1).

$^{45}Ca^{2+}$-efflux experiments.

The wash-out of $^{45}Ca^{2+}$ from the salivary fragments during superfusion with the control solution is presented in figure 5.3A. At the onset of superfusion, the glandular tissue contained 4.85 ± 0.75 pmol of $^{45}Ca^{2+}$ (mg tissue wet weight)$^{-1}$, (n=8) and the percentage of $^{45}Ca^{2+}$ retained after 45 minutes superfusion was calculated to be 39 ± 4%. These experiments show that the basal outflow of $^{45}Ca^{2+}$ can be resolved into two, kinetically distinct, monoexponential components with $t_{1/2}$ values of 3.7 ± 0.5 min and 41.3 ± 7.5 min, respectively (figure 5.3B). The slower phase was assumed to reflect the washout of $^{45}Ca^{2+}$ from a single cellular compartment. Therefore, to ensure that the $^{45}Ca^{2+}$ efflux rate was monoexponential in subsequent experiments, the salivary fragments were superfused with the control solution for 15 minutes prior to sample collection (figure 5.4A).

The removal of Na^{+} from the control solution caused a slight but statistically insignificant decrease in the $^{45}Ca^{2+}$ efflux rate (figure 5.4C).

When Ca^{2+} was removed from the control solution ($[Ca^{2+}]_o$ ~0.02 μmol l^{-1}), the basal efflux rate slightly increased (figure 5.4B). However, this increase

was essentially transient and after ~5 minutes superfusion the efflux rate was normal. Statistical analysis of this response indicated that this increase was insignificant.

DISCUSSION.

The basal $^{86}Rb^{+}$-efflux rate measured from batches of isolated human sweat glands during superfusion with the control solution were ~3 times greater than those measured in the rat submandibular gland. This difference could be due to the $^{86}Rb^{+}$ being washed out more easily from the smaller sweat glands than the salivary fragments. However, differences in membrane K^{+} permeability between the different types of exocrine gland could also account for this.

The present results established that the basal, transient and sustained increases in the $^{86}Rb^{+}$-efflux rate were significantly inhibited when human sweat glands were exposed to amiloride. Identical experiments undertaken on the rat submandibular gland, however, indicated that in this tissue all of these parameters were essentially normal in the presence of amiloride. Amiloride, therefore impairs the regulation of K^{+} permeability in the human sweat gland, but not the rat submandibular gland.

The principal pharmalogical action of amiloride is to evoke a fall in pH_i by inhibiting H^{+} extrusion via the Na^{+}-H^{+} exchanger (Aronson, 1985; Grinstein, Cohen & Rothstein, 1984). This agent can therefore impair the regulation of pH_i in exocrine cells. Recent patch-clamp experiments have identified voltage- and Ca^{2+}-activated K^{+} channels in cultured human sweat gland epithelial cells and it has been established that the conductance of these channels is inhibited if the pH_i is lowered (Henderson, Brayden, Roberts & Cuthbert, 1990). The above finding could therefore explain why amiloride reduced K^{+} permeability in the unstimulated human sweat glands and significantly inhibited the biphasic response to ACh. Similar K^{+} channels have also been localised in rodent submandibular acini (Maruyama, Gallacher & Petersen, 1983; Petersen & Maruyama, 1984, Petersen, 1986; Gallacher & Morris, 1986; Petersen & Gallacher, 1988), so it is not known why amiloride did not elicit similar effects in this tissue. There is recent experimental evidence however, that strongly indicates that the properties of selective K^{+} channels in different types of exocrine cell vary. For example, the K^{+} channels in mouse submandibular acini (Gallacher & Morris 1986) appear more sensitive to changes in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$ than those in cultured human sweat gland epithelial cells (Henderson, Brayden, Roberts & Cuthbert, 1990). Furthermore, the K^{+} channels in *Necturus* gall bladder epithelial cells, which have similar $[Ca^{2+}]_i$-sensitivity to

those found in submandibular acini are more resistant to decreases in pH_i than those found in sweat gland epithelial cells (Copello, Segal & Reuss, 1991; Henderson, Brayden, Roberts & Cuthbert, 1990; Gallacher & Morris, 1986). It is thus possible that the different effects elicited by amiloride in the rat submandibular and human sweat gland could be due to the K^+ channels having differential pH_i- sensitivity.

Isotopic-efflux experiments undertaken on human sweat glands have indicated that the basal, transient and sustained efflux rate are profoundly impaired when HCO_3^- is replaced with the buffer HEPES (Wilson, Bovell, Elder, Jenkinson & Pediani, 1990a). Contrary to this result, all of these parameters were only slightly affected in the rat submandibular gland during superfusion with the HCO_3^--free Na^+-containing solution (chapter 3). These findings, therefore provide further support for the proposal that the K^+ channels in the human sweat gland are more sensitive to a drop in pH_i than those in the rat submandibular gland.

In electrically excitable cells Ca^{2+}-entry occurs via voltage-activated Ca^{2+} channels (Reuter, 1983). However, exocrine secretory cells do not possess these channels and the mechanism by which $[Ca^{2+}]_o$ enters the cytosol is not known (Putney, 1986; Petersen & Gallacher, 1988; Gallacher, 1988). Patch-clamp studies on rodent submandibular acini have indicated that Ca^{2+}-influx is Na^+-dependent and possibly occurs via reversed Na^+-Ca^{2+} exchange (Gallacher & Morris, 1987).

Although the Na^+-Ca^{2+} counter-transporter is not universally distributed, it has been identified in nerve, muscle and some secretory epithelial cells (Blaustein, 1974; Lee, Taylor, Windhager, 1980). This exchanger extrudes Ca^{2+} from the cell against its large electrochemical gradient and it achieves this by using the steep electrochemical gradient for Na^+ entry into the cell to drive Ca^{2+} efflux (Reuter & Seitz, 1968; Blaustein & Hodgkin , 1969; Blaustein, 1974). This transporter can operate as a freely-reversible system depending on the prevailing electrochemical gradient for Na^+ and has a stoichiometry of three Na^+ for one Ca^{2+} in the normal or reversed mode (Blaustein & Hodgkin, 1969; Pitts, 1979).

When all the Na^+ present in the control solution was replaced with $NMDG^+$, the Na^+-Ca^{2+} exchanger would operate in the reversed direction ($[Na^+]_i$ exchanged for $[Ca^{2+}]_o$). Therefore, if the salivary fragments were pre-loaded with $^{45}Ca^{2+}$ and the Na^+-Ca^{2+} exchanger were regulating cellular Ca^{2+} we would expect a decrease in the basal $^{45}Ca^{2+}$ efflux rate on removal of $[Na^+]_o$. However, the data from the $^{45}Ca^{2+}$ efflux experiments demonstrated that there was no significant decrease in the basal $^{45}Ca^{2+}$ efflux rate when $[Na^+]_o$ was

removed from the control solution. Furthermore, there was no significant increase in the basal $^{45}Ca^{2+}$ efflux rate when Ca^{2+} was removed from the control solution which one would expect since the concentration gradient opposing Ca^{2+} extrusion under these experimental conditions would be greatly reduced. These latter results therefore demonstrate that Na^{+}-Ca^{2+} exchange does not play an important role in maintaining cellular Ca^{2+} in the rat submandibular gland and hence, suggest that Ca^{2+}-influx does not occur via reversed Na^{+}-Ca^{2+} exchange.

TABLE 5.1. The effects of amiloride (1 mmol l^{-1}) upon the basal, transient and sustained rate of $^{86}Rb^{+}$-efflux in the rat submandibular gland and human sweat gland are presented. Values of n refer to number of experiments. *, p<0.02; **p<0.01.

	Control.	**Amiloride.**
Human sweat gland.		
n	8	7
Basal efflux rate (min^{-1})	0.088 ± 0.006	0.065 ± 0.004**
Transient phase (Δ min^{-1})	0.077 ± 0.015	0.030 ± 0.004*
Sustained phase (Δ min^{-1})	0.125 ± 0.023	0.039 ± 0.007**
Rat submandibular gland.		
n	14	12
Basal efflux rate (min^{-1})	0.027 ± 0.002	0.025 ± 0.001
Transient phase (Δ min^{-1})	0.025 ± 0.002	0.019 ± 0.003
Sustained phase (Δ min^{-1})	0.026 ± 0.004	0.028 ± 0.003

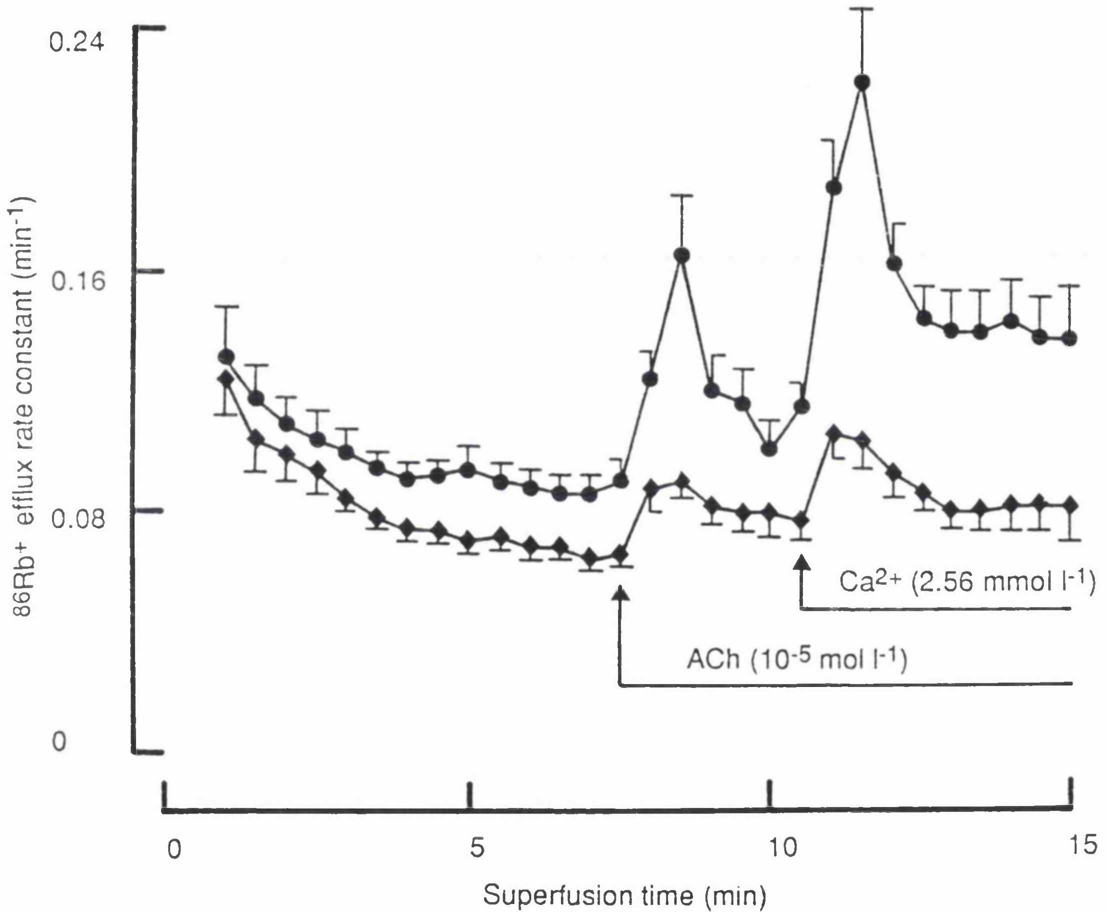

FIGURE 5.1. The cholinergic regulation of membrane K^+ permeability from batches of isolated human sweat glands in the absence (circles; n=8) and presence of 1 mmol l^{-1} amiloride (diamonds; n=7). Batches of between 30-50 glands were perfused with the Ca^{2+}-free, control solution to which ACh (10^{-5} mol l^{-1}) and $CaCl_2$ (2.56 mmol l^{-1}) were added as indicated.

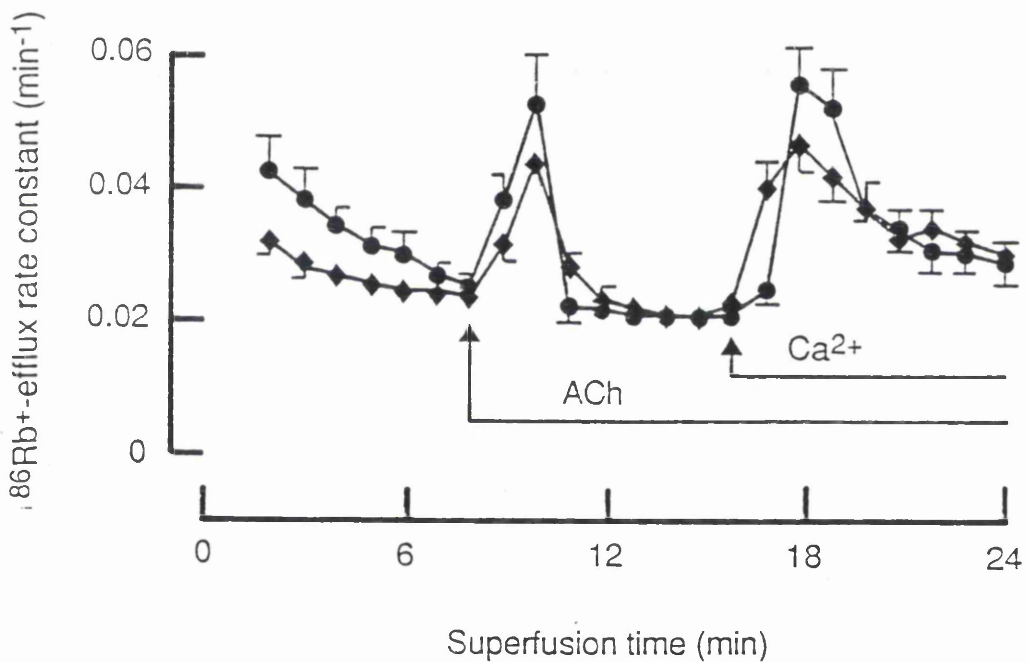

FIGURE 5.2. The effect of amiloride (1mmol l^{-1}) upon the efflux of $^{86}Rb^+$ from superfused rat submandibular fragments is shown. The salivary tissue was initially superfused with the nominally Ca^{2+}-free control solution ($[Ca^{2+}]_o$ ~20 μmol l^{-1}) to which ACh (10^{-5} mol l^{-1}) and Ca^{2+} (2 mmol l^{-1}) were added as indicated. The circles represent the control data (n=14) and the diamonds show the effects of amiloride (n=12).

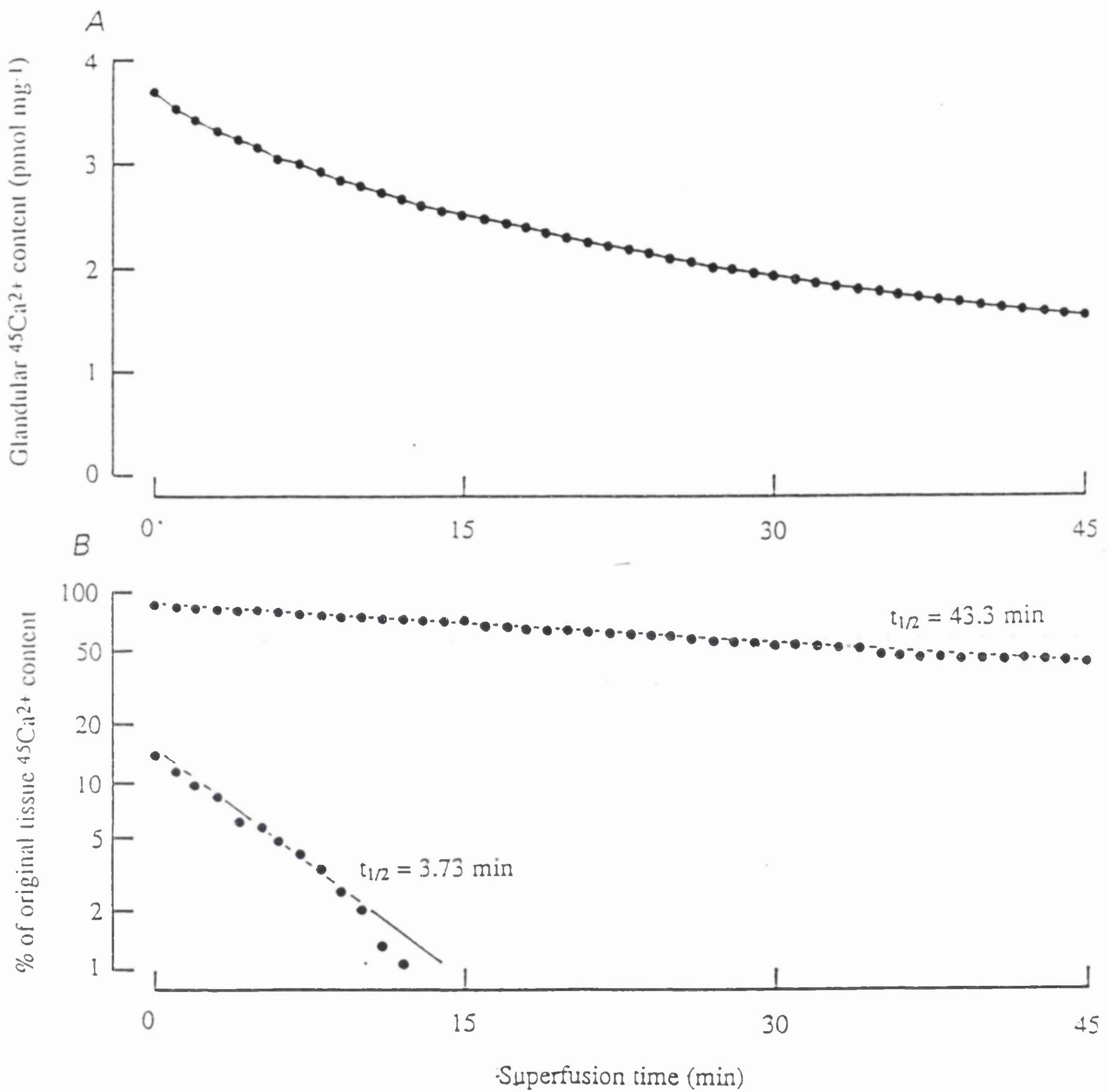

FIGURE 5.3 : shows the decline in the $^{45}Ca^{2+}$ content as the tracer is washed from the salivary fragments during superfusion with the control solution (figure 5.3A, n=8). Initial experiments demonstrated that the basal $^{45}Ca^{2+}$ efflux could be discerned into two, kinetically distinct, exponential components with $t^{1}/_{2}$ values of 3.7 ± 0.5 min and 41.3 ± 7.5 min respectively (figure 5.3B).

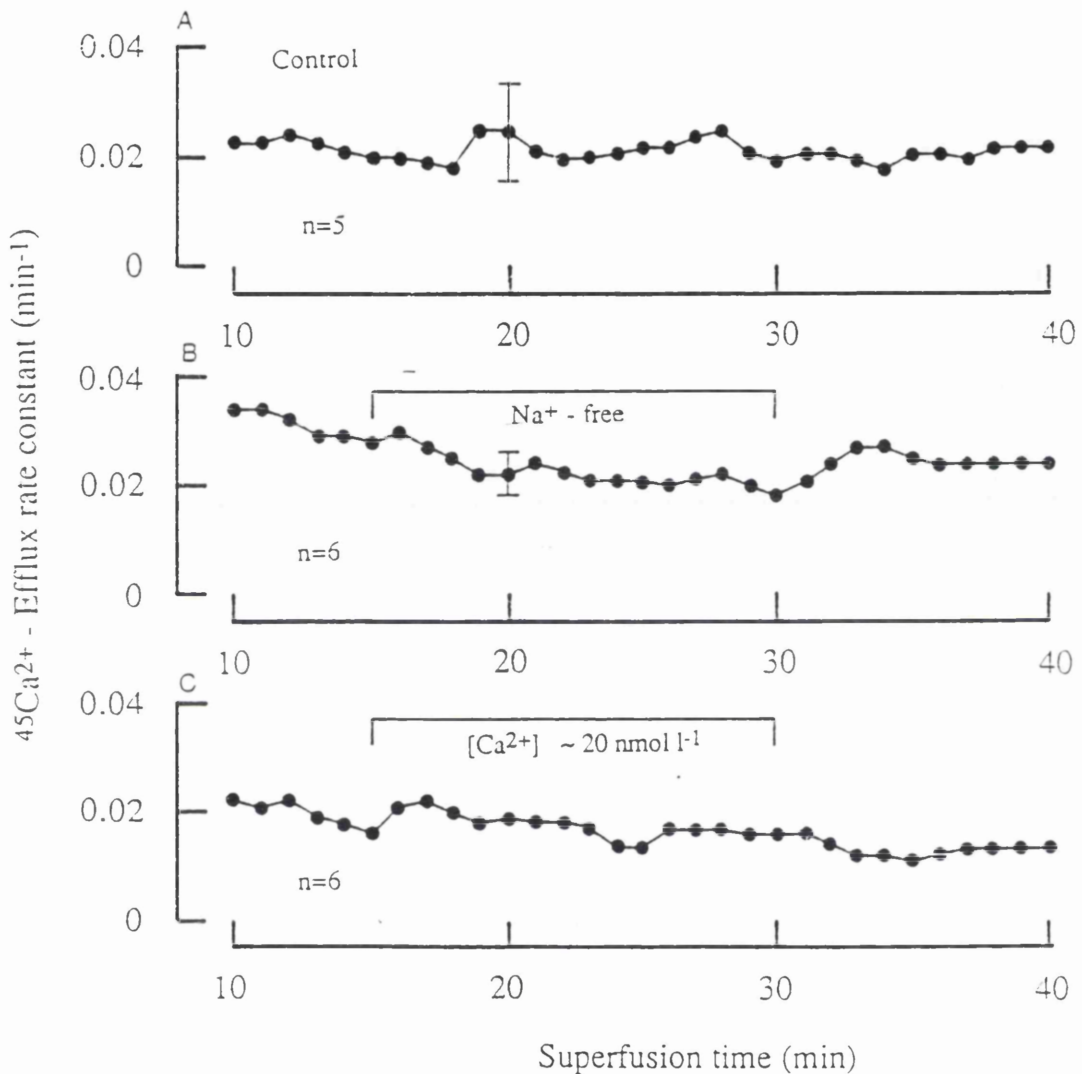

FIGURE 5.4 : (A) shows the monoexponential basal rate of $^{45}Ca^{2+}$ efflux during superfusion with the control solution (n=8). The effects of removing $[Na^{+}]_o$ (figure 5.4B, n=6) or $[Ca^{2+}]_o$ (figure 5.4C, n=6) from the control solution upon the basal efflux rate is also presented.

CHAPTER 6 : THE EFFECT OF THE PHORBOL ESTER TPA UPON THE CHOLINERGIC REGULATION OF MEMBRANE K^+ ($^{86}Rb^+$) PERMEABILITY IN THE RAT SUBMANDIBULAR GLAND *IN VITRO*.

INTRODUCTION.

The results from chapter 5 suggested that an inhibition of Na^+/H^+ exchange is not responsible for the inhibitory effect exerted by $NMDG^+$ on the transient and sustained components of the response to ACh in the rat submandibular gland. Furthermore, the findings from the previous chapter did not support Gallacher and Morris's hypothesis (1987) that Ca^{2+}-influx into rodent submandibular acini occurs via reversed Na^+-Ca^{2+} exchange. The mechanisms regulating Ca^{2+}-inflow into rodent submandibular acini therefore still remains unknown.

It is now well established that agonists that bind to muscarinic cholinoreceptors, such as ACh, are functionally coupled via a GTP-binding protein, to the enzyme phospholipase C. This enzyme catalyses the hydrolysis of the membrane phospholipid, phosphatidyl-inositol-bisphosphate (PIP_2) (Berridge & Irvine, 1984; 1989). The hydrophilic products of this reaction, and subsequent reactions, inositol-1,4,5-trisphosphate (IP_3) and inositol-1,2,4,5-tetrakisphosphate (IP_4), diffuse into the cytoplasm where they evoke a sustained increase in the internal free Ca^{2+} concentration ($[Ca^{2+}]_i$) by mobilising Ca^{2+} from internal and external pools (Streb, Irvine, Berridge & Schulz, 1983; Morris, Gallacher Irvine & Petersen, 1987; Berridge & Irvine, 1989). This rise in $[Ca^{2+}]_i$ causes the co-ordinated increases in Cl^- and K^+ membrane permeability and this is the underlying mechanism regulating fluid secretion in exocrine glands (Petersen, 1992).

The hydrophobic product of PIP_2 hydrolysis is diacylglycerol (DAG) that remains in the cell membrane where it allosterically activates the Ca^{2+} and phospholipid-dependent enzyme protein kinase C (PKC) (Kishimoto, Takai, Sawamura, Hoshijima, Fujikura & Nishizuka, 1980). The physiological significance of this event is not understood. It has been demonstrated in the perfused rabbit submandibular gland that the phorbol ester 12-0-tetra-decanoylphorbol-13-acetate (TPA), which can activate PKC (Nishizuka 1984, 1986, 1988), only inhibits the Ca^{2+}-dependent sustained phase of ACh-evoked salivary secretion (Rawlings, Brownbill, Steward & Case, 1990). This result therefore suggests that exogenous activation of PKC may limit the inflow of

$[Ca^{2+}]_o$ into stimulated cells. Therefore, to test this hypothesis the effects of TPA upon the cholinergic regulation of membrane K^+ permeability in the rat submandibular gland was examined.

METHODS.

Experimental protocol.

Rat submandibular gland fragments were preloaded with $^{86}Rb^+$ as described in the preceding chapters. All solutions were HCO_3^--buffered and again great care was taken to ensure that the salivary fragments were not exposed to an outwardly directed Ca^{2+} gradient during superfusion with Ca^{2+}-free solutions (details in chapter 4). In all the experiments, the fragments of salivary tissue were initially superfused with the Ca^{2+}-free control solution to which ACh (10^{-5} mol l^{-1}) was added directly. The $[Ca^{2+}]_o$ of the superfusing solution was then raised by switching to another ringer solution which contained 2 mmol l^{-1} $CaCl_2$ and fresh ACh (10^{-5} mol l^{-1}). The fresh ACh ensured that ACh would not be degraded during the time course of the experiment.

TPA (Sigma Chemical Company) was prepared as a stock solution (1.62 mmol l^{-1} in dimethyl sulphoxide) and was shielded from direct light. Superfusates containing TPA were prepared by adding aliquots from the TPA stock solution and ultrasonicating for ~30 seconds.

Data analysis.

The methods employed for calculating $^{86}Rb^+$-efflux rate constants, quantifying the basal, transient and sustained increases in the efflux rate are as detailed before. The data is given as the mean ± standard error, n refers to the number of individual experiments and differences between the means were determined either by using analysis of variance or a Student's unpaired t-test.

RESULTS.

In the experiments presented in figure 6.1A, the salivary fragments were initially perfused with the Ca^{2+}-free control solution ($[Ca^{2+}]_o$~0.2μmol l^{-1}). ACh (10^{-5} mol l^{-1}) was then added to this solution as indicated. In the continued presence of ACh, the $[Ca^{2+}]_o$ of the superfusing solution was raised to 2 mmol l^{-1} for each of the three five minute periods shown (figure 6.1A). Under Ca^{2+}-free conditions, ACh could elicit a transient increase in the rate of $^{86}Rb^+$-efflux (Δ min^{-1} = 0.026 ± 0.003, n=11). The efflux rate reached a peak after 1 min, from which it rapidly declined to the basal level. Further increases in the $^{86}Rb^+$-efflux rate constant could be evoked when the $[Ca^{2+}]_o$ of the superfusing solution

was raised to 2 mmol l^{-1} in the continued presence of ACh (figure 6.1A). The response to the first rise in $[Ca^{2+}]_o$ consisted of a rapid increase in the efflux rate to a peak (Δ min^{-1} = 0.029 ± 0.004, n=11), from which there was a rapid decline to a more sustained phase which persisted until $[Ca^{2+}]_o$ was lowered. Analysis of variance indicated that the increase in the efflux rate evoked during the second or third elevation in $[Ca^{2+}]_o$ (second elevation, Δ min^{-1} = 0.014 ± 0.002; third elevation, Δ min^{-1} = 0.012 ± 0.002) was significantly smaller ($P<0.01$ and $P<0.001$ respectively) than the increase evoked by the first initial rise in $[Ca^{2+}]_o$.

The experiments presented in figure 6.1B were designed to examine the effects of TPA upon the Ca^{2+}-dependent increases in $^{86}Rb^+$-efflux depicted in figure 6.1A. In an initial series of experiments the salivary tissue was first exposed to TPA (20 nmol l^{-1}) 2 minutes after the addition of ACh (10^{-5} mol l^{-1}). The response to the initial elevation of $[Ca^{2+}]_o$ (Δmin^{-1} = 0.020 ± 0.003, n=6) was slightly smaller but not statistically different, from the equivalent control value. The responses to subsequent increases in $[Ca^{2+}]_o$ (second, Δ min^{-1} = 0.013 ± 0.001; third, Δ min^{-1} = 0.011 ± 0.002; n=6) were also indistinguishable from their equivalent control values (see table 6.2). Similar results were obtained when the salivary fragments were exposed to a higher concentration of TPA (80 nmol l^{-1}) (diamonds, figure 6.2B). It would thus appear that TPA does not affect the Ca^{2+}-dependent component of the response to ACh.

Further experiments were undertaken in which the phorbol ester TPA (20 nmol l^{-1}) was present in the superfusing Ca^{2+}-free control solution from 5 minutes prior to the addition of ACh (figure 6.2). The data from these experiments confirmed that TPA does not exert an inhibitory effect on the Ca^{2+}-dependent component and showed that the basal efflux rate was also unaffected by TPA. The initial Ca^{2+}-independent component of the response to ACh (Δmin^{-1} = 0.014 ± 0.003, n=8), was however, significantly smaller ($p<0.01$) when compared to the value obtained in a series of control experiments (table 6.2). This latter finding suggests that TPA can exert an inhibitory effect on the Ca^{2+}-independent component of the response to ACh.

DISCUSSION.

The data from the control experiments confirmed that ACh evokes an increase in membrane K^+ permeability in rat submandibular acini and that this response could be resolved into a Ca^{2+}-independent transient and a Ca^{2+}-dependent sustained phase. These can be attributed to the mobilisation of Ca^{2+} from internal stores and to an inflow of Ca^{2+} from the external fluid respectively (Putney, 1976a; Petersen, 1992).

Cholinergic secretagogues are functionally coupled to the enzyme phospholipase C (Birdsall, Hulme & Stockton, 1984; Evans & Marty, 1986) which catalyses the hydrolysis of the membrane phospholipid PIP_2 (Berridge & Irvine, 1984; 1989) to form the second messengers IP_3 and DAG. IP_3 passes into the cytosol where it evokes the release of Ca^{2+} from internal stores (Berridge & Irvine, 1984; 1989; Streb, Irvine, Berridge & Schultz, 1983) and DAG remains in the cell membrane where it allosterically activates the Ca^{2+}-dependent enzyme PKC (Kishimoto, Takai, Sawamura, Hoshijima, Fujikura & Nishizuka, 1980). The physiological significance of this latter event is not fully understood.

Biochemical experiments investigating the role played by PKC in cell surface signal transduction and tumour promotion have demonstrated that tumour promoting phorbol esters such as TPA can activate PKC and thereby mimic the effect of endogenously produced DAG (Nishizuka 1984, 1986). In the present study, PKC was endogenously activated using TPA and the data from these experiments clearly demonstrated that TPA only inhibited the Ca^{2+}-independent component of the response to ACh. This finding indirectly suggests that PKC may be capable of exerting a negative feedback mechanism on the mobilisation of Ca^{2+} from intracellular stores in the rat submandibular gland. A study looking specifically at muscarinic responses in PC12 cells provides further support for this hypothesis, since both carbachol-evoked phosphoinositide hydrolysis and Ca^{2+} mobilisation are inhibited in the presence of phorbol esters (Vicentini, Di Virgilio, Ambrosini, Pozzan & Meldolesi, 1985). Electrophysiological, (Llano & Marty, 1987; Maruyama, Y. 1989) and microspectrofluorimetric, (Tan & Marty, 1991; Ko, O'Dowd, Pediani, Elder, Bovell, Jenkinson & Wilson, 1994) experiments performed on various cell types have also similarly demonstrated that TPA impairs receptor-regulated hydrolysis of PIP_2. There is evidence, however, which does not support the above hypothesis. For example, in the perfused rabbit submandibular gland, TPA inhibits ACh-evoked salivary secretion only during prolonged activation, suggesting, in contrast to the present data, that activation of PKC only impairs Ca^{2+}-influx (Rawlings, Brownbill, Steward & Case, 1990). It is thus possible that there are marked variations in the sensitivity of salivary glands from different species to phorbol esters.

TABLE 6.2. Effects of TPA upon the basal, transient and sustained $^{86}Rb^+$-efflux rate.

	Control (n=8)	**TPA** (n=6)
Basal efflux rate constant (min^{-1})	0.033 ± 0.002	0.027 ± 0.002
Transient response (Δ min^{-1})	0.024 ± 0.003	0.014 ± 0.003 *
Sustained response (Δ min^{-1})	0.024 ± 0.004	0.022 ± 0.006

* P<0.01 with respect to the control group.

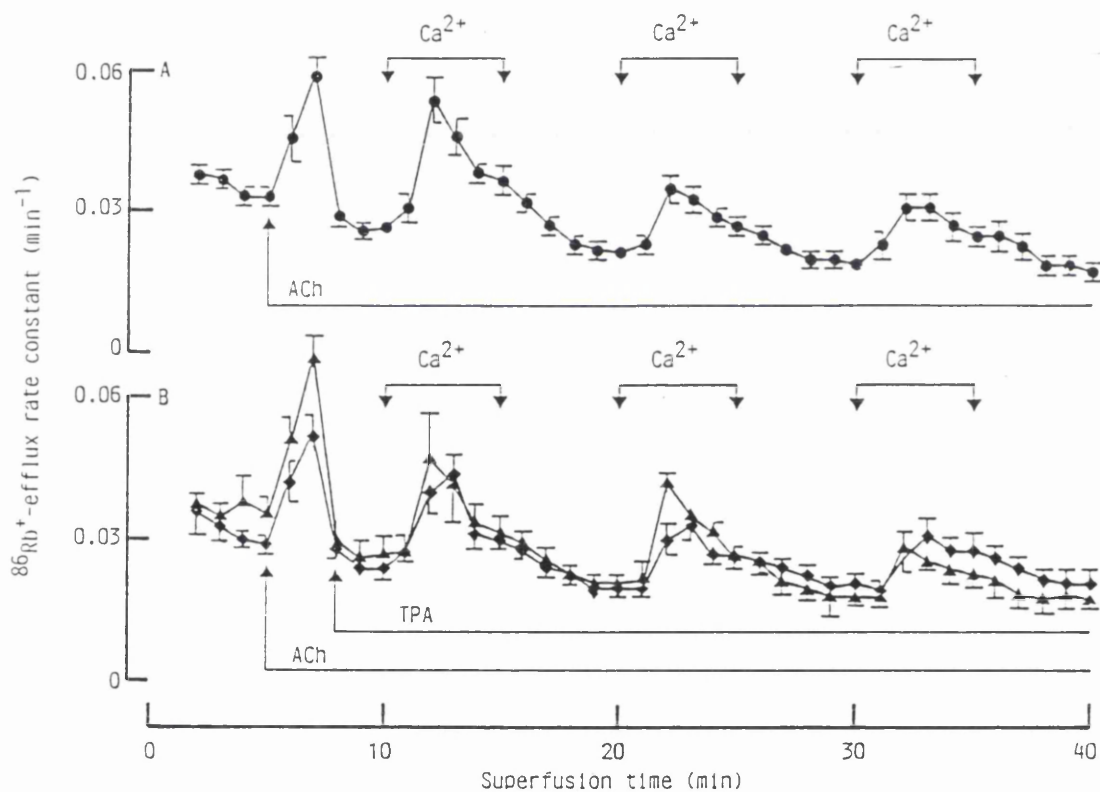

FIGURE 6.1. Effects of TPA upon the Ca^{2+}-dependent component of the response to ACh. Control data (n=11) are presented in A and B shows the effects of 20 nmol l^{-1} (triangles, n=6) and 80 nmol l^{-1} (diamonds, n=3) TPA.

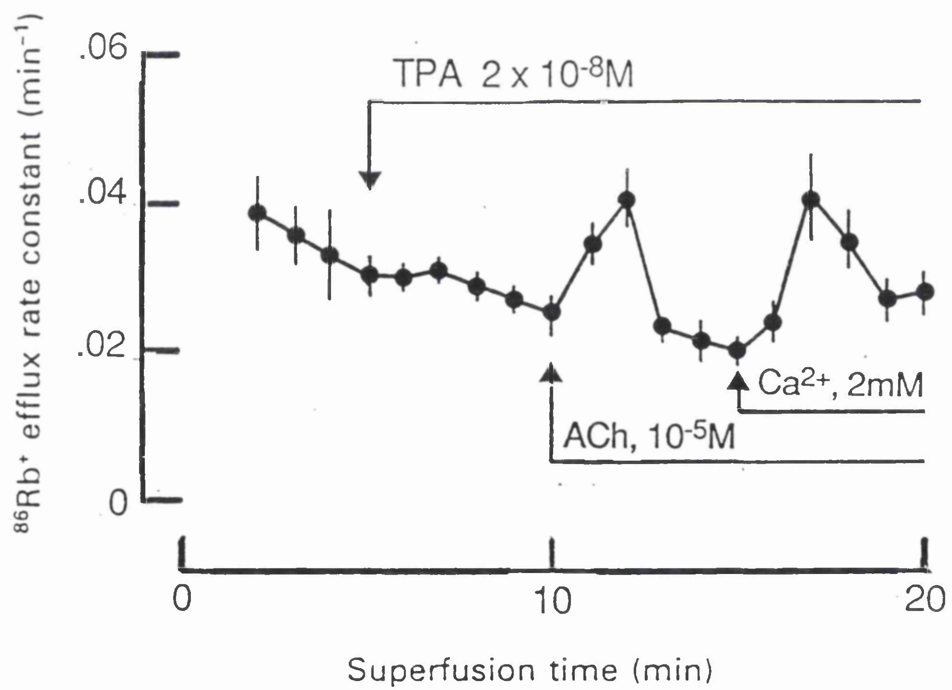

FIGURE 6.2. The effects of TPA (20 nmol l^{-1}) upon the basal, transient and sustained increases in the $^{86}Rb^+$-efflux rate (n=8).

CHAPTER 7 : GENERAL DISCUSSION.

Validity of $^{86}Rb^+$ as an indicator of membrane K^+ permeability.

The voltage-sensitive Ca^{2+}-activated K^+ channels found in rodent salivary acini are known to have an extremely low $^{86}Rb^+$ conductance (Gallacher, Maruyama & Peterson, 1984). However, in spite of this, it has been proposed that $^{86}Rb^+$ can still monitor changes in cellular K^+ permeability provided the cytosolic $^{86}Rb^+$ to K^+ ratio is low (Gallacher, Maruyama & Peterson, 1984). In this study, the measured cytoplasmic $^{86}Rb^+$ to K^+ ratio was always less than 0.002 (Bovell, Elder, Pediani & Wilson, 1989b; Wilson, Bovell, Elder, Jenkinson & Pediani, 1990a) and so I have therefore assumed that changes in the rate of $^{86}Rb^+$ efflux would reflect changes in membrane K^+ permeability.

Effects of ACh upon membrane K^+ permeability.

When $^{86}Rb^+$-loaded rat submandibular fragments or isolated human sweat glands were exposed to ACh in the presence of $[Na^+]_o$, there was a rapid and immediate increase in the $^{86}Rb^+$-efflux rate. This result confirmed that the secretory cells from both types of gland lose cellular K^+ on cholinergic activation and therefore supports the hypothesis that fluid secretion from exocrine glands is associated with an increase in basolateral membrane K^+ permeability (Burgen, 1956; Petersen, 1970, 1980; Nishiyama & Petersen, 1974; Roberts, Iwatsuki, & Petersen, 1978; Roberts & Petersen, 1978; Parod & Putney, 1978b; Gallacher & Petersen, 1980).

In common with the rat parotid and sublingual gland, this cholinergically-evoked K^+ efflux could be discriminated into two distinct phases (Putney, 1976a; Marier, Putney & van de Walle; Parod & Putney, 1978b; Putney, Leslie & Marier, 1978), a Ca^{2+}-independent transient phase followed by a Ca^{2+}-dependent sustained phase. It thus appears that the loss of K^+ from the rat submandibular and human sweat gland in response to ACh is mediated by an increase in $[Ca^{2+}]_i$, which is initiated by the mobilisation of Ca^{2+} from intracellular stores (Putney, 1976a, Aub, McKinney & Putney, 1982; Aub & Putney, 1987) and is sustained by an influx of Ca^{2+} from the extracellular fluid (Putney, 1976a; 1977; Marier, Putney, van de Walle, 1978). Using the highly fluorescent Ca^{2+} indicater dye Fura-2 (e.g. dual excitation fluorimetry technique), these events have been shown to occur in isolated rat parotid acinar cells (*e.g.* Merritt & Rink, 1987) and more recently in cultured human sweat gland epithelial cells (Wilson, Lee, Smith, Pediani, Brown & Elder, 1992).

Comparison of the effects of bicarbonate and HEPES.

^{31}P NMR spectroscopic measurements of pH_i in the unstimulated rabbit submandibular gland have indicated that the removal of HCO_3^- from phosphate-free Ringer's solution evokes a fall in the resting pH_i (Steward, Seo & Case, 1989). However, in rabbit submandibular acini (Lau, Elliott & Brown, 1989) and cultured horse sweat gland epithelial cells (Pediani, Stewart, Elder & Wilson, 1994), resting pH_i is elevated when HCO_3^- is removed from the perfusate.

It has been demonstrated in various cell types that the activity of Ca^{2+}-activated K^+ channels is reduced if pH_i is lowered (Cook, Ikeuchi & Fujimoto, 1984; Christensen & Zeuthen, 1987; Brown, Loo, Wright, 1988; Cornejo, Guggino & Guggino, 1989; Copello, Segal & Reuss, 1991). The data obtained from the rat submandibular gland indicated that the basal efflux rate was normal, and the transient and sustained increases in K^+ efflux were only slightly impaired in the absence of HCO_3^-. This finding is in contrast to directly analogous isotopic experiments undertaken using human sweat glands, where all of these parameters are severely impaired under HCO_3^--free conditions (Wilson *et al.*, 1990a). It is thus possible that the K^+ permeabilty coupling process in the human sweat gland is more sensitive to a fall in pH_i than the rat submandibular gland and hence, requires further investigation. Using the fluorescent H^+ and Ca^{2+} indicators BCECF and Fura-2 respectively, this possibility could be confirmed by looking at ACh-evoked changes in the cytosolic H^+ and Ca^{2+} concentration of secretory cells isolated or cultured from both gland types in the presence and absence of HCO_3^-.

Preliminary experiments recently undertaken on a cell line derived from the human sweat gland epithelium have demonstrated that the Ca^{2+}-mobilising agonist ATP can evoke an increase in $[Ca^{2+}]_i$ under HCO_3^--free conditions (Wilson, Whiteford, Bovell, Pediani, Ko, Smith, Lee & Elder, 1994). It was also shown in these cells that this rise in $[Ca^{2+}]_i$ could evoke corresponding changes in membrane anion permeability.

Effects of $NMDG^+$ upon membrane K^+ permeability.

It has been reported for unstimulated mouse submandibular acini, that exposure to $NMDG^+$-containing solutions reduces the basal activity of the Ca^{2+}-activated K^+ channels in this tissue (Gallacher & Morris, 1987). This finding therefore suggests that a reduction in the basal $^{86}Rb^+$ efflux rate from the rat submandibular gland would occur in the presence of $NMDG^+$. However, data obtained from initial (chapter 3) and subsequent experiments (chapter 4) did not support Gallacher & Morris's data (1987), and indicated that the basal efflux rate

was significantly elevated on exposure to $NMDG^+$. Since Gallacher & Morris's work (1987), various scientists have demonstrated that resting pH_i is decreased and resting $[Ca^{2+}]_i$ is elevated when salivary acini are bathed in $NMDG^+$-containing solutions (Arkle, Gillespie & Greenwell, 1988; Brown, Donohue, Elliott & Lau, 1989; Elliott, Lau & Brown, 1991). Increased $[Ca^{2+}]_i$ would activate K^+ channels (Maruyama, Gallacher & Petersen, 1983), thereby permitting an increase in the K^+-efflux rate, but it has also been reported that a fall in pH_i can have an opposing effect (Cook, Ikeuchi & Fujimoto, 1984; Christensen & Zeuthen, 1987; Brown, Loo, Wright, 1988 Cornejo, Guggino & Guggino, 1989; Copello, Segal & Reuss, 1991). It is thus very difficult to predict the consequences of Na^+ removal.

Initial experiments undertaken on the rat submandibular gland indicated that exposure to the impermeant cation $NMDG^+$ specifically inhibited the sustained component of the response to ACh (chapter 3). This finding therefore supported Gallacher & Morris's hypothesis that receptor-regulated Ca^{2+}-inflow into rodent submandibular acini, which supports the sustained increase in membrane K^+ permeability, is Na^+-dependent (Gallacher & Morris, 1987; Morris, Fuller & Gallacher, 1987). Using the fluorescent Ca^{2+} indicator Fura-2, Merritt & Rink, (1987) and more recently Elliott, Lau & Brown (1991) have reported that Ca^{2+}-influx into mammalian salivary acini is not Na^+-dependent and these findings therefore do not support Gallacher & Morris's hypothesis (1987). These conflicting results may be due to the fact that these authors have employed a direct method for assessing Ca^{2+}-inflow, whereas, Gallacher & Morris (1987) and I have used indirect methods which apparently can suffer from the problem of interference from changes in pH_i which can effect the activity of K^+ channels (Elliott, Lau & Brown, 1991). Morris, Fuller & Gallacher (1987), using the Ca^{2+}-sensitive fluorescent probe, Quin-2 to assess the cholinergic-receptor regulation of $[Ca^{2+}]_i$ in rat submandibular acini, however, reported that the Ca^{2+}-influx pathway in rat submandibular acini is Na^+-dependent.

Elliott, Lau & Brown (1991) demonstrated that in the presence of $NMDG^+$, resting pH_i is decreased in salivary acini and there is evidence that a fall in pH_i can reduce K^+ channel activity (Cook, Ikeuchi & Fujimoto, 1984; Christensen & Zeuthen, 1987; Brown, Loo, Wright, 1988; Cornejo, Guggino & Guggino, 1989; Copello, Segal & Reuss, 1991). It is thus possible that the decrease in pH_i which accompanies Na^+ removal may be responsible for at least some of the inhibitory effects of $NMDG^+$-containing solutions on Ca^{2+}-influx in rodent submandibular acini (Gallacher & Morris, 1987, chapter 3).The data from these initial $NMDG^+$-experiments also suggested that $NMDG^+$ may exert a small

inhibitory effect on the transient component (chapter 3). The possible factors which could be responsible for causing this slight inhibition are the buffer used to maintain the pH of these solutions and their Ca^{2+} composition. This may be because the submandibular gland fragments would have been exposed to an outwardly directed Ca^{2+} gradient when the initial $[Ca^{2+}]_o$ of the superfusing solution was ~0.02 μmol l^{-1} and the data suggested that the biphasic response to ACh could be slightly impaired under HCO_3^--free conditions (chapter 3).

In the light of these possibilities, the effects of $NMDG^+$ were re-examined using HCO_3^--buffered $NMDG^+$-solutions in which the $[Ca^{2+}]_o$ of the superfusing solution was raised to either ~0.2 or 20 μmol l^{-1} to ensure that the salivary fragments were not exposed to an outwardly directed Ca^{2+} gradient. The data obtained under these experimental conditions confirmed again that the sustained component of the ACh-evoked K^+ permeability increase was Na^+-dependent, but also indicated that $NMDG^+$ inhibits the transient increase in the efflux rate (chapter 4). Although the inhibitory effect exerted on the transient phase was much smaller than that exerted on the sustained phase it persisted when the $[Na^+]_o$ of the $NMDG^+$-solution was raised to 4.5 mmol l^{-1}, whereas, the sustained phase returned to normal under these conditions (chapter 4). It would thus appear in the rat submandibular gland that the transient and sustained increase in membrane K^+ permeability have differential sensitivity for $[Na^+]_o$. The data from these experiments also demonstrated that the inhibitory effect exerted on the sustained component of the cholinergic response was not readily reversible when the $[Na^+]_o$ of the $NMDG^+$-solution was subsequently raised to 36.1 mmol l^{-1}. This latter result is contrary to electrophysiological data obtained from isolated mouse submandibular acini which has shown that Ca^{2+}-activated K^+ channels which have become refractory to ACh in the presence of $NMDG^+$ are re-activated (~3 to 4 mins) when 20mmol l^{-1} Na^+ is introduced to the bathing solution (Gallacher & Morris, 1987). Gallacher & Morris (1987) suggested that the inhibitory effect exerted by $NMDG^+$ on these Ca^{2+}-activated K^+ channels may be due to a depletion of the intracellular Na^+ concentration ($[Na^+]_i$) which would occur under Na^+-free conditions. It is thus possible that elevations in $[Na^+]_o$ can raise $[Na^+]_i$ rapidly in isolated rodent salivary acini, but not in pieces of rodent salivary tissue.

Analogous $NMDG^+$-experiments were also performed using human sweat glands. These studies indicated that the cholinergic regulation of membrane K^+ permeability is also inhibited under Na^+-free conditions, but the pattern of inhibition observed is different to that seen in the rodent submandibular gland (Gallacher & Morris, 1987; Bovell *et al.*, 1989b). In the human sweat gland

$NMDG^+$ has little effect on the sustained component of the response, but essentially, abolishes the transient response (Wilson *et al.*, 1990a). Furthermore, this inhibitory effect can be rapidly reversed if Na^+ is introduced into the superfusing solution (Wilson *et al.*, 1990a). It would therefore appear that in the human sweat gland that only Ca^{2+} mobilisation is acutely dependent upon $[Na^+]_o$, whereas in the rat submandibular gland the mobilisation of Ca^{2+} from internal and external pools is Na^+-dependent (chapter 4). It is difficult to explain why $NMDG^+$ evokes different effects in the human sweat gland and rat submandibular gland since it has been proposed that both gland types share an essentially similar stimulus-secretion coupling mechanism (Petersen & Maruyama, 1984; Putney, 1986; Petersen & Gallacher, 1988; Saga, Sato & Sato, 1988; Bovell, Elder, Jenkinson & Wilson, 1989a; Bovell *et al.*, 1989b; Wilson *et al.*, 1990a).

The effects of amiloride.

The main pharmacological action of amiloride is to compromise H^+ extrusion via the Na^+-H^+ exchanger and so evoke a fall in pH_i (Aronson, 1985, Grinstein, Cohen & Rothstein, 1984). When the human sweat gland was exposed to this blocker, the basal, transient and sustained rates of $^{86}Rb^+$-efflux were significantly impaired (chapter 5). In contrast, when these experiments were repeated in the rat submandibular gland, all of these parameters were essentially normal on exposure to amiloride (Chapter 5).

Recent patch-clamp experiments have identified voltage- and Ca^{2+}-activated K^+ channels in cultured human sweat gland epithelial cells and it has been established that the conductance of these channels is inhibited if pH_i drops (Henderson, Brayden, Roberts & Cuthbert, 1990). This latter finding could well explain why amiloride reduced membrane K^+ permeability in the unstimulated human sweat glands and significantly inhibited the biphasic response to ACh. However, K^+ channels with similar electrophysiological properties appear to be present in rodent submandibular acini (Gallacher & Morris, 1986; Maruyama, Gallacher & Petersen, 1983; Petersen & Maruyama, 1984; Petersen, 1986; Petersen & Gallacher, 1988), so it is very difficult to rationalise why amiloride did not elicit a similar effect in this tissue.

Recent electrophysiological data have suggested that the K^+ channels in rodent submandibular acini (Gallacher & Morris, 1986) appear more sensitive to changes in the $[Ca^{2+}]_i$ than those in cultured human sweat gland epithelial cells (Henderson, Brayden, Roberts & Cuthbert, 1990). Furthermore, the K^+ channels in *Necturus* gall bladder epithelial cells, which have similar $[Ca^{2+}]_i$-sensitivity to

those found in rodent submandibular acini, are more resistant to a fall in pH_i than those found in sweat gland epithelial cells (Copello, Segal & Reuss, 1991; Henderson, Brayden, Roberts & Cuthbert, 1990; Gallacher & Morris, 1986). It is thus possible that the different effects elicited by amiloride in the rat submandibular and human sweat gland is due to the K^+ channels having differential pH_i sensitivity.

Calcium mobilising secretagogues (e.g. ACh) can increase the rate of proton extrusion via the Na^+-H^+ exchanger and hence have the capacity to raise pH_i (Dufresne, Bastie, Vaysse, Creach, Hollande & Ribet 1985; Soltoff, McMillian, Cantley, Cragoe & Talamo, 1989; Siffert & Akkerman, 1987, Steward, Seo & Case, 1989; Sage, Jobson & Rink, 1990). In rat thymocytes, it has been recently proposed that this rise in pH_i plays a significant physiological role in the stimulus-response coupling process by facilitating Ca^{2+}-influx (Grinstein & Goetz, 1985) or Ca^{2+}-mobilisation (Brass & Joseph, 1987) during cell activation. Furthermore,data from thrombin-stimulated human platelets suggest that Ca^{2+} mobilisation is abolished and Ca^{2+}-influx suppresed if Na^+-H^+ exchange is prevented, either by the removal of $[Na^+]_o$ or by pharmacological blockade (Siffert & Akkerman, 1987). As a result of these latter findings, it has been postulated that receptor-mediated activation of Na^+-H^+ exchange is an integral component of the stimulus-response coupling process, either by facilitating Ca^{2+} mobilisation or Ca^{2+}-influx (Siffert & Akkerman, 1987). However, it seems highly unlikely that a decrease in pH_i caused by an impairment of Na^+-H^+ exchange is solely responsible for the inhibition of cholinergic responsiveness in the rat submandibular and human sweat gland since amiloride did not duplicate the same effects when $[Na^+]_o$ was replaced with $NMDG^+$.

Not all studies, however, support the view that Na^+-H^+ exchange plays such a central role in the stimulus-response coupling process (Rink, 1987; Sage, Jobson & Rink, 1990; Simpson & Rink, 1987). It may therefore be possible to explain the inhibitory effects exerted by $NMDG^+$ solutions in terms of a less specific action. For example, it has recently been demonstrated in unstimulated salivary acini that in the presence of $NMDG^+$-containing solutions pH_i is decreased (Arkle, Gillespie & Greenwell, 1988; Brown, Donohue, Elliott & Lau, 1989) and it has been demonstrated in human platelets that a fall in pH_i can inhibit IP_3-evoked Ca^{2+} mobilisation (Brass & Joseph, 1985).

Changes in both $[Ca^{2+}]_i$ and pH_i have been measured in rat thymocytes (Grinstein & Goetz, 1985), pancreatic acini (Gillespie, Greenwell & Scratcherd, 1988) and squid axons (Mullins & Requena, 1987). These investigations have

shown that changes in the pH_i can result in two main effects. These are primarily to promote the sequestration of intracellular Ca^{2+} when the pH_i is increased and evoke the mobilisation of Ca^{2+} from internal stores when the pH_i is decreased. From these observations it has been proposed that an inverse relationship exists between $[Ca^{2+}]_i$ and pH_i (Gillespie, Greenwell & Scratcherd, 1988; Grinstein & Goetz, 1985; Grinstein, Goetz-Smith & Cohen, 1987; Mullins & Requena,1987). If this is so, then the inhibitory effects evoked when the pH_i is decreased could be reversed by a rise in $[Ca^{2+}]_i$ and may well explain why the regulation of K^+ permeability in the rat submandibular gland was insensitive to amiloride.

Does Ca^{2+}-influx in submandibular acini involve Na^+-Ca^{2+} exchange.

In electrically excitable cells Ca^{2+}-inflow occurs via voltage-activated Ca^{2+} channels (Reuter, 1983). These channels, however, have not been found in rodent salivary acini and the route by which Ca^{2+} passes from the extracellular fluid into the cytoplasm is not yet known (Putney, 1986; Petersen & Gallacher, 1988; Gallacher, 1988). Patch-clamp data recorded from mouse submandibular acini has indicated that Ca^{2+}-influx is Na^+-dependent and possibly occurs via reversed Na^+-Ca^{2+} exchange (Gallacher & Morris, 1987). The data from the $^{45}Ca^{2+}$-efflux experiments, however, indicated that there was no increase or decrease in the efflux rate when either Ca^{2+} or Na^+ was removed from the control solution. Such changes may be expected if Na^+-Ca^{2+} co-transport was responsible for regulating cellular Ca^{2+} in this tissue. These results therefore suggest that Ca^{2+}-inflow into rat submandibular acini does not occur via reversed Na^+-Ca^{2+} exchange, however, until a specific inhibitor for Na^+-Ca^{2+} exchange is found, these results can only be tentatively interpreted.

Possible roles for protein kinase C and diacylglycerol.

Agonists that bind to muscarinic cholinoreceptors are functionally coupled to the enzyme phospholipase C (Birdsall, Hulme & Stockton, 1984; Evans & Marty, 1986) which catalyses the hydrolysis of the membrane phospholipid PIP_2 (Berridge & Irvine, 1984; 1989) to form the second messengers IP_3 and DAG. IP_3 passes into the cytosol where it evokes the release of Ca^{2+} from internal stores (Berridge & Irvine, 1984; 1989; Streb, Irvine, Berridge & Schultz, 1983) and DAG remains in the cell membrane where it allosterically activates the $[Ca^{2+}]_i$-dependent enzyme PKC (Kishimoto, Takai, Sawamura, Hoshijima, Fujikura & Nishizuka, 1980). The physiological significance of this latter event is not fully understood. Biochemical experiments investigating the role played by PKC in cell surface signal transduction and

tumour promotion have demonstrated that exogenous, tumour promoting phorbol esters, such as TPA, can activate PKC and so mimic some of the effects of cholinergic secretagogues (Nishizuka 1984, 1986). In some of the experiments performed on the rat submandibular gland, PKC was activated using the phorbol ester TPA and the data from these experiments clearly showed that TPA only inhibited the Ca^{2+}-independent, transient increase in the $^{86}Rb^{+}$-efflux rate. This finding strongly suggests that PKC and, hence, DAG may be capable of exerting a negative feedback mechanism on the mobilisation of Ca^{2+} from intracellular stores in the rat submandibular gland. A study looking specifically at muscarinic responses in PC12 cells and smooth muscle cells provides further support for this hypothesis, since both carbachol-evoked phosphoinositide hydrolysis and Ca^{2+} mobilisation are inhibited in the presence of phorbol esters (Brock, Rittenhouse, Powers, Ekstein, Gimbrone & Alexander, 1985; Vicentini, Di Virgilio, Ambrosini, Pozzan & Meldolesi, 1985; McMillan, Chernow & Roth, 1986; Baron & Coburn, 1987b; Kotlikoff, Murray & Reynolds, 1987). In addition to these findings it has been demonstrated in rat lacrimal, rabbit submandibular and guinea pig pancreatic acini that TPA again inhibits Ca^{2+} mobilisation in response to cholinergic-agents (Ansah, Dho & Case, 1986; Llano & Marty, 1987; Maruyama, 1989). There is other experimental evidence, however, which does not agree with this hypothesis. For example, in the perfused rabbit submandibular gland, TPA inhibits ACh-evoked salivary secretion only during prolonged activation (Rawlings, Brownbill, Steward & Case, 1990), suggesting, in contrast to the present data from the rat submandibular gland, that activation of PKC impairs only Ca^{2+}-influx. In addition to this, it has been reported that TPA has no effect on the rise of the $[Ca^{2+}]_i$ evoked by cholinergic and catecholaminergic agonists in rat parotid acinar cells (Gray, 1988).

Using the dual excitation fluorimetry technique, it would thus be interesting to see what effect TPA has on ACh-evoked changes in $[Ca^{2+}]_i$ in single rat submandibular acini loaded with the fluorescent Ca^{2+} indicator Fura-2. Furthermore, using a specific binding assay to measure IP_3 (Palmer & Wakelam, 1990), it would also be useful to examine the effects of TPA upon ACh-elicited IP_3 production in these acini.

Further work.

Present experiments have suggested that there are complex actions between pH_i and $[Ca^{2+}]_i$ and both of these may be important to the regulation of membrane K^+ permeability during fluid secretion. Further experiments are required to address these problems. The fluorescent dyes BCECF and Fura-2 are

now being routinely used in this laboratory to measure the changes in pH_i and $[Ca^{2+}]_i$ that occur in stimulated epithelial cells.

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APPENDIX 1: Composition of physiological salt solutions.

The solutions used for isolating, loading and superfusing the batches of isolated human sweat glands and fragments of rat submandibular gland are detailed as follows :

HUMAN SWEAT GLAND ISOLATION SOLUTION.

Stock solution A.

NaCl (1M)	= 58.44g.
KCl (48mM)	= 3.578g.
KH_2PO_4 (12mM)	= 1.633g.
Distilled water	= 800 ml.

Dissolve.

Distilled water to 1 litre.

Stock solution B.

HEPES (250mM)	= 59.575g.
$NaHCO_3$ (200mM)	= 16.8g.
$MgSO_47H_2O$ (12mM)	= 2.96g.
Distilled water	= 800 ml.

Dissolve.

Distilled water to 1 litre.

Working solution.

Stock solution A	= 100ml.
Stock solution B	= 100ml.
Distilled water	= 600ml.
D-glucose	= 1.98g.

Gas working solution with 95% O_2/5% CO_2.

Add 1.3 ml of molar $CaCl_2$ whilst gassing and adjust the pH to 7.4 with normal NaOH.

Distilled water to 1 litre.

Gas solution with 95% O_2/5% CO_2 and check pH is 7.4.

HCO_3^--BUFFERED Na^+-CONTAINING SOLUTION (CONTROL).

Stock solution C.

NaCl (1.03M)	= 60.193g.
KCl (47mM)	= 3.504g.
1M $MgCl_27H_2O$ (11.3mM)	= 1.13ml.
Distilled water to 1 litre.	

Stock solution D.

$NaHCO_3$ (250mM)	= 21.003g.
NaH_2PO_4 (11.5mM)	= 1.38g.
Distilled water to 1 litre.	

Working solution.

Stock solution C	= 100ml.
Stock solution D	= 100ml.
Distilled water	= 600ml.
D-Glucose	= 0.55g.
Na-pyruvate (4.9mM)	= 0.539g.
Na_2-fumarate (2.7mM)	= 0.432g.
Na-glutamate (4.9mM)	= 0.828g.
Gas solution with 95% O_2/5% CO_2.	
Molar $CaCl_2$ (2.56mM)	= 2.56ml.
Distilled water to 1 litre.	

Gas working solution with 95% O_2/5% CO_2 and check pH = 7.4.

HEPES–BUFFERED Na^+–CONTAINING SOLUTION.

Stock solution E.

NaCl (1.03M)	= 60.193g.
KCl (47mM)	= 3.504g.
$MgCl_2$ (11.3mM)	= 1.13 ml of 1M solution.

Disilled water to 1 litre.

Working solution.

Stock solution E	= 100ml.
NaH_2PO_4 (1.15mM)	= 0.138g
HEPES (25mM)	= 5.958g.
Distilled water	= 600ml.
D-Glucose	= 0.55g.
Na-pyruvate (4.9mM)	= 0.539g.
Na_2-fumarate (2.7mM)	= 0.432g.
Na-glutamate (4.9mM)	= 0.828g.

Gas working solution with 100% O_2.

Adjust to pH7.4 with normal NaOH (~25ml.)

Add Molar $CaCl_2$	= 2.56ml.
Check pH	= 7.4.

Dist. water to 1 litre.

Gas solution with 100% O_2.

HEPES-BUFFERED, NMDG$^+$-CONTAINING SOLUTION.

1Molar Pyruvic acid solution.

Pyruvic acid.	= 3.467ml.
Distilled water	= to 50 ml.

From this volume, remove 5ml aliquots and freeze.

Working solution.

NMDG$^+$ (144.4mM)	= 28.19g.
KCl (3.55mM)	= 0.265g
KH_2PO_4 (1.15mM)	= 0.157g.
$MgCl_2$ (11.3mM)	= 1.13 ml of 1M solution.
HEPES (25mM)	= 5.958g.
D-Glucose (2.8mM)	= 0.556g.

Add the following metabolites (free acid form).

Pyruvic acid (4.9mM)	= 4.9ml of 1M solution.
Glutamic acid (4.9mM)	= 0.721g.
Fumaric acid (2.7mM)	= 0.313g.
Distilled water	= to 800ml.

Gas working solution with 100% O_2.

Adjust pH to 7.4 with normal HCl (~110 ml).

Molar $CaCl_2$	= 2.56ml.

Add distilled water to 1 litre.

Gas working solution with 100% O_2.

HEPES-BUFFERED Li^+-CONTAINING SOLUTION.

Working solution.

Li Cl (108mM)	= 4.578g.
KCl (3.55mM)	= 0.265g.
KH_2PO_4 (1.15mM)	= 0.157g.
$MgCl_2$ (11.3mM)	= 1.13 ml of 1M solution.
HEPES (25mM)	= 5.958g.
D-Glucose (2.8mM)	= 0.556g.
EGTA (0.1mM)	= 0.038g.

Add the following free acids.

Pyruvic acid. (4.9mM)	= 4.9ml of 1M solution.
Glutamic acid (4.9mM)	= 0.721g.
Fumaric acid (2.7mM)	= 0.313g.
Distilled water	= 800ml.

Gas working solution with 100% O_2.

Molar $CaCl_2$ = 2.56ml.

Adjust to pH 7.4 with 1M LiOH (~23-24 ml).

Add distilled water to 1 litre.

Gas working solution with 100% O_2.

HCO_3^--BUFFERED NMDG$^+$-CONTAINING SOLUTION.

Working solution.

KCl (3.55mM)	= 0.265g.
KH_2PO_4 (1.15mM)	= 0.157g.
Mg Cl_2 (1.13mM)	= 1.13 ml of 1M solution.
D-Glucose (2.8mM)	= 0.556g.

Add the following free acids.

Pyruvic acid (4.9mM)	= 4.9ml of 1M solution.
Glutamic acid (4.9mM)	= 0.721g.
Fumaric acid (2.7mM)	= 0.313g.
Distilled water	= 800ml.
EGTA (0.1mM)	= 0.038g.
NMDG$^+$ (119.4mM)	= 23.307g
Dist. water	= 800ml.

Adjust to pH 7.4 with normal HCl .

Add NMDG$^+$ (25mM) = 4.88g.

Gas working solution with 95%O_2/5%CO_2 until pH is 7.4.

Make solution up to 1 litre with distilled water.

PUBLISHED WORK

I also present for examination the following collection of published papers some of which describe the results of collaborative studies which are not described in the main part of this thesis.

Bovell, D.L., Elder, H.Y., Pediani, J.D. and Wilson, S.M. (1989). Potassium ($^{86}Rb^{+}$) efflux from the rat submandibular gland under sodium-free conditions *in vitro*. *J. Physiol.,* **416**, 503–515.

Wilson, S.M., Bovell, D.L., Elder, H.Y., Jenkinson, D.M. and Pediani, J.D. (1990). The effects of removing external sodium upon the control of potassium ($^{86}Rb^{+}$) permeability in the isolated human sweat gland. *Exp. Physiol.,* **75**, 649–656.

Wilson, S.M., Pediani, J.D., Cockburn, F., Bovell, D.L., Jenkinson, D.M., Paton, J.Y., Coutts, J., Davidson, R., Lamberts, J., Morris, G. and Elder, H.Y. (1991). The cholinergic regulation of potassium ($^{86}Rb^{+}$) permeability in sweat glands isolated from patients with cystic fibrosis. *Exp. Physiol.,* **76**, 573–578.

Wilson, S.M., Pediani, J.D., Jenkinson, D.M. and Elder, H.Y. (1992). Amiloride impairs the cholinergic regulation of potassium permeability in the human sweat gland but not in the rat submandibular gland. *Experientia,* **48**, 1115-1117.

Wilson, S.M., Pediani, J.D., Ko, W.H., Bovell, D.L., Kitson, S., Montgomery, I., Brown, U.M.O., Smith, G.L., Elder, H.Y. and Jenkinson, D.M. (1993). Investigation of stimulus–secretion coupling in equine sweat gland epithelia using cell culture techniques. *J. Exp. Biol.,* **183**, 179–199.

Ko, W.H., O'Dowd, J.J.M., Pediani, J.D., Elder, H.Y., Bovell, D.L., Jenkinson, D.M. and Wilson, S.M. (1994). Extracellular ATP can activate autonomic signal transduction pathways in cultured equine sweat gland epithelial cells. *J. Exp. Biol.,* **190**, 239–252.

Pediani, J.D., McEwan, P.E., Elder, H.Y. and Wilson, S.M. (1994). The effect of removing external sodium upon the cholinergic regulation of potassium permeability in the rat submandibular gland. *Comp. Biochem. Physiol.,* **107C**, 283–288.

Wilson, S. M., Whiteford, Bovell, D. L., Pediani, J. D., Ko, W. H., Smith, G. L., Lee, C. M. & Elder, H. Y. (1994). The regulation of membrane $^{125}I^{-}$ and $^{86}Rb^{+}$ permeability in a permanent cell line (NCL-SG3) derived from the human sweat gland epithelium. *Exp. Physiol.,* **79**, 445-459.

Pediani, J.D. and Wilson, S.M. (1994). The effect of a phorbol ester upon the cholinergic regulation of potassium permeability in the rat submandibular gland. *Experientia,* in press.