THE ELECTROPHYSIOLOGY OF MAMMALIAN SALIVARY GLANDS

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This Thesis is dedicated to my mother and father

Jane and Ron Morris

CONTENTS.

SUMMARY.

Cholinergic muscarinic receptor stimulation of salivary and lacrimal acinar cell secretion is associated with an increase in the basolateral membrane permeability towards K^+ ions. The patch-clamp technique has been employed to demonstrate a voltage and Ca^{++} sensitive large conductance K^+ channel within these membranes. The conductive properties and activities of this channel were investigated in both insitu cell-attached and excised patches and were shown capable of regulating the K^+ conductance in resting acinar cells. The cell-attached recording configuration was employed to demonstrate that cholinergic receptor regulation of K^+ channel activity is a messenger mediated effect i.e. activation of the channel occurs secondary to a) release of Ca^{++} from intracellular stores and b) influx of Ca^{++} into the cell. Activation of the intracellular Ca^{++} rise was found to be dependent upon $[Na^+]_{ext}$ in both electrophysiological and in parallel intracellular dye studies using the Ca^{++} indicator quin-2.

Pilot studies, undertaken to investigate the relevance of a rodent model for salivary acinar cell secretion based upon the electrophysiological evidence of the K^+ selective channel were corroborated with the finding of a similar voltage and Ca^{++} sensitive channel in human salivary acinar cells. This K^+ conductance could likewise be stimulated by the cholinergic agonist acetylcholine.

The whole-cell technique was employed to investigate the presence of cationic and anionic conductive pathways in both salivary and lacrimal acinar cells. A method for internal

pipette perfusion which enables the composition of the intracellular solution to be changed during the course of the experiment was developed. When used in conjunction with whole-cell recording, by monitoring changes in the voltage and Ca^{++} sensitive current, the secondary messenger function of Ca^{++} in lacrimal acinar cells was directly demonstrated. The mechanisms underlying the receptor transduction of the Ca^{++} signal in acinar cells was investigated. The studies demonstrate that $Ins(1,4,5)P_3$ promotes intracellular Ca^{++} release and that $Ins(1,3,4,5)P_4$ in combination with $Ins(1,4,5)P_3$ promotes a rise in intracellular Ca^{++} attributable to Ca^{++} influx.

INTRODUCTION.

The studies outlined in this thesis commenced in January 1984 only a short time after the patch-clamp technique had been introduced in order to research exocrine secretory mechanisms. To place the electrophysiological data which forms the basis of these investigations into context with our understanding of fluid and electrolyte secretion in exocrine glands, I will review the earlier developments within the fields of electrophysiology and ion transport.

Early Non-Electrophysiological Studies of the Events Accompanying Salivary Secretion.

Salivary glands secrete protein and electrolytes as saliva into the oral cavity in response to neural stimulation. Salivary glands are diverse both in the types of gland found within an animal and even more so between species (Young and Van Lennep, 1978). In man the volume of saliva secreted can reach 0.75L/day which is equivalent to one fifth the plasma volume (Schneyer, 1955). This secretion in man does not play a role in the regulation of whole-body fluid and electrolyte balance as most is reabsorbed later by the alimentary canal. In animals lacking sweatglands, evaporative cooling through fluid loss from the oral cavity is an important thermoregulatory mechanism. In man Saliva plays an important role in oral health, lubrication, deglutination, speech and taste as well as the digestion of starches and glycogen by the presence of salivary amylase found in many species.

The primary secretion of electrolytes and water takes place at the most proximal unit of the secretory element, the acinus. A generalised view of an endpiece element from a

serous gland such as the parotid is seen in Fig.1.

Heidenhain in 1878 noted the correlation between intensity of parasymaphetic nerve stimulation and change in salivary output from dog submaxillary and parotid salivary glands. As the strength of the stimulus increases, salivary flow rate increases to a plateau and so does the total concentration of salts present in saliva. Early workers looking at the composition of saliva in cat submaxillary (Langstroth, 1938), dog parotid (Gregersen, 1931) and the major human salivary glands (Thaysen et al., 1954; Hilde, 1955) presented a model for the relationship described by Heidenmain. They demonstrated that sodium and potassium were the major cations found in saliva. This secretion had greater levels of K^+ and HCO_3^- and lower levels of Na^+ and Cl^- than those found in the serum (this holds true for all hypotonic salivas Schneyer, 1969). Thaysen, Thorn and Schwartz (1954) proposed two stages in the formation of saliva. This model consisted of a primary acinar secretion isotonic with the plasma which subsequently underwent modification in the ductal region to form saliva. This hypothesis was based upon the observation that at progressively higher rates of secretion the Na^+ concentration of saliva increased whilst the K^+ concentration remained constant. This effect was explained as saturation of the secondary Na^+ reabsorb tive mechanism and not ionic concentration by water reuptake.

Support for this model was subsequently found using micropuncture and microperfusion techniques and is reviewed by Burgen (1967) and Schneyer and colleagues (1972); the two stage hypothesis of salivary formation still holds true today.

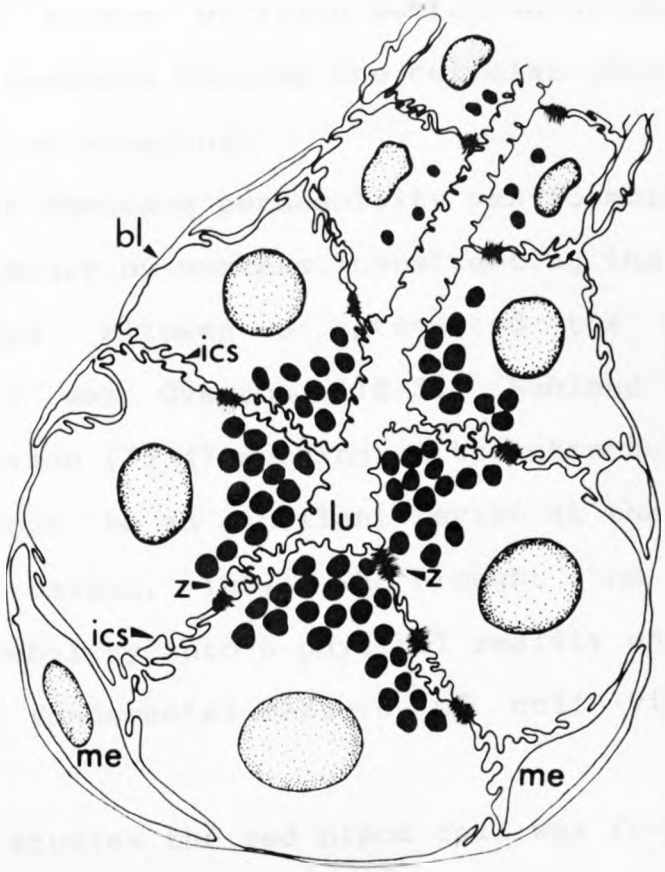


Fig.1. Diagram of an salivary acinus. The acinus is composed generally of large pyramidal cells which delineate a small lumen (lu). Tight junctions (z) separate lateral intracellular spaces (ics) from secretory caniculi (s) and the lumen. The cell appears to have a distinct polarity with secretory granules clustered near to the small luminal or apical membrane. Acinar units also have supporting myoepithelial cells associated with them (me). Each acinus is connected by short tubules (intercalated ducts) to a system of longer more complex tubules, (the striated ducts of rodent salivary gland) which in turn connect to small excretory ducts. As saliva passes through the gland the number of canals decrease by convergence into one main excretory duct which empties into the oral cavity. Reprinted from Young & Van Lennep (1978).

Localising the source of fluid secretion allowed for the channelling of research towards the cellular understanding of stimulus-secretion coupling.

The concept of membrane permeability was formulated at the turn of the century by workers investigating the degree to which membranes allowed or prevented the passage of substances. It was Overton (1895), Ruhland (1908) and Danielli and Davson (1934) working on the structure of plasma membrane (subject to an excellent review at that time by Dan ielli and Davson, 1952) who brought the concept of membrane permeability into a physical reality where today it remains as a fundamental measure of cell viability and function.

In these early studies the red blood cell was frequently used as an experimental system. Davson in 1941 established that the K^+ permeability could be changed by poisoning with fluoride leading to a loss of K^+ from the cell. Gardos in 1958 discovered using red blood cells that the fluoride induced K^+ efflux, brought about by metabolically inhibiting the cell was dependent upon Ca^{++} since external chelation inhibited the effect. The significance of this result was not to be fully understood until the development of modern day electrophysiological techniques.

In salivary glands Douglas and Poisner (1963), using a cannulated and perfused submaxillary gland preparation of the cat showed that arterial perfusion of acetylcholine into the isolated gland elicited a copious salivary secretion. Omission of Ca^{++} from the perfusate profoundly inhibited the secretory response. This established an important connection between Ca^{++} and electrolyte/water secetion from salivary

glands. In the 1970's it was demonstrated that agonists which activate either cholinergic or a-adrenergic receptors in rat parotid gland slices caused a release of K^+ and that Ca^{++} was an obligatory intermediate in this response (Schramm et al., 1974, 1975). Using the Ca^{++} ionophore A23187 to artifically elevate $[Ca^{++}]_i$ an increased membrane permeability to the K^+ ion was seen to occur (Selinger et al., 1973, 1974). In contrast these investigators discovered that b-adrenergic stimulation of these salivary tissue slices was not associated with an efflux of K^+. This receptor is linked to c-AMP generation, a system shown to have little effect on the salivary gland secretory potential (Petersen, 1980), discussed shortly.

Radioisotopes have been a valuable tool for monitoring ion fluxes. Dreisbach in 1964 demonstrated uptake of $^{45}Ca^{++}$ in cat submandibular gland in response to cholinergic agonist. Using the same intact gland preparation Nielsen and Petersen (1972) later reported an enhanced $^{45}Ca^{++}$ efflux in response to cholinergic and adrenergic stimulation corresponding to increased mobilisation and loss of cytosolic Ca^{++}. Secretagogue evoked Ca^{++} uptake was also shown to occur in submaxillary (Martinez, 1976; Kangasuntheram & Randle, 1976) and sublingual (Putney, 1978a) and parotid gland slices (Putney, 1976, 1978; Miller, 1977).

The K^+ flux across parotid membranes was also followed by radioisotpoe flux measurements when Putney (1976) demonstrated that Rb^+ was an ideal substitute for K^+. Cholinergic stimulation of parotid gland slices resulted in $^{86}Rb^+$ efflux from preloaded tissue. In addition in another

preparation of the same gland under identical stimulation, uptake of $^{22}Na^{+}$ was show to be enhanced (Putney, 1978b) and ouabain (which inhibits the Na^{+}/K^{+} pump) in the continued presence of agonist appeared to cause an additional uptake of $^{22}Na^{+}$. Simultaneous agonist evoked K^{+} loss and Na^{+} uptake were later demonstrated within the same tissue (Landis and Putney, 1979).

DEVELOPMENTS IN ELECTROPHYSIOLOGY.

The first paper citing a recording of an electrical current across a biological membrane was published in the mid eighteenth century by Du Bois-Reymond (1857) in frog skin. Bayliss and Bradford two decades later (1886) expanded these experiments to show that the current could be inhibited by atropine and suggested that its origin was from glandular structures within the skin.

Using dog submaxillary gland and electrodes placed within the salivary duct and on its surface they also recorded an electrical potential associated with secretion which could be inhibited by atropine. Following the development of microelectrode techniques in the 1950's for measuring changes in transmembrane potential in single cells (Ling & Gerrard, 1949; Nastuk & Hodgkin, 1950), the first intracellular recording of a salivary acinar cell potential was made by Anders Lundberg in 1955. Using cat submaxillary gland he recorded membrane potentials across the contraluminal membrane of the cell to be around -20mV with a hyperpolarisation upon parasympathetic nerve stimulation of approximately -20 to -30mV. These early inestigations into the electrical nature of the salivary response, coined by Lundberg as the secretory potential were flawed by poor membrane potential measurements. Two other types of electrical responses were recorded which from their position on a morphological basis he attributed to no acinar cell types.

In trying to understand the correlation between fluid and electrolyte transport and the secretory potential Lundberg examined the effect of ion substitution on fluid secretion

and the secretory potential. He believed the electrical response to be a manifestation of an active transport of Cl^- occurring across the basolateral membrane. In a series of experiments using cat sublingual gland (1957a/b), parasympathetically evoked hyperpolarisations of similar magnitude to earlier studies were obtained. The intracellular concentrations for K^+, Na^+ and Cl^- ions determined by Krnjevic and himself (cited as unpublished data in 1958) enabled Nernst potentials of -97, +29 and -12 to be calculated respectively. The resting membrane potential found in these cells was slightly higher than submaxillary gland approximated to -33mV (1957a). Thus none of these ions were in equilibrium. By applying the technique of double-barrelled intracellular microelectrode recording the presence of passive ionic membrane permeability changes were looked for. By using one electrode as a monitor of the membrane potential and the another to inject current, a measure of the resistance and hence conductance of the membrane was obtained. He found a very small reduction in membrane resistance upon agonist challenge and obtained only hyperpolarising secretory responses at all membrane potentials. These two observations led him to propose that the secretory potential was independent of transmembrane potential. He concluded that passive membrane permeability changes played no role in the generation of membrane potential. He concluded that an active inward transport of Cl^- across the basolateral membrane occurred to move Cl^- in the direction of secretion (1957b). This assumption fitted with his earlier electrophysiological data which showed an inability to obtain secretory potentials in Cl^- free (nitrate

replaced) perfusion media (1957a).

During the same period Burgen (1956) established that parasympathetic stimulation of a perfused canine submandibular gland resulted in a marked efflux of K^+ into the blood. Reuptake of K^+ followed upon the cessation of stimulation. This phenomenon was discussed in a physiological review written by Lundberg (1958) but the idea of K^+ efflux being coupled to the secretory potential was rejected because it did not conform to the existing electrophysiological data. This mistake in an otherwise pioneering work was to inhibit the understanding of the true nature of the secretory potential for over two decades. Imai (1965a/b) working on perfused submandibular gland of the dog investigated the effects of ion substitution on parasympathetically induced secretory potentials and saliva production. Nerve stimulation resulted in a net increase in perfusate (blood) K^+ concentration and a decrease in perfusate Na^+ concentration. Upon removing the stimulus there was a period of K^+ reuptake into the glandular tissue and Na^+ release. The previous findings of Burgen on the movement of K^+ and Na^+ were confirmed. Using intracellular microelectrodes the stimulus induced hyperpolarisation in basolateral membrane potential was seen together with more persist nt reductions in membrane resistance. This suggested that the secretory potential was in part associated with changes in passive ion conductance. The cholinergically evoked electrical response was unaffected by replacement of Cl^- in the perfusate with sulphate, although saliva formation was reduced (1965b). In contrast to Lundberg both secretory potential and saliva secretion were strongly dependent upon the perfusate K^+ concentration.

From the magnitude of the secretory potential for a given perfusate K^+ concentration Imai calculated a 3 to 4 fold increase in K^+ permeability during stimulation (1965b). This led to the conclusion that the secretory potential was more easily explained by a change in the membrane permeability towards K^+ and that the active uptake of Cl^- was not part of the response. During most of the following decade emphasis on the control of the secretory potential shifted towards a role for K^+.

Between 1967 and 1968 Petersen and Poulsen emphasised this point in a paper (1968) where they extended the work of Imai with Cl^- substituted solutions. They demonstrated that replacement of Cl^- with nitrate likewise left the electrical events as normal but severely curtailed secretion (1969). Petersen (1970a/b) found that an elevation in external K^+ concentration reduced the secretory potential and that external Na^+ concentration also affected its magnitude. This suggested that the movement of both ions was responsible for the generation of the secretory potential.

The breakthrough in understanding of the secretory potential came with improved measurements in resting membrane potential. Petersen in 1973 using isolated segments of mouse salivary gland was able to show that the secretory potential evoked by cholinergic agonist stimulation was markedly dependent upon the cells resting membrane potential. Biphasic changes in secretory potential were recorded at previously unobtainable, higher membrane potentials. Membrane potentials similar to those obtained by Lundberg gave only agonist evoked hyperpolarising changes. By correlating the nature of the responses at different levels of membrane

potential this author derived a value for the reversal potential of the agonist evoked secretory potential. This occurred at a transmembrane potential of around -50mV. Nishiyama and Kagayama working in a different laboratory at the same time confirmed these findings in rabbit and cat submandibular glands (1974, 1973).

Having established the effect of the transmembrane potential on the secretory potential Nishiyama and Petersen (1974) demonstrated a more pronounced reduction in membrane resistance upon ACh challenge in isolated superfused mouse, cat and rabbit submandibular glands. Finally, contary to the findings of Lundberg 20 years earlier, it was accepted that passive K^+ movement contributed to the generation of the secretory potential. The original discovery by Burgen of a K^+ efflux could be explained, but what of the reuptake seen upon cessation of stimulation?.

Salivary glands were known to have Na^+/K^+ATPase activity (Schwartz et al., 1963, 1968) inhibitable by ouabain. Petersen (1971a/b) measuring acetylcholine evoked K^+ efflux from superfused submandibular glands of the cat demonstrated that ouabain whilst inhibiting the reuptake of K^+ normally seen upon withdrawl of agonist from the perfusate had no effect on the secretory potential. Further to this Petersen and Nishiyama (1974) when Na^+ loading the submandibular cells and then challenging with K^+ demonstrated a hyperpolarisation in membrane potential which was not associated with a change in membrane resistance. This was the first evidence of an electrogenic Na^+/K^+ exchange in this tissue. Roberts, Iwatsuki and Petersen (1978) investigating the ionic dependency of the secretory potential using intracellular

microelectrode impalement of parotid acinar cells showed that the delayed hyperpolarisation evoked upon cholinergic stimulation could be abolished by removing extracellular Na^+ or by the addition of ouabain. This can be explained as inhibition of the Na^+/K^+ pump. Further evidence for the role of this exchange mechanism was reported by Putney in 1978 using radiolabelled $^{22}Na^+$ uptake in rat parotid gland slices (see earlier). The localisation of the Na^+/K^+ATPase to the basolateral membrane was made by Bundgaard and co-workers in cat salivary gland (1977).

Much of the electrophysiology of the late seventies concentrated on discovering the receptor subtypes present within salivary acinar cells which coincided with similar investigations described earlier using radiolabelled ions for flux studies.

The first demonstration of K^+ release from perfused cat submandibular gland evoked by a-adrenergic stimulation was made by Petersen in 1970. Petersen and Pedersen (1974) described a similar secretory potential for both adrenaline and acetylcholine in superfused mouse parotid gland. Roberts and Petersen (1978) confirmed these agonist responses within a single acinar cell. In 1976 Ruddich and Butcher demonstrated that Substance P caused profuse K^+ release from rat parotid gland slices, this was confirmed by $^{86}Rb^+$ flux studies (Putney, 1977) and by $^{22}Na^+$ uptake studies in the same preparation (Landis and Putney, 1979). Gallacher and Petersen measured agonist evoked equilibrium potentials in mouse and rat parotid cells (1980) to show that Substance P also acted through a similar permeability-coupled mechanism. This effect was later demonstrated (1981a/b) to occur through

two mechanisms a) release of ACh from existing neural attachments, an indirect effect and b) activation of a Substance P receptor, a direct effect in rat parotid acinar cells. A purinergic receptor was also been demonstrated in superfused mouse parotid gland (Gallacher, 1982). The evoked electrophysiological response from this receptor was similar to that obtained from adrenergic or cholinergic agonists. It was recognised that a number of control systems impinging on acinar cells use similar ion permeability changes to evoke secretion.

Ca^{++} AND ITS ROLE IN STIMULUS-PERMEABILITY COUPLING.

Brief hyperpolarisations recorded in parotid acinar cells during cholinergic or adrenergic agonist stimulation were found to be independent of extracellular Ca^{++} (Petersen & Pedersen, 1973, 1974). This contradicted the results of Schramm & Selimger that K^+ release was dependent upon extracellular Ca^{++}. This apparent anomaly was solved by Putney (1976) using $^{86}Rb^+$ loaded rat parotid gland slices. He showed that cholinergic or α-adrenergic agonists evoke a release of K^+ which can be separated into two distinct phases by their external Ca^{++} dependency. An initial phase where $^{86}Rb^+$ release peaked and was independent upon extracellular Ca^{++} followed by a lower Ca^{++} dependant $^{86}Rb^+$ release. If agonist was added for a second time to Ca^{++}-free perfused slices, no second transient release was seen (even if an agonist acting on a different receptor was used). However if external Ca^{++} was readmitted to the tissue, even in the absence of agonist the preparation could sustain a further transient release. This important observation led Putney

(1977) to propose that the transient phase of the secretory response was the result of a release of Ca^{++} from a receptor mediated pool, inaccessible to EGTA but capable of being refilled by extracellular Ca^{++}. The lower more sustained response was probably due to Ca^{++} influx. Thus the studies by Petersen and Pedersen were looking at the effects of brief, short acting stimuli whilst the ion flux studies were looking at changes in membrane permeability during the sustained phase of secretion on a much longer time scale.

APPLICATION OF THE PATCH-CLAMP TECHNIQUE TO SALIVARY ACINAR CELLS.

The ionic basis of the secretory potential by the early 1980's was in part defined; however, the actual mechanism underlying the change in membrane permeability towards K^+ or Cl^- was not investigated until the advent of the patch-clamp technique.

In 1983 Maruyama, Gallacher and Petersen published the first single channel recordings for a discrete membrane bound pore which conducted K^+ across membranes of mouse parotid acinar cells. The location of this channel was almost definitely basolateral as seals were made on the exterior surface of cell clusters. The channel was more active at depolarising membrane potentials in both excised and cell-attached patch recording configurations and could be activated within the excised patch by increasing the concentration of Ca^{++} bathing the internal membrane face from $10^{-9}M$ to $10^{-7}M$. Changing the external membrane Ca^{++} concentration had no effect on channel opening. The conductance of this channel under symmetrically high (140mM) KCl gradients was 250pS (for an explanation of

conductance see Chapter Three). A possible candidate channel to explain the ionic basis for potassiums role in the secretory potential was found. The transmembrane channel offered a link between changes in $[Ca^{++}]_i$ and membrane permeability. Petersen and Maruyama (1984) postulated a role for activation of this channel in the control of fluid and electrolyte transport in exocrine acinar cells. This model for secretion will be covered in detail in the concluding chapter. Another model proposed during the same period was electrolyte secretion driven by agonist activation of a basolateral Na^+/Cl^- co-transporter (Poulsen et al., 1982). Its similarity with the hypothesis originally conceived by Lundberg had the drawback of assuming no role for the agonist evoked passive membrane permeability changes which had been shown to underlie the secretory potential. This was discredited by the finding of $Na^+/Cl^-/K^+$ co-transport system in salivary acinar cells (Exley & Gallacher, 1984).

In this thesis I have set out to investigate the following:

a) Can quantitative data be obtained from the K^+ channels behaviour in cells and excised patches to support the hypothesis that this channel was indeed the major K^+ conductive pathway in acinar cells?

b) Can the agonists established to cause the stimulus-permeability changes resulting in K^+ efflux in salivary acinar cells likewise affect the kinetics of the K^+ channel in an intact cell?

c) Can a better understanding of the regulation of intracellular Ca^{++} be obtained in exocrine acinar cells?

d) Which other channels are present in salivary acinar cells and how do they relate to fluid secretion?

2i) THE PATCH-CLAMP TECHNIQUE.

A) Description of Apparatus.

Experiments were performed throughout this study, using the following apparatus:

LM-EPC7 (List electronics, Darmstadt, FRG) patch clamp amplifier.

VBF/4 (Kemo Limited, England) dual channel variable filter.

5113 (Tecktronics, USA) dual beam storage oscilloscope.

Store 4DS (Racal recorders, England) magnetic tape recorder.

S88 (Grass instruments, USA) dual channel stimulater.

Narishige (Japan) hydraulic manipulator.

CK (Olympus instruments, Japan) inverted microscope.

Leitz (Leitz instruments, FRG) manipulator.

The patch-clamp apparatus was set up in rack form next to a Faraday cage enclosing the experimental working area containing the microscope on a solid table support, together with manipulation devices for amplifier head stage/pipette holder and ancillary pipette/bath perfusion equipment.

The Pipette Fabrication Set-Up.

Glass microelectrode recording pipettes were pulled using the following apparatus:

Model 700C (D.K.I., U S A) vertical pipette puller.

Adapted horizontal electrode burnisher with a Vickers-A.E.1, W6618, long focus x40 microscope lens; operating magnification of the complete system is x600.

Sylgard coating rig comprising of electrode rack, heating coil and low magnification electrode viewing device.

Pipettes were made out of CEE BEE precision capillary tubes, type 101-PB (Bardman Laboratory supplies, Denmark); a soft glass. Starting with an initial diameter of approximately 1.5mm, pipettes were pulled in two stages. This consisted of a primary pull to approximately 400µm diameter, re-centering of the heated area of glass and then a secondary pull to a separating (tip) diameter for the two recording pipettes of approximately 1µm. Electrodes were then coated with the hydrophobic Sylgard resin (to reduce signal noise) and heat-cured before being fire polished ready for use.

Patch-Clamp Pipette Holder Set-Up.

For work not requiring internal pipette perfusion of the recording electrode, the pipette holder supplied with the list amplifier was used. In cases where pipette perfusion was carried out in order to control the composition of the intracellular solution of whole-cell recordings, a modified version of the original pipette holder (Hamill et al., 1981) was developed, see later in this section.

B) Theory of Patch-Clamp.

The first developments in the extracellular patch clamp method (Neher & Sakmann, 1976; Neher et al., 1978) relied on placing the recording electrode tip against a cell and forming mega ohm (10^6) electrical resistance seals. The seal resistance was sufficient to observe large single channel currents when the recording electrode was balanced at the bath potential but did not allow for manipulation of electrical potential across the patch. By the early 1980's electrophysiologists had refined the technique to produce a powerful new tool.

The paper published in 1981 (Hamill et al.) using heat polished and sylgard coated pipette electrodes achieved a greater resolution of single channel currents by utilising higher electrical resistance, giga ohm membrane/pipette seals. At such resistances the leakage current generated by clamping the pipette at potentials other than the bath potential became small enough so as not to interfere with the current recording apparatus. This allowed manipulation of the patch membrane potential within a physiological range. In order to achieve this precautions had to be taken to ensure a clean pipette tip, filtration of experimental solutions, use of the pipette only once and minimising the precipitation of salts within the pipette. The cell preparation methods described later in this chapter are all designed to produce relatively "clean" i.e. debris free, cell plasma membrane surfaces. This requirement of patch clamping determines the success rate of pipette/membrane sealing and is the most difficult part of the technique to reproduce on a day to day basis with acutely dissociated cells.

Upon formation of a giga seal the recording electrode confines a small patch of plasma membrane that can be both chemically and electrically isolated from the rest of the cell i.e. the pipette can contain a solution of widely different composition from that bathing the exterior of the cell and it can also be voltage clamped. A typical submandibular acinar cell of 15-20μM diameter has a total plasma membrane surface area of approximately 1.7 to $3.2 \times 10^{-10} m^2$. This value is very large with respect to the area of membrane sealed within the recording pipette. A 5 to 10 mega ohm resistance pipette has been calculated to capture

approximately 3 to $5 \times 10^{-12} m^2$ of plasma membrane (Sakman & Neher, 1983). With the exception of the enterocyte preparation voltage clamping the patch at various potentials and recording the small currents generated did not effect the cells resting membrane potential. For this reason clumps of acinar cells were preferred in all preparations.

A circuit diagram (Sigworth, 1983) of the recording electronics used in the patch-clamp technique is shown in Fig. 2. The various patch recording configurations are shown in Fig. 3.

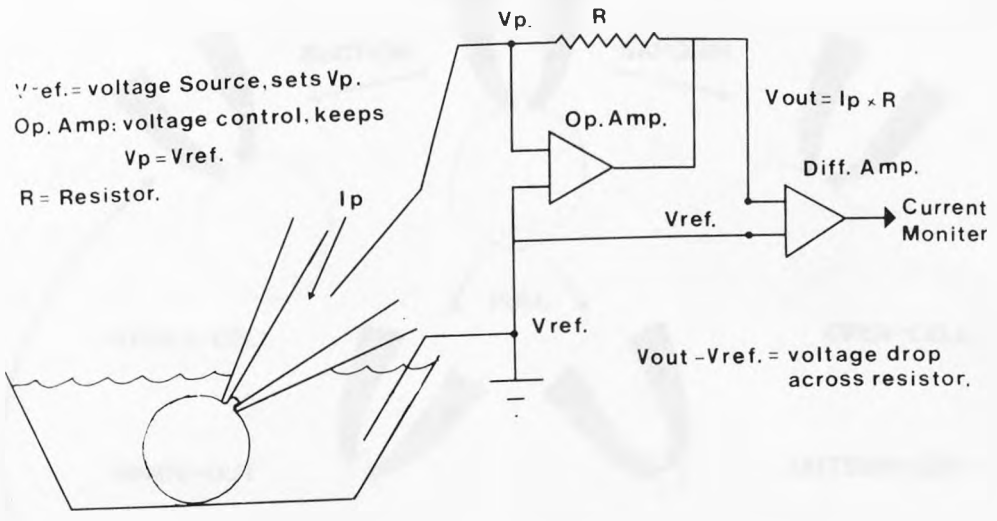


Fig. 2 A basic electrical circuit diagram used in the patch-clamp amplifier. The basic circuit consists of a current to voltage converter in the head-stage on which the pipette holder is attached. The pipette current (Ip) is monitored as the voltage drop across a high valued input resistor (R). Also included in the design are circuits for capacitance transient and series resistance cancellation (of particular use when monitoring whole cell currents) and inbuilt low noise circuitry, required for better time resolution of channel function.

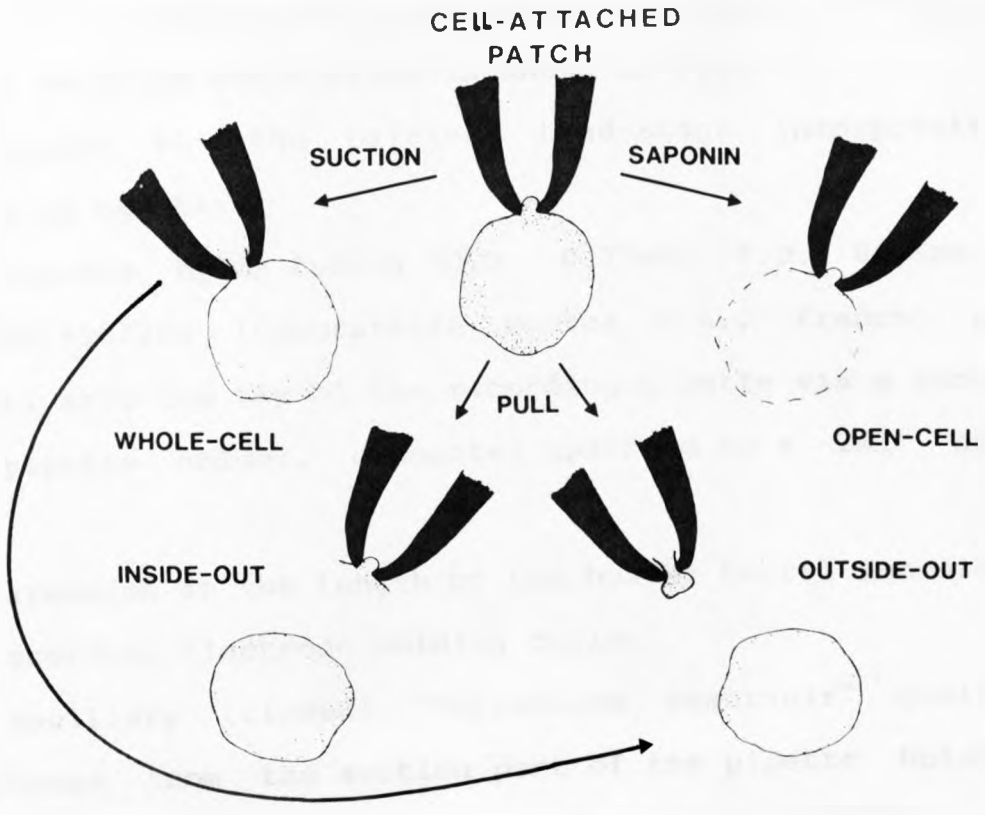


Fig. 3. Schematic reprsentation of the various patch clamp membrane geometries. The stability of the giga ohm range cell membrane/pipette seal is such that the recording pipette can be either drawn away from the cell surface forming one of two excised patch configurations or alternatively, the patch may be ripped away from the cell membrane whilst retaining the cell-attached configuration to produce the whole cell recording configuration. This latter configuration is achieved by producing a brief suction pulse within the interior of the recording pipette. The permeabilised cell recording configuration has been added in recent years. This is achieved by exposing the cell briefly to a low concentration of membrane detergent such as saponin (0.05%) whilst in the insitu cell-attached patch recording mode and results in an open cell, inside-out like patch being formed.

C) Internal Perfusion of the Patch Pipette.

The recently developed pipette holder is shown in Fig. 4 and a more detailed description is shown in Fig. 5.

Refinements to the original head-stage incorporate the following aspects:

A flexible nylon tubing (O.D. 0.75mm, I.D. 0.25mm) REF 800/200/125/200 (Laboratoire Portex S.A., France) passed coaxial into the tip of the recording pipette via a tunnel in the pipette holder, connected upstream to a 2ml "supply" vial.

An extension in the length of the holder barrel immediate to the recording electrode holding collar.

An ancillary (closed) "Collection reservoir" positioned downstream from the suction port of the pipette holder by similar thin nylon tubing and at a height of between 5-10cm below the supply fluid. The collection reservoir has a suction and drainage port and serves to dampen oscillations in pressure within the system as well as allowing (once hydraulic lock is achieved) the test solutions to siphon between supply and collection reservoir.

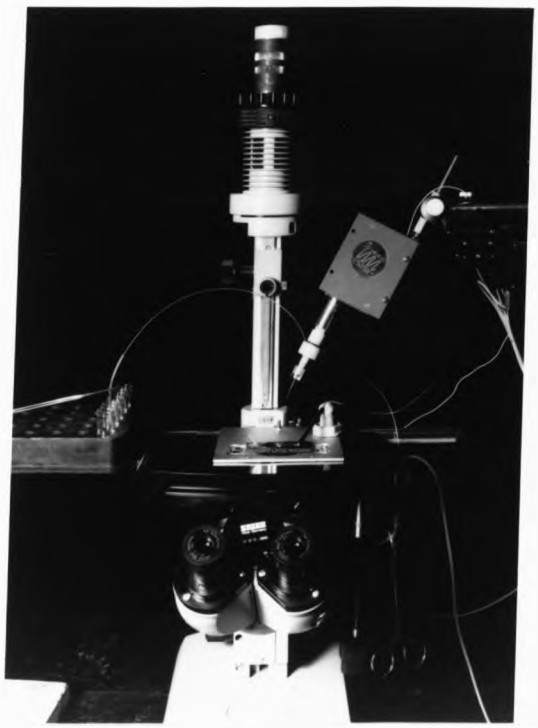
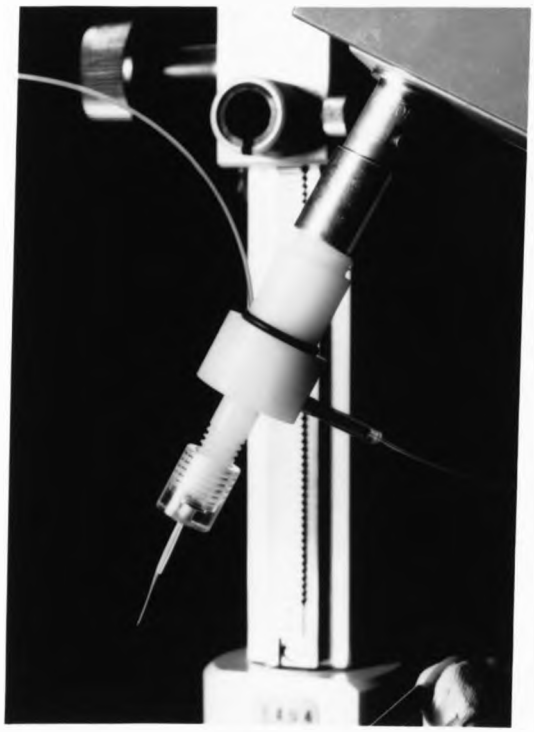


Fig. 4. Photograph of the recording pipette perfusion system attached to the patch-clamp rig. The redesigned pipette holder is attached in the normal manner to the patch-clamp amplifier head-stage. To the left of the photograph are supply vials and the clamped intake line for the pipette holder can be seen. To the right the outflow line which feeds a collection reservoir is displayed.



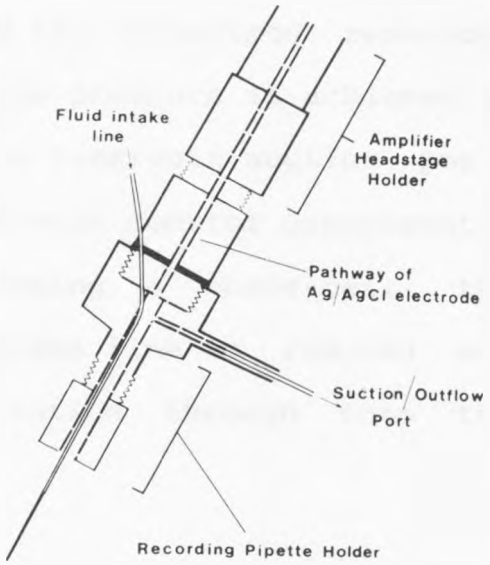


Fig. 5. The photograph and diagram provide a close-up view and illustration resectively of the developed head-stage. The pipette holder was fabricated from nylon and built to be easily dismantled. "O" ring seals were needed to ensure no pressure leaks occured. With adequate shielding, variance in noise levels using this method could be kept within 0.25pA of that experienced with the conventional head-stage. Nylon tubing of thin external diameter but relatively thick walled was chosen for the supply line so that it could be drawn down to a tip diameter of approximately 200-300μm. The corresponding internal diameter for the bore of the perfusion line tip was approximatley 70-100μm. The total volume of the perfusion system with an average length glass recording pipette in place is approximately 90μl. Suction pressures typically in the range of 10-15cm^3 H_2O induced a flow of approximately 150-250μl through the system. A recycling of the systems total volume occurred within 30sec.

To operate the system whilst obtaining a giga ohm membrane/pipette electrical resistance seal both the intake line from the supply reservoir and the collection reservoir drainage port were closed. Negative pressure is achieved in the pipette holder by the collection reservoir suction port. The intake line needs to be primed with control experimental saline prior to use. Upon forming a whole-cell the restriction to flow through the intake line is removed and gentle suction can then draw solution through into the pipette tip.

D) Bath Jet-Perfusion System.

Membrane patches or cells could be jet-perfused with bathing media by means of the set up shown in the Fig. 6. This method was employed for acetlycholine stimulation of whole-cell preparations. For other patch configurations acetylcholine stimulation was achieved by bolus application to the experimental chamber. Using both methods the final concentration of agonist was 10^{-5}M. Muscarinic antagonism by Atropine was likewise achieved in with experimental procedure adopted.

E) Data Acquisition and Analysis.

Data was stored on an analog tape recorder (Racal 4DS) inherently filtered at 1kHz. Analysis of channel opening was carried out subsequent to data acquisition by digitising the signal at 2kHz and using a channel open-state probability program designed within the Liverpool group (Findlay & Furlong, 1984) written for a microcomputer (B.B.C., Model B; Acorn computers, Cambridge). The total number of channels within a patch was determined by progressive depolarisation

of the patch membrane until no further recruitment of channels was seen. Open-state analysis was carried out for either 10s or 30s of continuous current activity. Multiple channel analysis was carried out by using separate and pre-selected thresholds for opening. The time spent open at each preselected level was then averaged to determine mean single channel open-state probability

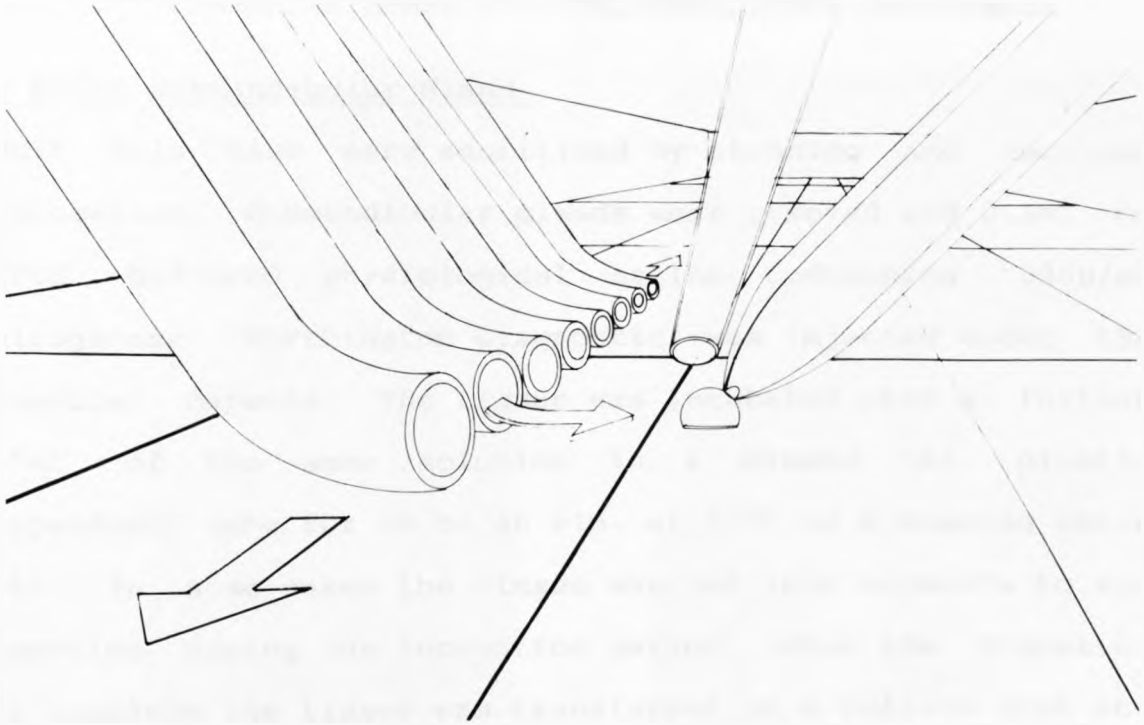


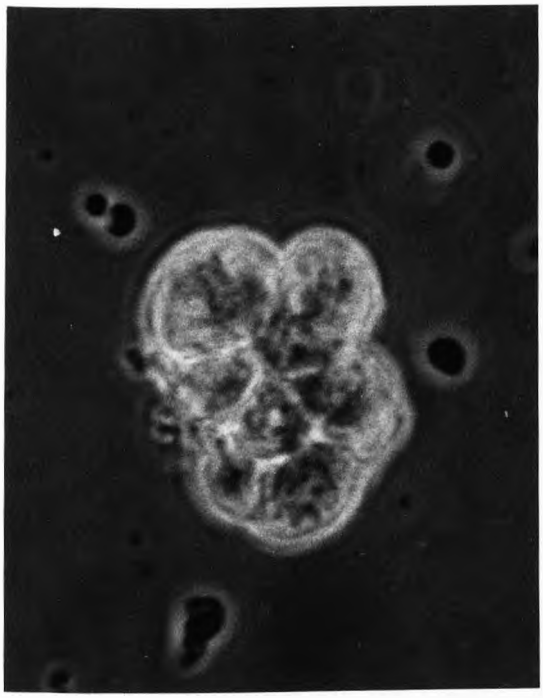
Fig. 6. Schematic diagram of the external jet perfusion system. The supply tubes were positioned in the experimental bath and the perfusate passed by gravitational pressure over the recording pipette tip. This system allows multiple changes of the bathing solution whilst recording from any of the patch clamp configurations. Polythene tubing (1.5mm O.D., 0.5mm I.D.) pulled to 700μm tip diameters was used for the jet perfusion lines.

2ii) PREPARATION OF CELLS FOR THE PATCH-CLAMP TECHNIQUE.

A) Mouse Submandibular Gland.

Adult male mice were sacrificed by stunning and cervical dislocation. Submandibular glands were removed and 0.5ml of HEPES buffered physiological saline containing 200U/ml collagenase (Worthington Diagnostic) was injected under the glandular capsule. The tissue was incubated with a further 0.5ml of the same solution in a closed 1ml plastic (eppendorf) tube for 30 to 60 min. at 37°C in a shaking water bath. In some cases the tissue was cut into segments to aid digestion during the incubation period. When the digestion was complete the tissue was transferred to a culture dish and allowed to settle before being washed by superfusion with physiological saline at room temperature. Small aliquots of the cells were then transferred to the experimental bath situated on the microscope. Fig. 7 depicts acinar cells isolated by this protocol. The majority of cells harvested remain viable for up to five hours following preparation when kept at room temperature and washed constantly with iso-osmotic physiological saline containing 1.2mM $CaCl_2$. Experiments were performed typically within the first 1-3 hours after cell preparation.

A)



B)

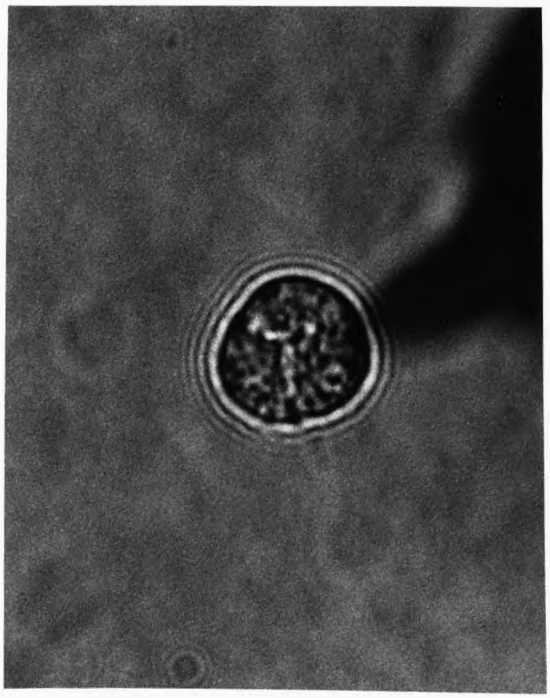


Fig. 7A. Photograph (x400 magnification) of acinar cells isolated from mouse submandibular glands, scale bar is 50μm. Fig. 7B. A single acinar cell with recording pipette sealed onto the plasma membrane. The pipette is seen as the dark area to the right of the picture.

B) Mouse Lacrimal gland.

Adult male mice were sacrificed as described previously. The exorbital lacrimal glands were removed and injected with and bathed in a prewarmed 1.0ml aliquot of physiological saline containing 4mg/ml trypsin at 37°C. After incubation with intermittent agitation at this temperature for 5min, 1.0ml HEPES buffered physiological saline containing 4mg/ml trypsin inhibitor was added and incubation continued for a further 5min. The glands retain their capsule using this proceedure, they were then digested as described previously with Worthington Collagenase (100U/ml) for a period of 30 to 40min. As with dissociated submandibular gland these cells were then washed with physiological saline at room temperature before use.

C) Human Submandibular Gland.

Tissue was obtained within 60min of removal from patients undergoing block neck dissections for residual carcinoma of the larynx, oropharynx or oral cavity. Following surgical excision glands were transferred immediately to cold (2°C to 4°C) physiological saline and transported to the laboratory. Tissue was obtained from 5 patients including both male and female sexes between the ages of 56 and 72 years. Histological examination of the tissue showed no evidence of carcinoma, although some showed fibrosis and chronic inflammatory infiltration consistent with radiation sialadentis. Lobules close to the surface of the gland were used for experimentation. Cubes of approximately 250mg of tissue were digested with 200U/ml collagenase as described for the mouse submandibular gland.

D) Rat Duodenum/Jejunum.

Male Wistar rats (180 to 200gm) fed ad libitum, were sacrificed by cervical dislocation. The upper two thirds of the small intestine were removed and flushed out with cold physiological saline. The bowel was then inverted and the exposed mucosal surface distended by forming a ligated, saline containing serosal sac. The intestinal segment was then incubated in an Na-citrate, phosphate-buffered physiological saline for 10min at 36°C in a shaking waterbath. Following this the incubation medium was discarded and fresh HEPES buffered physiological saline containing 1.5mM EDTA, 0.5mM dithiothreitol (DDT) and 0.1% bovine serum albumin (BSA) was added to the cells. After 10 to 15min at 37°C enough epithelial cells for experimentation were freed into the supernatent. Small clumps and single cells were separated from the undigested tissue mass by passage through a coarse nylon mesh. The cell suspension was then pelleted and washed (3x) by light centrifugation (1000rpm) in HEPES buffered saline at 37°C containing 3% BSA. A final incubation of the cell suspension with hyaluronidase 1mg/ml for 5min prior to experimentation in HEPES-buffered saline was found to improve giga seal formation. The cells were maintained and investigated at room temperature 22-26°C, immediatley after preparation. A typical isolated cell is depicted in Fig. 8:

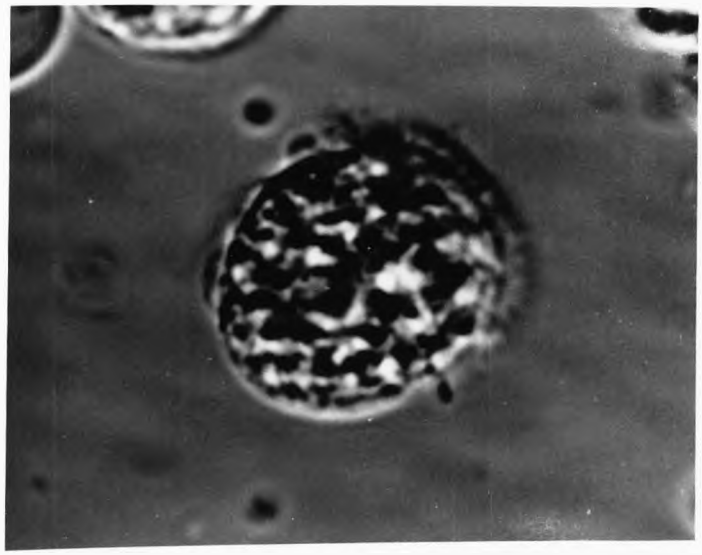


Fig. 8 . A single enterocyte isolated from the small bowel of the rat viewed at x600 magnification with phase optics. A distinct polarity can be ascribed to the cell due to the presence of an apical brush border. Bar = 20μm.

The isolated enterocytes were more difficult to maintain once isolated compared with salivary or lacrimal cells and most experiments were carried out within the first hour of isolation. For detailed biochemical evidence of cell viability and various methodologies for isolation see Kimmich (1975). The method described here has been developed as a modification of the citrate-chelation method (Stern and Jensen, 1966) and hyaluronidase method (Perris, 1965; Kimmich, 1970).

E) Human sweat gland cells.

The preparation of these will be discussed later in this chapter under the heading of tissue culture techniques.

2iii) SOLUTIONS USED WITH THE PATCH-CLAMP TECHNIQUE.

Throughout all experimental proceedures unless otherwise stated, the following HEPES buffered salines were used:

A) Standard Extracellular Saline.

Na-HEPES: extracellular saline, composition (mM): 140, NaCl; 4.5, KCl; 1.13, $MgCl_2$; 10, glucose; 10, HEPES, titrated to pH 7.2 with KOH. The concentration of free-Ca^{++} was 1.2mM unless otherwise stated.

B) Standard Intracellular Saline.

K-HEPES: intracellular saline, composition (mM): 145, KCl; 1.13, $MgCl_2$; 10, glucose; 10, HEPES, titrated to pH 7.2 with KOH. This saline contained no added Ca^{++} and 0.5 to 1.0mM EGTA. The osmolality of both the above solutions was checked and evaluated at 290mosM.

All experiments were carried out at room temperature, 22-26°C.

C) Modifications to the Extracellular Saline.

Na^+-free HEPES.

This was prepared using the large membrane impermeant cation, N-methyl,D-glutamine (NMDG) as a substitute for Na^+. $NMDG^+$ Cl^- was produced by acid titration (HCl) of the free salt. This saline had the composition (mM): 140, NMDG; 1.13, $MgCl_2$; 4.7 KCl; 10, glucose; 10, HEPES; 1.2, $CaCl_2$; pH titrated with HCl to 7.2; osmolality 290mosM.

In solutions where some Na^+ was desirable the relative proportions of NMDGCl and added NaCl were adjusted accordingly.

Another Na^+-free HEPES used was Isotonic Barium-HEPES: Ba^{++} replaced both Na^+ and K^+ in extracellular saline, the composition of which was (mM): 88, $BaCl_2$; 1.13, $MgCl_2$; 10, glucose; 10, HEPES; pH 7.2 with Tris base; 290mosM. The concentration of Ba^{++} and other cations was also varied according to need.

Cl⁻-free Extracellular Salines.

Cl^- was replaced with either SO_4^{--} or glutamine anions. Glutamine was preferred for most protocols. The composition of the monosodium-glutamine substitute was (mM): 140, $C_5H_8NNaO_4$; 1.13, $MgCl_2$; 4.7, K-Glutamine; 10, glucose; 10, HEPES; 1.2 $CaCl_2$; pH 7.2 with NaOH; osmolality 290mosM. For solutions of intermediate Cl^- concentration the proportions of added glutamine : chloride were adjusted accordingly.

D) Modifications to Standard Intracellular Saline.

Cl⁻-free Intracellular Saline.

As with the Na^+ containing extracellular salines chloride was replaced with either SO_4^{--} or Glutamine$^-$ i.e. monopotassium-glutamine substitution (mM): 140, $C_5H_8NKO_4$; 1.15, $MgCl_2$; 10, glucose; 10, HEPES; pH 7.2 with KOH; 290mosM.

Whole-Cell Intracellular Saline

This medium contained (mM): 20, NaCl; 40, KCl; 80, Kglutamine; 5, $MgCl_2$; 4, ATP; $2x10^{-5}$, GTP; 10, glucose; 10, HEPES; pH 7.6; 290mosM.

The free-Ca^{++} concentrations were adjusted for according to need for all solutions.

E) Specialised Cell Digestion Media.

Enterocyte, Citrate-Buffered Salines.

The Na-citrate, phosphate buffered solution contained (mM): 27, Na-citrate; 96, NaCl; 1.5, KCl; 1.8, KH_2PO_6; 5.6, Na_2HPO_4; pH 7.4; 290mosM.

The modified HEPES buffered solution contained (mM): 120, NaCl; 5, KCl; 1, $MgCl_2$; 10, glucose; 10, pyruvate: 10, ascorbate; 1, $MgCl_2$; 20, HEPES; pH 7.3; 300mosM.

F) The Calculation of Free-Ca^{++} Concentration in Solutions.

The free-Ca^{++} concentration in salines was determined by addition of $CaCl_2$ and EGTA buffer mixtures (Findlay et al., 1985) and calculated by an iterative microcomputer procedure (Martel and Smith, 1974) using stability constants for all reactions involving Ca^{++}, Mg^{++}, H^+, EGTA and when required ATP and GTP. (IUPAC Stability constants, Pergamon press). Ca^{++}-EGTA buffers were prepared by pH titration (Miller and Smith, 1984). In the case of the H^+ ion, concentration constants were redefined in order to account for the fact that pH is measured as activity (Fabiato, 1981). pH meters were selected which did not show errors due to liquid/junction potentials (Illingworth, 1981). The total EGTA concentrations varied between 1 and 2mM in Ca^{++} containing solutions unless otherwise stated. 0.1 to 0.5mM EGTA was added to nominally Ca^{++}-free solutions to achieve a resting Ca^{++} concentration of approximately 10^{-7} to 10^{-8}M in the presence of 1.2mM Mg^{++} and the abscence of ATP.

2iv) INTRACELLULAR DYE TECHNIQUES.

The Ca^{++} sensitive intracellular fluorescent dye, quin-2 (Tsien et al., 1982) was used to monitor the magnitude and rate of change of cytosolic free-Ca^{++} concentration in an acinar cell preparation derived from rodent submandibular gland. The more recently developed form of the indicator, fura-2 (Grynkiewicz et al., 1985) was likewise used in a similar manner.

A) Experimental Apparatus.

Dye fluorescence was measured on a Perkin-Elmer LS-5 fluorimeter (Perkin-Elmer Ltd., Buckinghamshire, England) a single wavelength fluorescence monitor. The dyes were excited with light of predetermined wavelength and monitored at their optimum emission wavelength. The instrument consists of two reflection grating monochromaters which are set i) between source (a pulsed neon flash tube) and sample: The excitation monochrometer and ii) between sample and detection photomultiplier: The emission monochrometer. A reference photomultiplier measures the incident light prior to its passing through the sample by means of a beam splitter. The output from the photomultipliers are processed by ratio recording electronics to produce a displayed output that is proportional to the level of emission from the sample. The signal from the ratiometer was displayed on an analogue chart recorder and all analysis carried out by hand (see results chapter).

The experiments were conducted at 30°C with constant stirring of the cell population by a small magnetic flea at the bottom of the curvette. Background autofluorescence was measured in

control suspensions and subtracted at the beginning of each experiment.

Incident and collected wavelengths were set for each dye as below:

	quin-2	fura-2
Excitation (nM)	340	340 or 380
Emission (nM)	490	510

Excitation slit : 10nM

Emission slit : 10nM

Excitation spectra optima for both dyes were generated when a wavelength scan was performed. These are shown in Fig. 8.

B) Cell Preparation

Single acinar cells were isolated by modifying the existing collagenase digestion method and incorporating hyaluronidase into the incubations. Submandibular glands were removed from two adult male rats (180-200gm). The tissue was finely chopped to approximately $1mm^3$ and incubated in a shaking water bath at 37°C in O_2 gassed media containing collagenase (2.7U/mg tissue weight) and hyaluronidase (10U/mg tissue weight) for 50mins. The glass incubation flask was silicon coated proir to reduce cell adhesion. The pH of the digestion media was checked at 5, 15, 30, 40 and 50min and adjusted when necessary with saturated Na_2HPO_4. At 30 and 40min the digest was mechanically disrupted by several passages through a broken and heat polished tip of a 10cc plastic pipette. Cells were harvested at 50min after passage through a regular 10cc plastic pipette. The resultant cell suspension was spun down by centrifugation for 1.5min at 1000rpm and the supernatant discarded. The cells were resuspended with nominally Ca^{++}-free saline containing

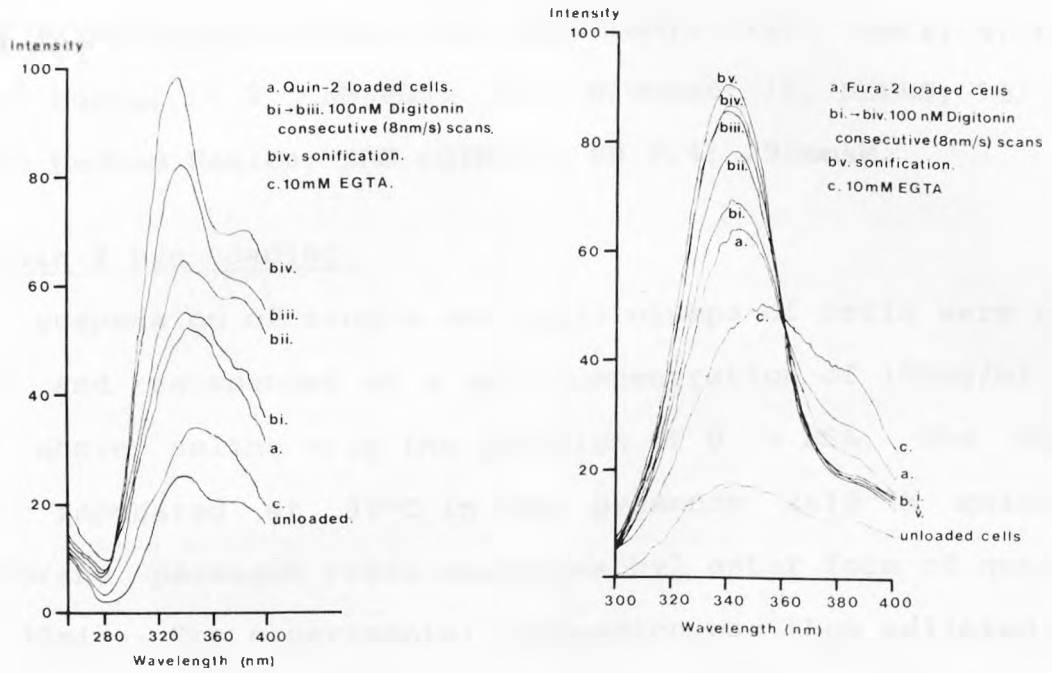


Fig. 9. Fluorescence spectra for quin-2 and fura-2. Note that the vertical scale is a measure of intensity. On an absolute scale equivalent concentrations of fura-2 give x30 more signal than quin-2. These curves differ slightly in their peak emission spectra from absolute values (Tsien, 1980; 1982, Grynkiewicz et al., 1985) and reflect uncorrected lamp and photomultiplier characteristics associated with this machine.

2% BSA and passed through a coarse nylon mesh. Two further centrifugations were carried out to remove cell/tissue debris from the preparation and the cells finally resuspended in Ca^{++}-containing saline.

The extracellular saline contained (mM): 120, NaCl; 4, KCl; 1.2, $MgSO_4$, 1.2, KH_2PO_4; 10, glucose; 15, HEPES; 1%/vol. Basal Medium Eagles, BME (GIBCO); pH 7.4; 290mosM.

C) Quin-2 Dye Loading.

The suspension of single and small clumps of cells were spun down and resuspended at a cell concentration of 100mg/ml in the above saline with the addition of 0.1% BSA. The cells were incubated at 37°C in the presence $2x10^{-5}M$ quin-2AM (membrane permeant tetra-acetoxymethyl ester form of quin-2) for 30min. The experimental suspension was then pelleted and resuspended in fresh buffer at a cell concentration of 20mg/ml. After incubation for 40mins at 37°C (to remove extracellular dye) a further centrifugation into the same volume of saline was performed. 100mg cell aliquots were then separated, resuspended in extracellular saline and used for experimentation.

D) Fura-2 Dye Loading.

Cells were incubated with fura-2AM at $4x10^{-6}M$ for 30min. before being washed and used as above in the fluorimeter.

E) Extracellular Dye Fluorescence Calibration.

None of the experimental procedures resulted in any significant change (<1%) in autofluorescense of the cell suspensions. The dye loading efficiency was calculated for quin-2 loaded cells. A suspension of cells at concentration

of 100mg/ml was found to contain approximately 10^7 cells/ml when measured with a cytocrit. The fluorescence of loaded cells (-autofluoresecence of lysed unloaded cells) was compared with the fluorescence of known Ca^{++}-quin-2 standards. Loading efficiency was expressed as the percentage of dye trapped in the cells and was typically 30-40%.

Dye leakage during the experiment was monitored by addition of 50-100uM Mn^{++}. This divalent cation binds to quin-2 with a greater affinity than Ca^{++} and has a fluorescence peak far removed from the 490nM Ca^{++}-quin-2 emission peak. Thus it is an effective means of quenching extracellular dye fluorescence (Hesketh et al., 1982). Mn^{++} was found not cross the plasma membrane of cells very easily hence quenching of the intracellular Ca^{++}-quin-2 signal with this cation was not a problem. The membrane impermeant, heavy metal chelator CaDPTA (diethylene-triamepentacetic acid) when added after Mn^{++} addition at slightly higher concentrations (200μM) provided an effective means of removing the metalic cation from solution before it could permeate the cells. Recently it has been confirmed in fura-2 loaded parotid acinar cells that ACh stimulation does not increase the membrane permeability to Mn++ (Merrit & Hallam, 1988). This non invasive intervention used to cell viability could be carried out anytime during the experiment.

F) Calibration of the Intracellular Ca^{++} Rise.

i) Quin-2.

Quin-2 was originally synthesised from the Ca^{++} chelator BAPTA (Tsien, 1980) a double aromatic analogue of EGTA. 'It can therefore be considered as a Ca^{++} chelator but with

improved spectral properties which lend themselves particularly useful for measuring intracellular [Ca^{++}]. The membrane-permeant form of the dye, quin-2AM is hydrophobic and readily crosses membranes (Tsien et al., 1982). An important prerequisite for a cytosolic calcium indicator is that it remains in the cytoplasm of the cell. Since only the cell cytoplasm contains the relevant esterases to convert quin-2AM to active quin-2 this requirement is fullfilled (Tsien, 1981). Free quin-2 binds Ca^{++} in a 1:1 stoichiometry, as seen from the excitation spectra (Fig.9) saturation of the dye with Ca^{++} produces hardly any shift in spectral peak but an approximate x6 fold increase in the fluorescence signal.

Thus by calculating the fluorescence of free and Ca^{++}-bound dye within the cells and by using the dissociation constant (kd) for Ca^{++}-interaction (Tsien, 1982) an equation for intracellular Ca^{++} concentration can be formulated:

$$[Ca^{++}] = Kd \frac{(F-Fmin)}{(Fmax-F)}$$

where:

Fmax= fluorescence value measured when all of the dye is saturated with Ca^{++}. This is achieved either by sonification of the sample in the curvette or treating the cells with membrane detergents i.e. saponin or digitonin followed by saturation of the quin-2 pool with extracellular Ca^{++}.

Fmin= fluorescence value measured when the total dye concentration in the system remains uncomplexed. This is achieved by treating the broken cell suspension with excess EGTA (100mM) at pH9.0 (alkaline pH increases the binding

affinity for the Ca^{++}-EGTA complex).

F= experimental value of fluorescence, measured in intact cells under resting and agonist stimulated conditions.

Kd= 117nM as measured at 37°C in a solution mimicking the intracellular milieu within cells (Tsien et al., 1982).

Values for the fluorescent signal were obtained from the output of the analog printer. Preceeding an experiment an estimate of extracellular dye was made and in some cases another MnDPTA treatment or EGTA chelation repeated at the end of the experiment but prior to the Ca^{++} calibration procedure. This further control which was sometimes incorporated, determines the degree of cytolysis occuring during the experimental proccedure. Son ication was found to be more reliable than digitonin at releasing all the intracellular quin-2.

ii) Fura-2.

A similar calibration procedure was carried out for the intracellular Ca^{++} concentration estimation. Fura-2 has a different K_d=224nM. This dye has improved spectral properties when compared with quin-2. Its better absorption coefficient and quantum yield make it approximately 30 times brighter (Grynkiewicz et al., 1985). Hence lower dye loadings achieve a similar fluorescence output, the main reason for its use. Another property of this dye is its lower affinity hence greater accuracy of measurement over a higher range of Ca^{++} levels and its better Mg^{++} selectivity (K_d=6-10mM). Mn^{++} also quenches the fluorescence of fura-2.

G) Solutions used with the Dye Techniques.

i) control saline consists of standard Na-HEPES buffered solution, pH7.4, gassed with 100% O_2, containing 1.2 mM $CaCl_2$ and prewarmed to 37°C.

ii) Na^+-free saline was prepared by substituting Na^+ for NMDG or K^+ as described earlier in this chapter.

iii) Ca^{++}-free control saline was nonimally Ca^{++} free with 0.1mM EGTA added.

2v) TISSUE CULTURE TECHNIQUES.

A) Mouse Submandibular Acinar Cells.

Short-term static culture of single isolated acinar cells was performed in a defined (serum free) media. These conditions cannot support cell division but can maintain cell viablity for up to a week following isolation. Single submandibular acinar cells were maintained by this method for use in whole-cell patch-clamp studies. Once the whole-cell currents were characterised this technique was superc eded by the easier to perform acute isolation of acinar cells from lacrimal glands.

Isolation Procedure

Cells were isolated by a scaled down version of the rat submandibular digestion procedure. Two submandibular glands were excised from an adult male mouse (200mg tissue) and subjected to a collagenase/hyaluronidase digestion (see section 2iv/B).

Single cells were preferentially selected during centrifugation by increasing the viscosity of the final saline wash (3% BSA) and spinning the suspension at 200-400 rpm. This separated the less dense single cells from the heavier cell clumps in the supernatant. Precautions were taken to miminise the chance of bacterial/fungal infection by using autoclaved instruments, sterile testtubes and penicillin (100U/ml), streptomycin (100µg/ml) and gentamycin (4µg/ml) in the saline washes.

Culture Media for Salivary Acinar Cells.

Cells were maintained in a 50% mix of Dulbecco's modified Eagles medium and Hams F-12 growth medium (Sens et al., 1985) containing 10% fetal calf serum, insuline/transferrin/Na-selenite (ITS) media supplement at 10µg/ml, 10µg/ml and 10ng/ml respectively (Barns & Sato, 1980) and pennicillin, steptomycin/gentamycin at the predetermined concentrations. A number of substratum was experimented with to promote cell adhesion. Cells were found to prefer either collagen gel (rat tail collagen) or matrigel (Flow laboratories) to plastic cell culture dishes. Cells were diluted to 100mg/40mls with incubation media, placed in coated 2ml sterile petridishes and kept in a sterile LEEC mkII incubator at 37°C in the presence of 5% CO_2-95% O_2.

B) Human Sweat Gland Cells.

Primary cultures established from whole-gland, secretory coil and collecting-duct of normal eccrine sweat glands (Lee et al., 1986) were donated from C.M. LEE, University of Newcastle.

Cells were passaged with Williams E medium (GIBCO) supplemented with 10% fetal calf serum, insulin (10µg/ml), transferrin (10µg/ml), epidermal growth factor (10ng/ml), hydrocortisone (10ng/ml), penicillin (100U/ml), streptomycin (100µg/ml) and amphotericin B (2.5µg/ml). They were then seeded onto tissue culture coverslips in wells 5mm in diameter and left to recover for 24hrs. The best patch-clamp sealing rate was found to occur on the second and third day following passage although the cells were used up to five or six days after seeding. By this time the cells had grown to

confluence and were almost certainly polarised into apical and basolateral domains (Lee et al., 1985). However due to their extremely thin cross sectional area during all periods of incubation it was found necessary to round up the cells with hypertonic KCl, so destroying polarity.

3i) THE K^+ PERMEABILITY OF BASOLATERAL MEMBRANES EXCISED FROM MOUSE SUBMANDIBULAR GLAND.

A) The Voltage and Ca^{++} Sensitive "Maxi-K^+" Channel.

The patch clamp technique was employed to record single channel currents in a large number of excised, inside-out (see methods), basolateral membrane patches from submandibular acinar cells. The membrane polarity of cells was known since recording pipette seals were made only on the exterior surface of acini clumps. In the following experiments unless otherwse stated the recording pipette contains the intracellular KCl HEPES-buffered saline with no added Ca^{++} and 1.0mM EGTA (10^{-9}M free-Ca^{++}). Upon excision of the patch from the cell the bathing solution was substituted for KCl HEPES-buffered saline containing no added Ca^{++} and 1mM EGTA (10^{-9}M free-Ca^{++}). When voltage clamping the patch at bath potential i.e. the imposed transmembrane potential at zero, no chemical or electrical gradients exist and no single channel currents were recorded. When hyperpolarising voltages (negative with respect to the cytosolic face of the membrane) were applied across the patch inward currents were observed. Similary when depolarising voltages (positive with respect to the cytosolic aspect of the membrane) were applied across the patch outwardly directed currents were seen. The frequency and duration of opening for these currents was strongly dependant upon the membrane potential (MP). At increasingly negative (hyperpolarising) membrane potentials the channel displayed

brief and infrequent openings. At increasingly positive (depolarising) membrane potentials an increase in both the duration and frequency of channel opening was seen.

Typical single channel currents recorded under these conditions and the corresponding current-voltage relationship needed to calculate single channel conductance are shown in Fig. 10. The channels within the membrane patch are clearly voltage sensitive. The i-v relationship is measured as the amplitude of a single channel opening at the test membrane potential. It was apparent that a linear relationship exists for the channel under symmetrical KCl gradients, the current reversing at zero MP. Conductance is a measure of the amount of current carried across the membrane for a given potential difference. The single channel conductance was calculated for Fig.10 as:

$$\text{conductance} = \frac{\text{amplitude of single channel current}}{\text{applied transmembrane potential}} \quad \text{(slope)}$$

$$= 300\text{pS (pico Semens)}$$

The average conductance for this channel in excised patches under symmetrical high-KCl gradients was found to be 245+-4.8 pS with a range of 220 to 310pS (n=12).

By changing the concentration of free-Ca^{++} within the bathing saline its effect on the inside-out patch was investigated. The influence of increasing the ionised Ca^{++} concentration on the cytoplasmic face of the plasma membrane was monitored. The patches were again exposed to symmetrical high KCl solutions on both sides of the membrane. The activity of the channels within the patch during this series of experiments are summarised in Fig. 11.

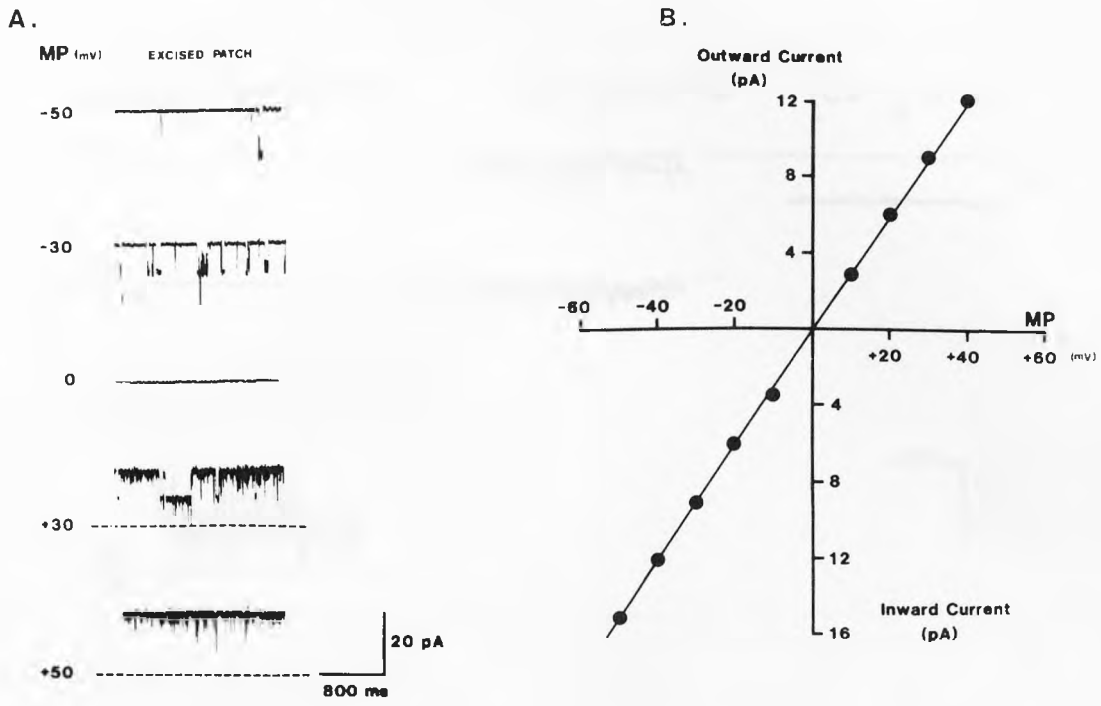


Fig.10. Single channel currents recorded from an excised inside-out patch under symmetrical high KCl, low-Ca^{++} ($<10^{-9}$M) gradients. A) Two channels were seen in this patch. Negative (hyperpolarising) membrane potentials generated inwardly directed currents, these are shown as downward deflections. Positive (depolarising) membrane potentials generated outwardly directed currents, these were seen as upward deflections. This nomenclature for current direction is used throughout the thesis. Broken lines where seen indicate the closed current level i.e. all channels closed. B) The current-voltage relationship for the channel.

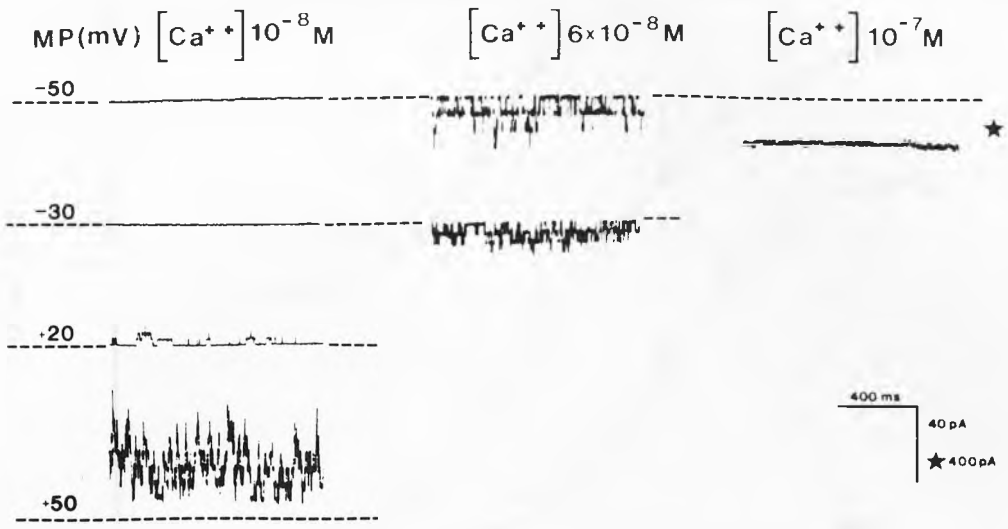


Fig.11 A). Sections of continuous current recordings from a single excised inside-out patch. The recording pipette was filled with high-KCl saline, 1mM EGTA and no added Ca^{++}. The bathing solution contained an identical high KCl saline but with the free-Ca^{++} concentration buffered (see Methods.) between 10^{-8} and 10^{-7}M. Three changes in bath [Ca^{++}] are seen in this figure.

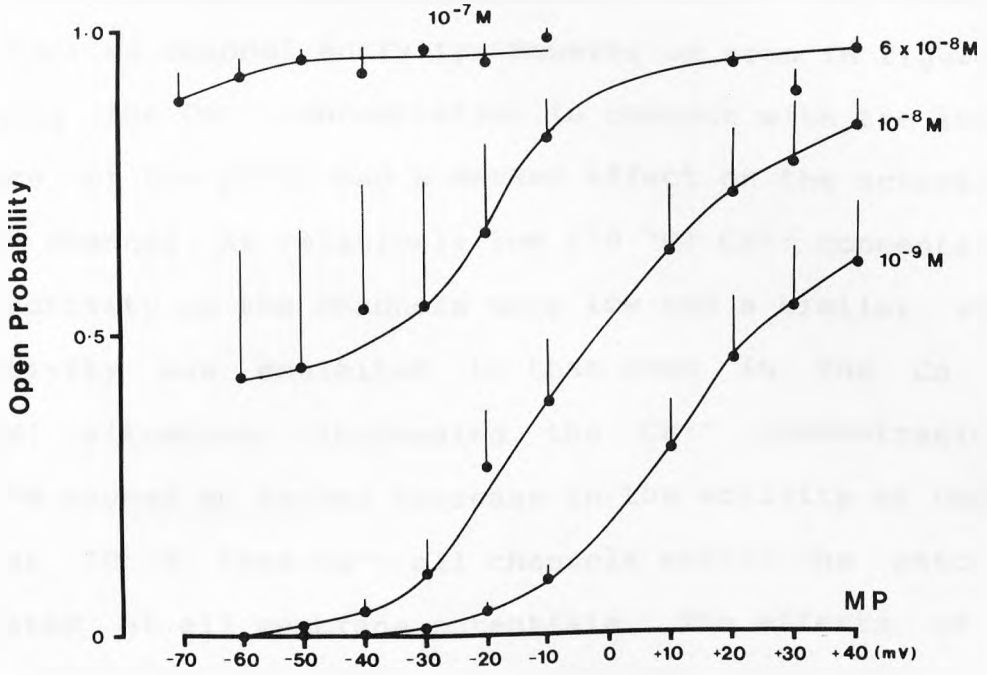


Fig.11 B). Displays the effect of changing both transmembrane potential and Ca^{++} on the single channel open-state probability in excised inside-out patches. Data collected for this figure only includes those patches of membrane where at least three random changes in Ca^{++} concentration was achieved. The effects were freely reversible. All lines were fitted by eye. The data was collected from a number of experiments where n = 11, 9, 3 and 6 for 10^{-9}, 10^{-8}, $6x10^{-8}$ and 10^{-7} M-Ca^{++} respectively.

Changing the Ca^{++} concentration bathing the external patch membrane (achieved using outside-out patches, see later) had no effect on channel activity. However as seen in figure 11A, changing the Ca^{++} concentration in contact with the internal surface of the patch had a marked effect on the activity of the K^+ channel. At relatively low ($10^{-8}M$) Ca^{++} concentrations the activity of the channels were low and a similar voltage sensitivity was exhibited to that seen in the Ca^{++}-free ($10^{-9}M$) situation. Increasing the Ca^{++} concentration to $6x10^{-8}M$ caused an marked increase in the activity of channels and at $10^{-7}M$ free-Ca^{++} all channels within the patch are activated at all membrane potentials. The effects of Ca^{++} were sustained and rapidly reversible. Fig.11B summarises the effects of both MP and Ca^{++} on the large conductance (250pS) K^+ channel. Open-state probability (o/p) is a measure of the time the channel spends open i.e a fully open channel has a value of 1.0. It is apparent that Ca^{++} fascillitates the effects of depolarising membrane potentials in opening this channel, hence the channel is described as being both voltage and Ca^{++} sensitive.

B) The Ionic Selectivity of the Ca^{++} and Voltage Activated K^+ Channel.

The ionic selectivity of the large conductance channel found in these membranes was investigated by placing asymmetrical cationic gradients across either excised inside-out or outside-out patches. A typical result taken from an outside-out patch i.e. external membrane surface in continuity with the bathing saline is shown in Fig. 12:

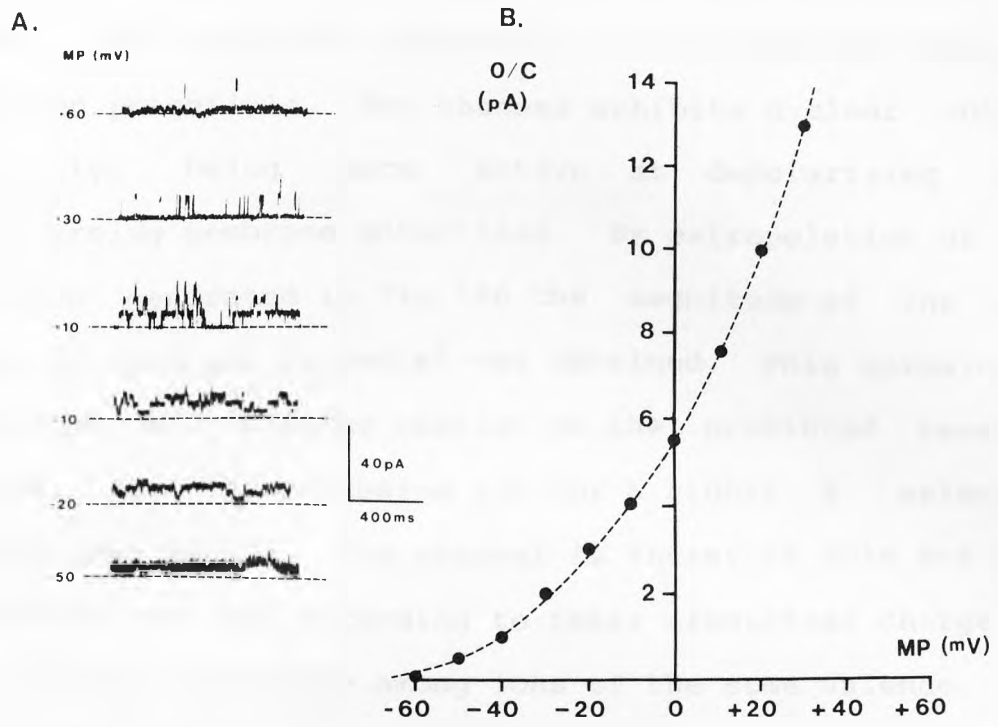


Fig.12. Single channel currents recorded from an outside-out patch under conditions where the recording pipette contained high KCl and the bath high NaCl HEPES-bufered saline. Under these conditions the patch was exposed to quasiphysiological chemical gradients. Both solutions had a free-Ca^{++} concentration of around 10^{-9}M, (1mM EGTA added). A) only outwardly directed single channel currents were seen B) The corresponding current-voltage relationship for the large conductance K^{+} channel under quasiphysiological gradients.

Single channel currents were recorded at bath potential i.e. 0mV MP. Only outwardly directed current steps are seen at all tested potentials. The channel exhibits a clear voltage sensitivity, being more active at depolarising than hyperpolarsing membrane potentials. By extrapolation of the i-v curve generated in Fig.12B the magnitude of the zero current or reversal potential was obtained. This aproximated to 80-90mV MP, a value similar to the predicted reversal potential for the potassium ion for a highly K^+ selective membrane (see later). The channel is therefore able not only to distinguish ions according to their electrical charge but also to show selectivity among ions of the same valence.

C) The Effects of Inorganic Cations and Blockers on the Ca^{++} and Voltage Activated K^+ channel.

The Actions of Barium on the Maxi-K^+ Channel.

Ba^{++} is a well established inhibitor of K^+ conductance in a wide variety of cell types i.e. aphysia, Pacifico (1969); frog skin, Nagel (1978); tutle colon, Kirk (1983) and pig pancreatic acinar cells; Iwatsuki & Petersen (1985). This divalent cation was tested for its effect on the voltage and Ca^{++} sensitive maxi-K^+ channel. A 5mM Ba^{++} containing bathing saline was substituted for normal saline whilst recording from an excised outside-out patch under symmetrical high KCl, low-Ca^{++} (10^{-9}M) conditions. The effects of Ba^{++} block on single channel currents recorded in a basolateral membrane patch are shown in Fig 13:

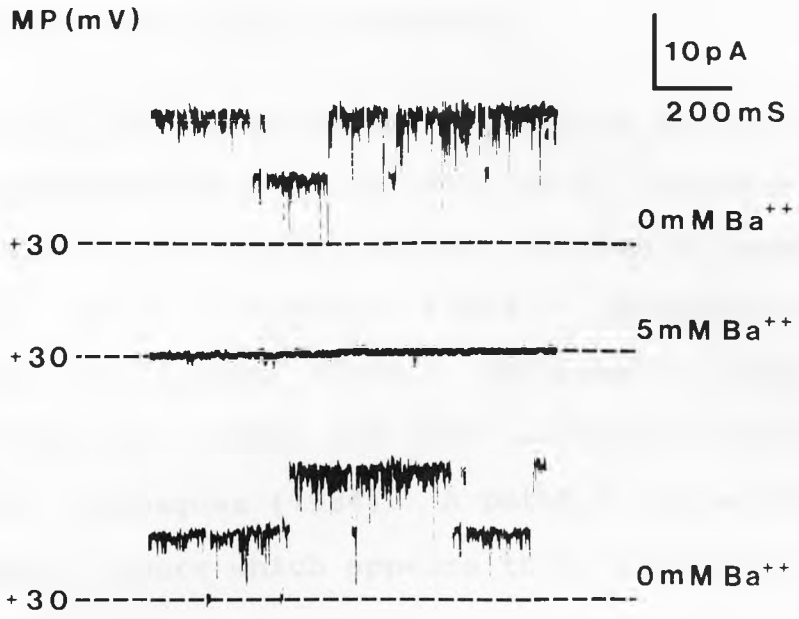


Fig.13. Single channel currents recorded from an excised outside-out membrane patch of basolateral membrane from a mouse submandibular acinar cell. This figure shows the effect of externally applied 5mM Ba^{++} to the patch under identical KCl gradients. The patchwas clamped at +30mV MP.

When applied to the external surface of the patch Ba^{++} markedly inhibited the large conductance K^+ channel, this effect as shown was freely reversible.

The actions of Tetraethylammonium (TEA) on the K^+ Channel.

TEA is a pharmacological inhibitor of K^+ currents in many cell types i.e. molluscan neurone, Herman & Gorman (1981); rat muscle, Blatz & Magleby (1984); pancreatic B-cells, Findlay and colleagues (1985); pancreatic acinar cells, Iwatsuki & Petersen (1985) and also in planar lipid bilayers, Vergara and colleagues (1984). A pattern of sensitivity to TEA blockade occurs which appears to be dependant upon the size of the K^+ channel present (see discussion).

Outside-out patches were used to test for effects of TEA on the external membrane surface. Excised patches were bathed in symmetrical high KCl, low-Ca^{++} (1.0mM EGTA) HEPES-buffered saline. The effects of TEA are shown in Fig. 14. TEA reduced the current amplitude in a dose dependant manner. The open-state probability of the K^+ channel during TEA block remained unaffected (n=14). TEA blockade of channel amplitude was also seen to persist over a wide range of membrane potentials (+50 to -50 mV MP) i.e. extracellular TEA blockade of the K^+ channel is voltage independent. The effects of TEA blockade were freely reversible. As seen in Fig.14B, single channel conductance rapidly declines over a very narrow dose range. 1mM TEA inhibits the channel by 98% of its total conductance under these conditions.

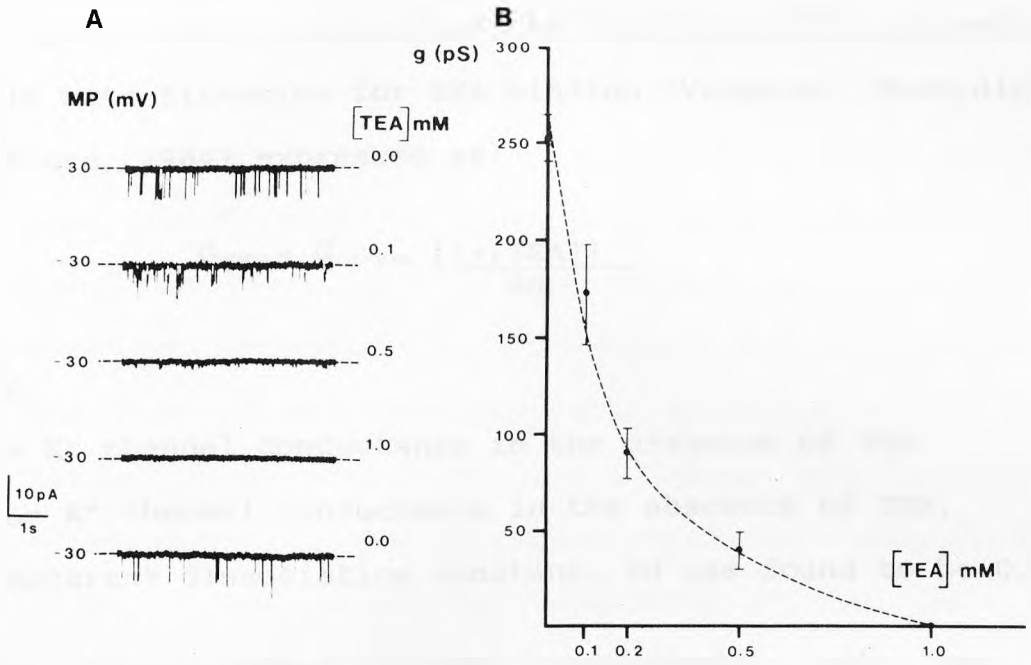


Fig.14 Single channel currents recorded from an excised outside-out membrane patch held at -30 mV MP under symmetrical KCl and 10^{-9}M Ca^{++} (no-added Ca^{++} and 1mM EGTA) gradients. A) Depicts the effect of varying concentrations of TEA (0.1, 0.2, 0.5 and 1.0 mM) on K^+ channel amplitude within the patch. B) Mean single channel conductance for the K^+ channel. Single channel current amplitudes were converted to conductance values at all membrane potentials between -50 and +50mV MP and then meaned for six outside-out patches. The K^+ channel blocker was applied to the extracellular membrane surface at concentrations ranging between 0.1 and 2mM. At least two different TEA concentrations were applied at random to the external surface of any patch, all effects were reversible.

A dissociation constant for TEA inhibition was found by fitting a single exponential to the data points assuming a single site titration for TEA binding (Vergara, Moczydlowski & Lattore, 1984) expressed as:

$$G_{TEA} = G_{norm} \frac{(1+[TEA])^{-1}}{Kd}$$

where:

G_{TEA}= K^+ channel conductance in the presence of TEA.

G_{norm}= K^+ channel conductance in the abscence of TEA.

The apparent dissociation constant, Kd was found to be 0.23mM

Since the single channel conductance but not the open-probability of the channel is sensitive to the binding of TEA, the binding of this molecule must occur significantly faster than the channel kinetics.

Insufficient data was collected for the internal TEA block of the channel but it appears that a significantly higher concentration of inhibitor is needed to reproduce similar effects which are also voltage dependant (Vergara, Moczydlowski & Lattore, 1984) This suggests that TEA binds at a different site when applied to the intracellular aspect of the membrane.

In both Ba^{++} and TEA block protocols residual conductances were seen. These conductances have not been investigated in detail but are distinct from the non-selective channel present in these membranes (Chapter Six). Excised patches possess a mini-K^+ selective channel with a conductance of approximatley 40pS (n=6) which occasionally appears (n=3) during the initial stages of secretagogue induced cell-attached patch activation (Chapter Four).

3ii) THE CONDUCTANCE OF THE K^+ CHANNEL IN THE CELL-ATTACHED PATCH.

The insitu cell-attached recording configuration (see methods) is a non-invasive approach used for the investigation of currents contained within a small patch of membrane which is functionally still part of the intact acinar cell. This recording configuration prec edes either form of excised patch configurations or the formation of a whole-cell. Once a high resistance seal has been established the electrical enviroment existing across the patch can be influenced in isolation without affecting the rest of the cell. The notation ΔMP is used for all cell-attached patch recordings.

High-K^+ Pipettes

Using pipettes containing high-KCl saline and low-Ca^{++} (1.0mM EGTA) single channel currents were obtained from cells bathed in high-NaCl saline and 1.2mM-Ca^{++} (extracellular saline). By voltage clamping the pipette at bath potential (ΔMP=0mV), single channel currents were recorded across the patch. These channels were inwardly directed, a consequence of the cells resting membrane potential and exhibited a low frequency and duration of opening. Altering the transmembrane potential of the patch towards positive values (a depolarising shift in patch potential) resulted in a greater degree of activation of channels within the patch. A negative change in transmembrane patch potential (a hyperpolarising shift in patch potential) reduced channel activity within the patch. The channels seen in the cell-attached patch under the conditions stated above (K^+-pipette/Na^+-bath) together with

those recorded when the acinar cell is bathed in an identical high-KCl solution to that contained within the recording pipette (K^+-pipette/K^+-bath) are shown in Fig. 15. Basolateral membrane patches from K^+-depolarised cells contained no currents when the pipette was clamped at the bath potential (ΔMP=0mV). However voltage clamping the patch between -50 and +50mV ΔMP elicited voltage sensitive currents very similar in magnitude and frequency of opening (n=8) to those seen in the excised patch under symmetrical KCl, low-Ca^{++} gradients (Fig. 10). The currents seen in cells bathed in extracellular like saline were also voltage sensitive. By depolarising the patch transmembrane potential by some 50mV, all current activity was lost within the patch. This potential corresponds to the reversal potential for the cell. Analysis of the current amplitude at the spontaneous membrane potential (ΔMP=0mV) and the current reversal potential in normally bathed cells gave a mean resting membrane potential of 43 ± 1.8mV (n=16). Depolarising the cell-attached patch of a normally bathed cell further than the resting membrane potential resulted in outwardly directed currents. The corresponding current-voltage plots Fig.15C for both conditions are linear with conductances of 257 ± 9.0 pS for the K^+ depolarised acinar cells and 264 ± 11.0 pS for the normally bathed cells. The parallel shift in the i-v relationship reflects the presence of a resting membrane potential.

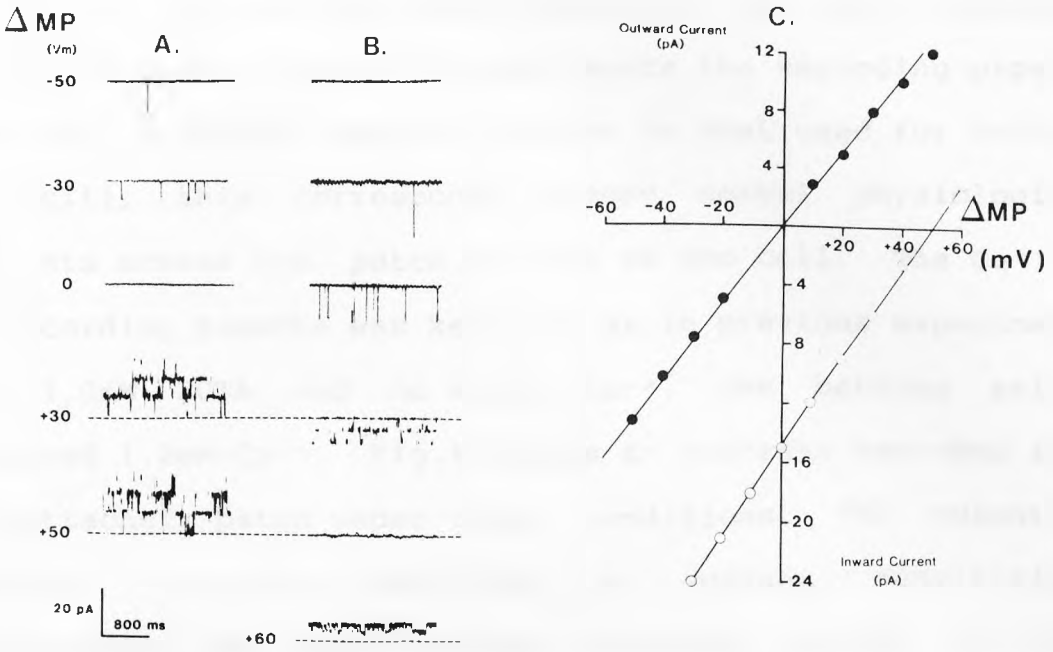


Fig.15. Single-channel currents recorded from a cell-attached patch of mouse submandibular acinar cell membrane. The patch pipette was filled with high-KCl solution and 1mM EGTA but no added Ca^{++}. A) and B) show the effect of removing the cells membrane potential. The cell in A) was bathed in a high-KCl solution containing 1.2mM-Ca^{++} i.e. was K^{+} depolarised, whilst B) represents the same cell but bathed in high-NaCl saline containing 1.2mM-Ca^{++} mimicking the normal extracellular fluid. The corresponding current-voltage relationships are seen in Fig.15C. The K^{+}-depolarised cell is represented as closed circles, the Na^{+}-bathed cell the open circles high Na^{+} pipettes.

In the experiments described above high-KCl filled the patch pipette i.e. the extracellular surface of the patch membrane. In the following series of experiments the recording pipette contained high-NaCl saline (similar to that used for bathing the cell), this corresponds to more normal physiological gradients across the patch as well as the cell. The Ca^{++} in the recording pipette was kept low as in previous experiments with 1.0mM EGTA and no added Ca^{++}. The bathing saline contained 1.2mM-Ca^{++}. Fig.16 shows K^+ currents recorded in a cell-attached patch under these conditions. The outwardly directed currents displayed a voltage sensitivity. Depolarising the patch membrane enhanced channel activity within the patch. The mean single-channel current amplitude when plotted as a function of change in patch membrane potential (ΔMP) displays an increasingly pronounced rectification at more negative changes in patch potential. The K^+ current does not undergo reversal but zero current appears to occur (by extrapolation) for a hyperpolarising shift in MP of some 40-50 mV. If we assume the membrane potential of Na^+ bathed cells is around -40mV (as defined in the previous experiments), the predicted reversal potential is close to -90 mV. The K^+ channel recorded <u>insitu</u> with the cell-attached configuration displays a similar high-K^+ selectivity to that found in the excised patch (Fig.12).

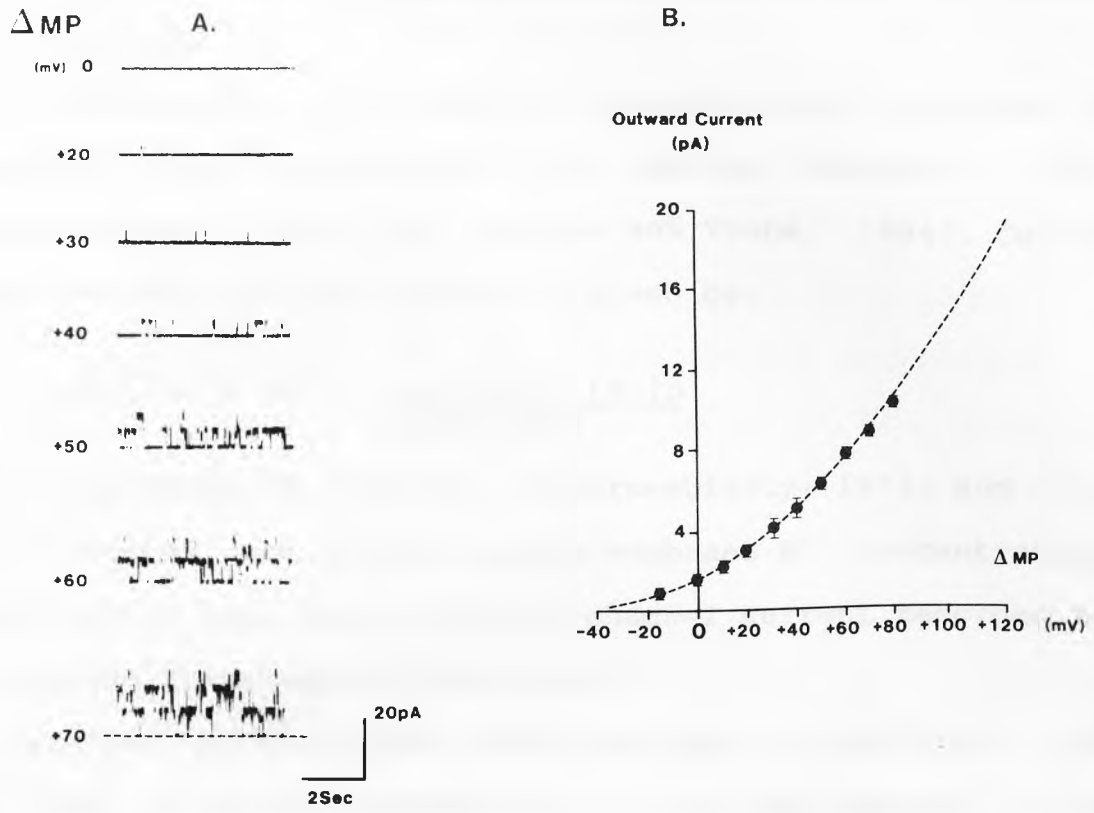


Fig.16. Single-channel current recording and current-voltage relationhip for a cell-attached insitu membrane patch. The pipette was filled with high NaCl, low-Ca^{++} (1.0mM EGTA and no added Ca^{++}) HEPES buffered saline. The cell bathed in a similar high-NaCl containing saline with a normal extracellular Ca^{++} concentration (1.2mM). A) Outwardly directed currents were seen at all potentials. B) Shows the mean i-v relationship for six experiments displayed as closed circles, the broken line is a plot of the i-v relationship as predicted by the constant field equation.

A) The External K^+ Concentration and its Effects on Channel Permeability.

The permeability of the open K^+ channel can be estimated if constant field assumptions are applied (Goldman, 1943; Sakmann et al., 1983 and Sakmann and Trube, 1984). Current flow through the open channel is given by:

$$I = (VF^2/RT)\, P\, [K+]i\, \frac{\exp(VF/RT) - [K+]o}{\exp(VF/RT) - 1}$$

F,R T are standard constants, P permeability, $[K^+]_i$ and $[K^+]_o$ are internal and external patch membrane K^+ concentrations and I is the amplitude of the K^+ channel current recorded for an applied transmembrane potential V.

In excised patches under identical high K^+ conditions (145 mM) the calculated permeability of the open channel P was $4.59 \times 10^{-13} cm^3/s$. The permeability of the channel in K^+ depolarised cell-attached patches, assuming symmetrical K^+ gradients was $4.7 \times 10^{-13} cm^3/s$. In cell-attached patches where the bath contained high NaCl an estimate of P can also be made by correcting V for a magnitude equal to the cells resting membrane potential (-45mV). In Na^+-bathed acinar cells where the pipette contained high NaCl ($[k^+]_o$=4.5mM) the permeability P was $4.29 \times 10^{-13} cm^3/s$. For the high KCl ($[K^+]_o$=145mM) pipette, Na^+-bathed insitu P was 4.55×10^{-13} cm^3/s. Thus the permeability of the channel is little affected by the concentration of extracellular K^+.

The current-voltage relationship for the Na^+-pipette/Na^+-bath insitu cell-attached patch has previously been shown (Fig.16B). A marked rectification in current is seen when compared with the K^+-pipette/Na^+-bath cell-attached patch (Fig.15B). Using the constant field equation a theoretical

line describing rectification under conditions of $[K^+]_o$=4.5mM was drawn and can be seen as the broken line in Fig.16B. This assumes the calculated value for P=4.3x10^{-13}cm^3/s and resting membrane potential V=-45mV. Since the measured and predicted currents are almost identical, it is clear that the K^+ channel conductance can be modelled by the constant field equation.

The maxi-K^+ channel was found to attain a conductance of around 250pS in experimental conditions where the recording pipette contains 145mM KCl. However when physiological ion gradients are imposed across the K^+ channel the single channel conductance especially at the resting membrane potential was much smaller. The amplitude of outward K^+ current recorded at the cells resting membrane potential (ΔMP=0mV) when <u>insitu</u> with the Na^+-pipette/Na^+-bath cell-attached patch configuration was 1.6 $\pm$ 0.18pA. Assuming the spontaneous resting membrane potential to be -45mV, the single channel conductance at this potential predicted by the constant field equation was 35pS. Thus K^+ channel conductance was markedly dependant upon $[K^+]_o$.

<u>B) An Estimation of $[Ca^{++}]_i$ from the O-P of the K^+ Channel in the Cell-Attached Patch.</u>

The mean single channel open-state probability for the maxi-K^+ channel measured as a function of membrane potential for all the previous cell-attached patch recording configurations is seen in Fig.17. The mean single channel o-p was identical for NaCl bathed acini in both high-KCl and high-NaCl pipette conditions, i.e. the channel open-state probability is independant of the external K^+ concentration. The slope of the open-state probability relationship for the

K^+ depolarised cell-attached patch was identical to the others but shifted to the right by 40mV. This effect is due to the absence of any resting membrane potential.

The mean o-p curve of the channel obtained from the K^+-depolarised insitu cell-attached patch was compared with o-p curves generated at different bath-Ca^{++} concentrations from excised inside-out patches under symmetrical high-KCl gradients (Fig.11). This has enabled an indirect estimation of $[Ca^{++}]_i$ under resting conditions to be made (assumming that no other factors affecting the activity of the K^+ channel exist). This estimate of channel activity suggests that $[Ca^{++}]_i$ is regulated in submandibular acinar cells to values between 10^{-8} and 10^{-7}M.

C) Voltage Activation of the K^+ Channel at Physiological Membrane Potentials.

In all three cell-attached patch situations the o-p of the channel was close to zero at the physiological membrane potentials i.e. for Na^+-bathed cells at ΔMP=0mV and similarly for K^+-bathed (depolarised cells) at MP=-40 to -50mV. Under physiological ion gradients of high-Na^+ solutions in both recording pipette and bath the open probability of the channel was 1.7 ± 0.6 ($x10^{-3}$). Depolarising the membrane 10 and 20 mV caused an increase in single channel open-state probability of 8.5 ± 4.2 and 28 ± 13 ($x10^{-3}$) respectively. These very low values for single channel open-state probability can be misread as the K^+ channel is found to be exquisitely sensitive to changes in potential around the resting membrane potential. Membrane K^+ conductance is determined not only by the mean open-state

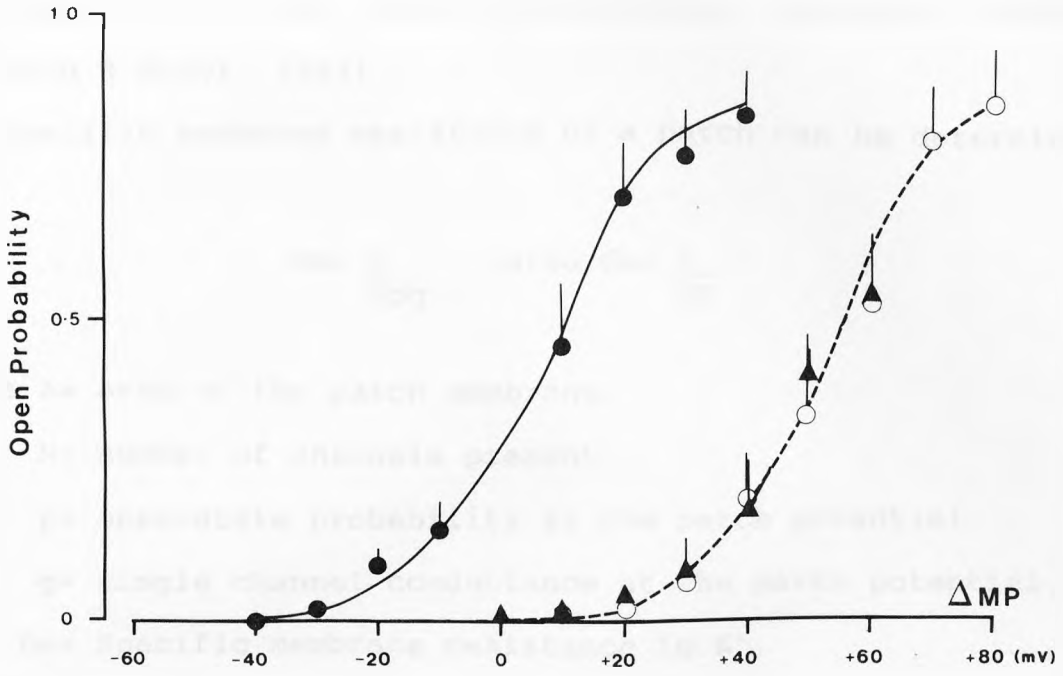


Fig.17. The effects of membrane potential on the open-state probability of the K^+ channel recorded from cell-attached patches under the conditions of: K^+-pipette/K^+-bath (closed circles); K^+-pipette/Na^+-bath (open circles) and Na^+-pipette/Na^+-bath (closed triangles). The recording pipette always contained no added Ca^{++} and 1mM EGTA, the bathing saline contained 1.2mM free-Ca^{++}.

probability of channels within the patch but also by the single channel conductance under the prevailing ionic gradients and by the number of functional channels present (Sakmann & Neher, 1983).

The specific membrane resistance of a patch can be determined as:

$$Rm = \frac{A}{Npg} \qquad \text{also } Gm = \frac{1}{Rm}$$

where A= area of the patch membrane.

N= number of channels present.

p= open-state probability at the patch potential.

g= single channel conductance at the patch potential.

Rm= Specific membrane resistance to K^+.

Gm= Specific membrane conductance to K^+.

Sakmann & Neher (1983) have reported a correlation between the patch-pipette resistance and the area of the free membrane patch. They have estimated that for 2-5 Mega ohm pipettes (used in this study) the area of membrane trapped inside a recording pipette is approximately 5 um^2.

The mean number of channels (N) per patch was found by analysis of a large number of patches to be 3.26 ± 0.17 (n=81).

The experimental values obtained for the above parameters enable the relationship between the transmembrane resistance to K^+ movement and the membrane potential to be drawn, Fig.18. The parallel shift in the plots was due almost entirely to the different conductances of the channel at identical patch potentials for K^+ and Na^+ filled pipettes. In the high K^+ situation (symmetrical 145mM-K^+ gradients) the channel attains the maximal 250 pS conductance. In the high

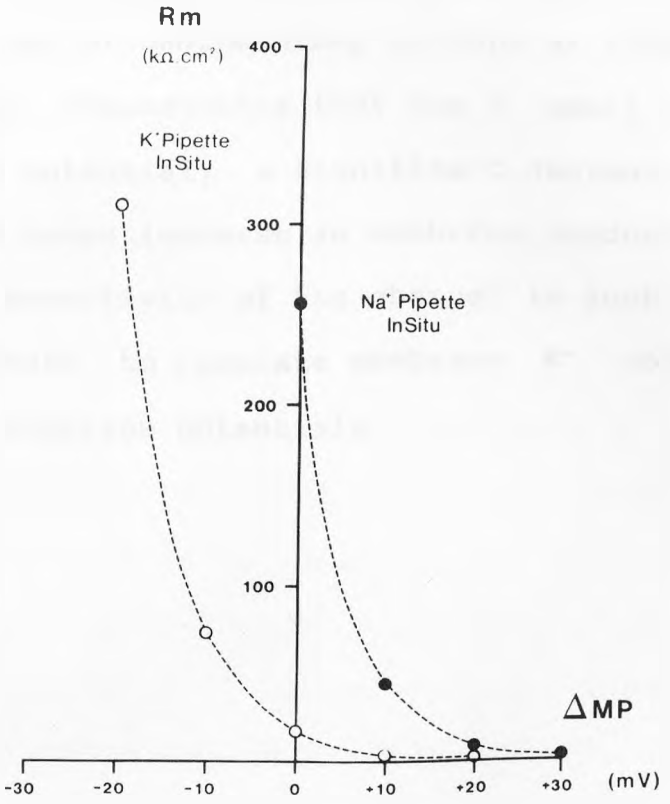


Fig.18. Calculated membrane resistance as a function of membrane potential for acinar cells bathed in high-NaCl saline (140mM-NaCl; 4.5mMKCl) with either KCl (open-circles) or a identical high-NaCl saline (closed circles) in the recording pipette.

Na^+ condition (4.5mM-K^+ present) the conductance of the channel was 35ps at 0mVΔMP rising to 70pS at +30mV ΔMP. The figure clearly demonstrates that for a small depolarising shift in patch potential, a significant decrease in membrane resistance and hence increase in membrane conductance occurs. The voltage sensitivity of the channel is such that it can certainly operate to regulate membrane K^+ conductance at physiological membrane potentials.

3iii) RODENTS AS MODELS FOR HUMAN SALIVARY FUNCTION.

Patch clamp studies were undertaken to corroberate the electrophysiological evidence recorded from mouse submandibular glands by completing similar experimental protocols on basolateral membrane patches from acutely digested human submandibular gland acinar cells (see methods).

Single channel currents were first recorded in cell-attached patches of basolateral membrane. The recording pipette contained high-K^+ and 10^{-9}M ionised-Ca^{++} in all experimental protocols. The pipette was then withdrawn from the cell and transferred into high-K^+, low-Ca^{++} media. Typical currents for both situations are recorded in Fig.19. The channels within the cell-attached patch were clearly voltage sensitive. A depolarising shift in patch MP increases the frequency and duration of K^+ channel opening and a hyperpolarising shift in patch potential has the converse effect. In the excised patch under symmetrical high-KCl gradients no currents were recorded at zero pipette potential (MP=0mV). By imposing a membrane potential a clear voltage sensitivity was seen; hyperpolarising potentials (negative MP's) produced inwardly directed currents which became progressively less active. Depolarising potentials (positive MP's) produced outwardly directed currents which became progressively more active. Both i-v relationships are linear with conductances close to 250 pS. The parallel shift in membrane potential can be attributed to the cells resting membrane potential in the cell-attached situation and was found to have a mean value of -48.6 ± 2.4mV (n=6).

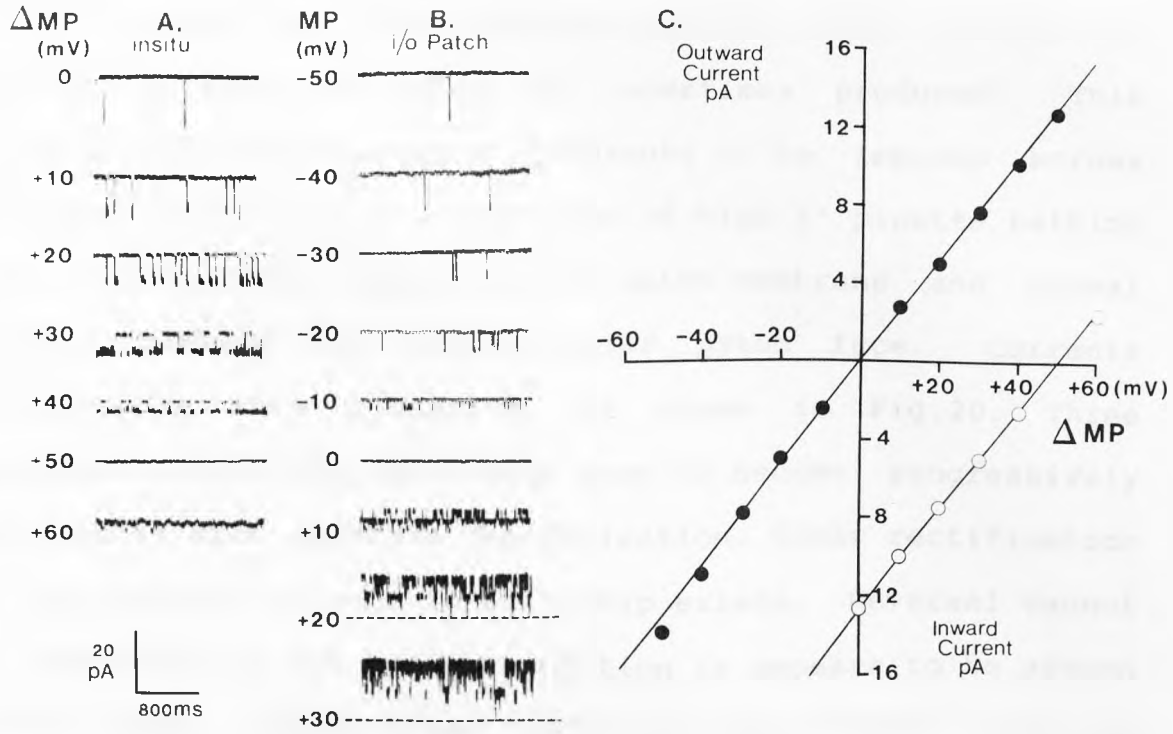


Fig. 19. K^+ channels recorded in basolateral membrane patches of human submandibular acinar cells. A) The insitu cell-attached patch was bathed in normal extracellular saline (1.2mM-Ca^{++}). The recording pipette contained high-KCl HEPES-buffered saline with no added Ca^{++} and 1mM EGTA. At zero patch potential (ΔMP=0mV) inward currents were seen due to the spontaneous cell membrane potential. B) Upon withdrawal of the pipette from the cell and its transfer into high KCl, low-Ca^{++} (1mM EGTA) bathing media, single channel currents were recorded from an inside-out membrane patch. C) Accompanying current-voltage relationships. Excised patch (closed circles) and cell-attached patch (open circles).

Upon withdrawl of the recording pipette from a cell an excised outside-out patch was sometimes produced. This allowed pseudophysiological gradients to be imposed across the patch membrane i.e. conditions of high-K^+ pipette bathing the intracellular aspect of the patch membrane and normal saline bathing the extracellular patch face. Currents recorded in this situation are shown in Fig.20. Three channels within the patch were seen to become progressively more active upon membrane depolarisation. Clear rectification of the current-voltage relationship exists. Reversal cannot be demonstrated but by extrapolation it appears to be around -70 to -90mV. Under these conditions where the Cl^- ions are in chemical equilibrium, the channel must clearly be K^+ selective (the Na^+ ion has a less negative reversal potential).

The inside-out patch recording configuration was used under symmetrical K^+ gradients to look for Ca^{++} sensitivity of this K^+ channel. A typical result is shown in Fig.21. Increasing the Ca^{++} bathing the intracellular surface of the patch clearly activates the K^+ channel. The K^+ channel in submandibular human basolateral membranes is both voltage and Ca^{++} sensitive and shares all the basic properties of the mouse submandibular K^+ channel.

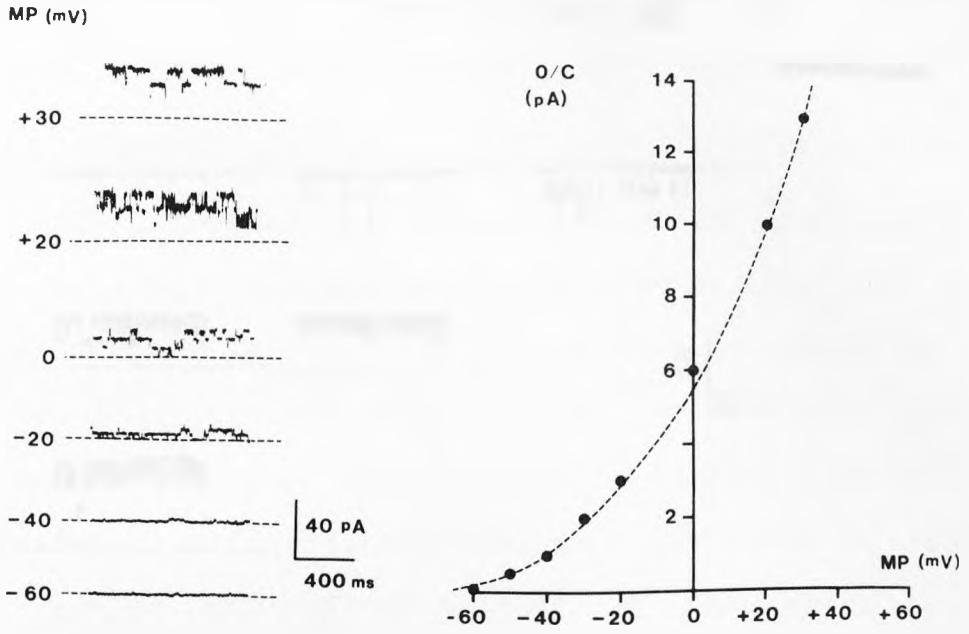


Fig.20. Single channel current recorded in an excised outside-out patch under physiological cation gradients from human submandibular acinar cell. The pipette contained high-KCl HEPES buffered saline with no added Ca^{++} and 1mM EGTA, the bath high-NaCl HEPES-buffered saline with 1.2mM Ca^{++}. A) Only outwardly directed currents were seen. B) The corresponding i-v relationship.

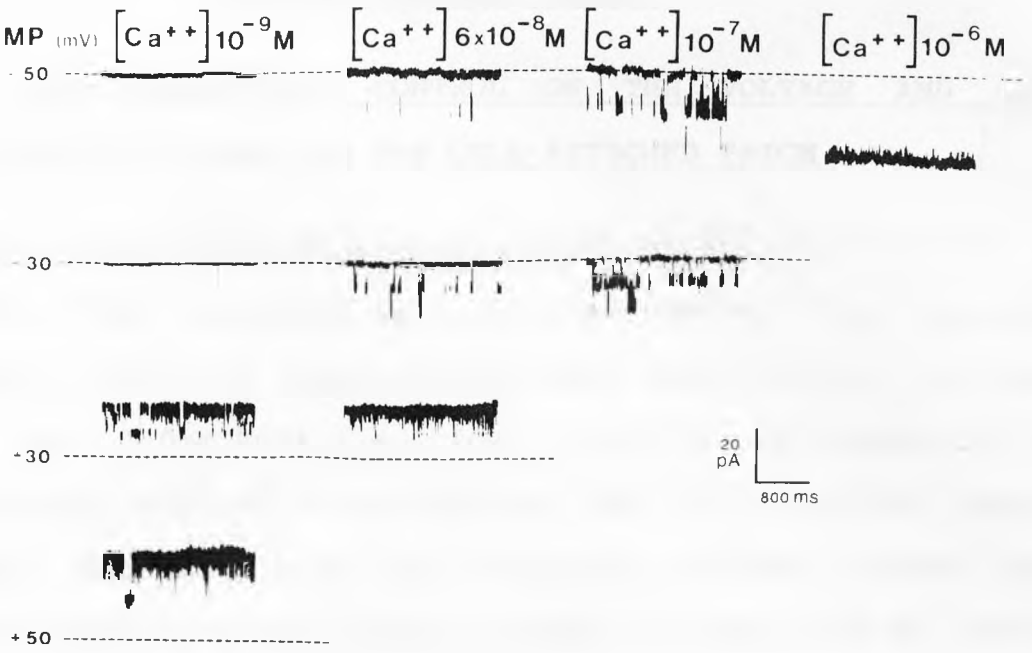


Fig.21. Sections of continuous current recordings taken from a human submandibular acinar cell inside-out patch under symmetrical KCl gradients. The recording pipettae contained 10^{-9}M Ca^{++} (no added Ca^{++} and 1.0mM EGTA), the bath ionised-Ca^{++} concentration was varied between 10^{-9} and 10^{-7}M.

4i) NEUROTRANSMITTER CONTROL OF THE VOLTAGE AND CA^{++} SENSITIVE K^+ CHANNEL IN THE CELL-ATTACHED PATCH.

A) Activation of K^+ Channel by Acetylcholine.

In all the following cell-attached patches the recording pipette contained high-KCl HEPES-buffered saline, no added Ca^{++} and 1.0mM EGTA i.e. $[Ca^{++}]_i=10^{-9}M$. Inclusion of the cholinergic agonist acetylcholine (ACh) $10^{-5}M$ to the pipette during recording from cell-attached patches bathed with normal extracellular saline (1.2mM Ca^{++}-bath) had no effect on the resting K^+ channel activity of the patch (n=4). Likewise ACh addition to the extracellular surface of the patch also failed to evoke an increase in single channel currents in excised, inside-out patches under symmetrical high KCl, low-Ca^{++} (1.0mM EGTA) gradients (n=3). However addition of ACh to the bathing saline of a Na^+-bathed cell (final bath concentration of $10^{-5}M$) whilst recording from a cell-attached patch resulted in a dramatic activation of K^+ currents within the patch Fig.22:

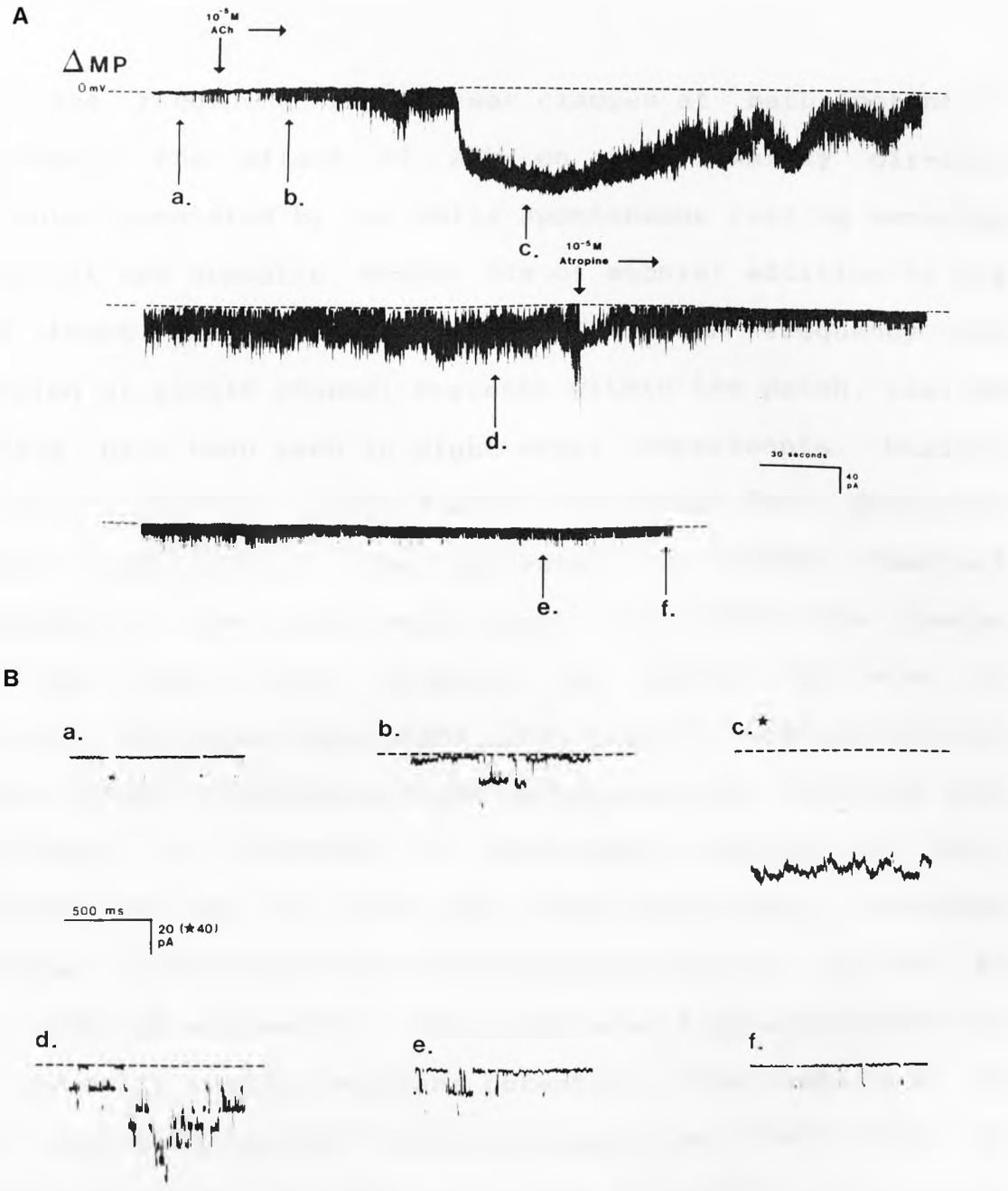


Fig.22. A) continuous current recording from a cell-attached membrane patch. The cell was bathed in extracellular saline (high-NaCl, 1.2mM-Ca^{++}) and the recording pipette contained high-KCl saline with 1mM EGTA and no added Ca^{++}. At the point indicated ACh was added to the bathing medium to achieve a final concentration of 10^{-5}M. ACh was then present throughout the duration of the experiment. The cholinergic antagonist Atropine was added when indicated at a similar bath concentration (10^{-5}M). B) Sections of the single channel current recording displayed on a faster time scale. a) resting currents; b),c) and d) different times following ACh addition; e) and f) ACh together with Atropine.

When the recording pipette was clamped at bath potential (ΔMP=0mV), the effect of ACh on the inwardly directed currents generated by the cells spontaneous resting membrane potential was dramatic. Within 30s of agonist addition to the bath there was a marked increase in both frequency and duration of single channel currents within the patch. Similar results have been seen in eight other experiments. Maximal current activation occured within the first 2min following agonist application. The activated K^+ current remained sustained (in some experiments up to 17min before the pipette seal was lost) until atropine was added. Inclusion of atropine in the bathing media (n=4) prior to addition of ACh had no effect on resting single channel current activity but abolished the response to subsequent addition of ACh. Sections (a) and (b) of Fig.22B reveal an initial transient increase in the amplitude of the single channel current at the onset of activation. This indicates a hyperpolarisation of the cells resting membrane potential. The amplitude of this hyperpolarisation varied between 5 and 15mV change in the cells tested. At peak activation (c) individual currents cannot be resolved but during the sustained phase (d) it becomes clear that the recorded current is due to the opening of the large conductance K^+ channel. Sections (b) and (e) reveal that at the onset of ACh activation and atropine inhibition of the channel activity, in addition to the large conductance steps, there are currents of smaller conductance present. These conductances appear to reflect the presence of the smaller K^+ conductive pathway mentioned earlier in Chapter Three.

B) The Effect of Extracellular Ca^{++} on the Agonist Evoked K^+ Current in the Cell-Attached Patch.

Continuous insitu current recordings were made from cell-attached patches during application of ACh (10^{-5}M) to cells bathed in high-NaCl HEPES-buffered saline containing no added Ca^{++} and 1mM EGTA (Ca^{++}-free extracellular saline). An agonist evoked K^+-channel response is shown in Fig. 23. Under Ca^{++}-free, high-NaCl extracellular conditions inward K^+ currents generated by the cells resting membrane potential were not seen to increase in activity upon addition of ACh to the bathing saline. There was if anything a slight hyperpolarisation in the cells membrane potential, seen as a small increase in single channel amplitude and decrease in open probability for the channel after the second ACh addition. When Ca^{++} (final concentration 1mM) was reintroduced to the bath in the continued presence of Ach a sustained activation of single channel currents occurred. The reintroduction of Ca^{++} to Ca^{++}-free bathing saline in the absence of agonist had no effect on single channel activity in the cell-attached patch.

Of the 16 cell-attached patches tested at the cells spontaneous membrane potential, 4 gave large activated currents (over 4 channel levels fully activated; mean single channel o-p=1.0), 7 gave modest activated currents (1 to 4 channel levels activated; mean single channel o-p>0.5), 3 gave low activated currents (1 to 2 channel levels activated; mean single channel o-p=<0.5) and 2 gave no increase in single channel current activity. Of the patches giving low or no increase in K^+ channel activity all the recorded single channel currents underwent a transient hyperpolarisation or

increased their current amplitude (sustained hyperpolarisation) throughout the experiment. The current amplitude recorded was 16.0+-2.0pA compared with 10.8+-1.6pA under resting conditions (n=5). These results suggest differences in access to the patch for an intracellular messenger. Atropine was found to inhibit the agonist induced hyperpolarisation seen in cells in the abs ence of extracellular Ca^{++} (n=4). The peak amplitude for hyperpolarisation occured with a mean latency of 33+-14sec (n=13).

A total of 14 out of 16 cell-attached patches displayed activated K^+ channels within the patch when extracellular Ca^{++} was readmitted in the presence of agonist. Reintroduction of extracellular Ca^{++} in the abs ence of agonist had no effect on single channel current activity (o-p or current amplitude) in any of the patches.

C) The Effect of Extracellular Na^+ Removal on Secretagogue Evoked K^+ Channel Activity in the Cell-Attached Patch.

Replacement of Extracellular NaCl with KCl Saline.

The effects of ACh on acinar cells bathed in high-KCl HEPES bufered saline containing 1.2mM-Ca^{++} was investigated. Under these conditions of K^+-pipette/K^+-bath the cell is depolarised (Chapter Three). The resting membrane potential of the electrically isolated patch of membrane under these conditions is zero. Hence a membrane potential was restored by voltage clamping the patch at -45mV (the average MP determined for these cells, see Chapter Three). In a series of control insitu recordings (n=7) the open-state probability for the channel was determined at this patch potential and

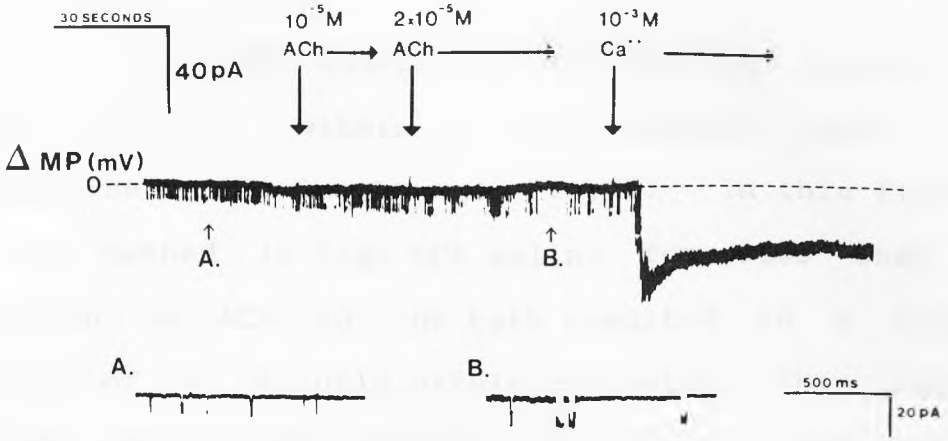


Fig. 23. Continous current recording from a cell-attached patch bathed in Ca^{++}-free (0.1mM EGTA) extracellular saline. The recording pipette contained high KCl, low-Ca^{++} (1mM EGTA) and was clamped at bath potential (ΔMP=0mV). ACh where indicated was applied twice in succession at progressively higher concentrations (10^{-5}M & $2x10^{-5}$M). The effect of Ca^{++} reintroduction to the bath (final concentration 1mM) in the continous presence of ACh is also shown.

found to be $2.3 \pm 0.9 \times 10^{-3}$. This o-p value was not significantly different to that obtained at the spontaneous membrane potential for cells bathed in normal extracellular saline ($1.9 \pm 0.7 \times 10^{-3}$).

The effect of ACh addition to the bathing saline on K^+ channel activity within a cell-attached patch of a K^+-depolarised cell is shown in Fig.24. In this figure the cell was bathed in high KCl saline for less than 5min. Application of ACh to the bath resulted in a transient activation of K^+ channels within the patch. This recording represents the longest duration of activation achieved in a K^+-depolarised cell. When responses were elicited single channel activity always returned to prestimulus levels. Inhibition of the response by the cholinergic antagonist atropine was not required. The transient response was obtained when cells were exposed to K^+-depolarising conditions for less than 10 min (n=2). Preincubation for longer periods gave no increase in channel activity within the patch (n=5). The conductance of the single channel events can be seen more clearly in the expanded time scale photographs (Fig.24B) and was clearly that of the large K^+ channel. Again there was evidence (c) that a smaller conductance channel is likewise activated.

To test whether the K^+ channel or the Ca^{++} influx pathway was refractory in K^+-depolarised cells, the Ca^{++} ionophore A23187 (10^{-6}M) was added to the bathing saline of the <u>insitu</u> cell-attached patch after agonist application (n=4). A typical example is shown in Fig.25.

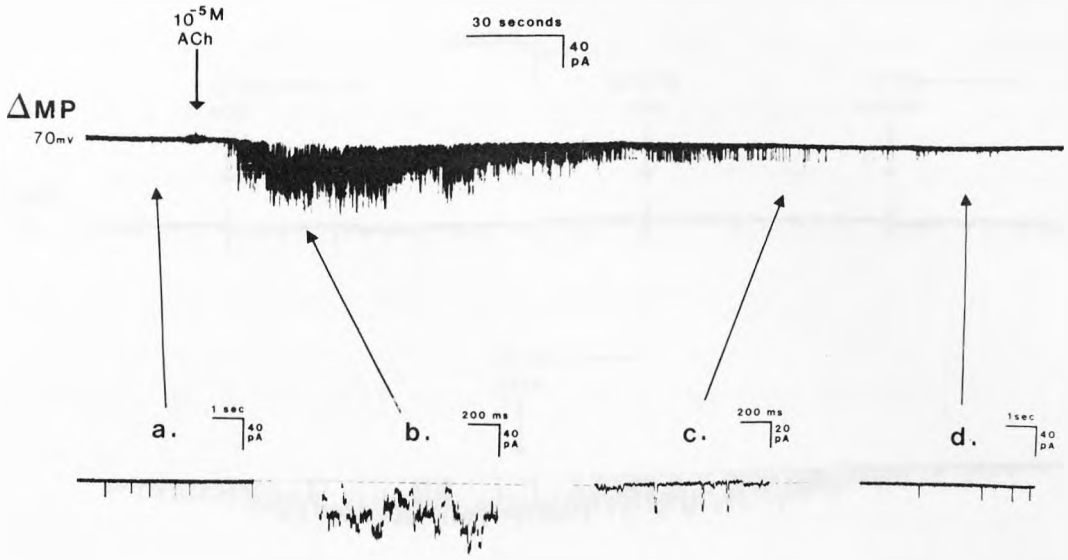


Fig.24. Single channel currents recorded from a K^+-depolarised cell-attached patch. Both pipette and bath contained an identical high-KCl HEPES-buffered saline. The bath contained 1.2mM-Ca^{++} and the pipette solution is Ca^{++}-free with 1mM EGTA added. A) In this figure a transmembrane potential (Δ MP) of -70mV has been restored across the patch. Clear inward K^+ currents were seen with low frequency of opening. ACh (10^{-5}M final bath concentration) was added to the bathing saline at the point indicated. B) Sections of the above single channel current recording displayed on a faster time scale. a) Unstimulated currents; b), c) and d) currents recorded in the presence of ACh.

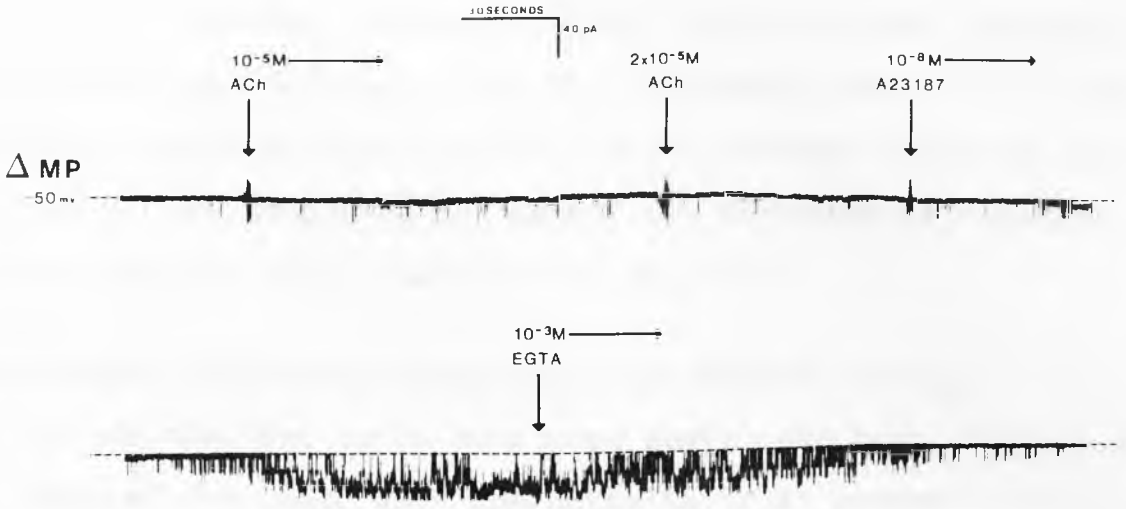


Fig.25. Sections of continous recording taken from a K^+-depolarised cell. The recording pipette contained high-KCl saline but had no added Ca^{++} and 1mM EGTA. The cell-attached patch had in this example a voltage clamped potential of -50mV. Clear inward single channel currents were seen. This cell was preincubated in Na^+-free, Ca^{++}-containing (1.2mM) saline for longer than 10min. ACh was applied at increasing bath concentrations (10^{-5}M and $2x10^{-5}$M) at the points indicated. The Ca^{++} ionophore A23187 was then applied at the point indicated at a bath concentration of 10^{-8}M.

In Fig.25 and in similar experimental protocols (n=4), repeated application of ACh had no effect on the o-p of single K^+ channel currents within cell-attached patches. Subsequent application of the Ca^{++} ionophore A23187 to the bathing saline however resulted in an increase in K^+ channel activity. The effect of ionophore was reversed by chelation of Ca^{++} by addition of 1mM EGTA to the bath.

Replacement of Extracellular NaCl with NMDGCl Saline.

The K^+ depolarised cells described above gave poor responses to agonist challenge when the activity of K^+ channels within cell-attached patches was monitored. Evidence demonstrating that no voltage gated Ca^{++} influx occurs in these cells was suggested by two observations: i) The similarities in o-p curves between normally bathed and high-KCl bathed acinar cells when the mean membrane potential is applied across the latter. ii) The corresponding parallel shift in i-v curves was entirely due to lack of the spontaneous membrane potential (Chapter Three, Fig.15 & 17.). To investigate whether cell depolarisation was having a converse effect and limiting the influx of Ca^{++} or that Na^+ removal underlies this phenomenon, N-methyl,D-glutamine (NMDG) was used as an extracellular Na^+ replacement.

A typical agonist evoked response is shown in Fig.26. Inward currents were seen due to the cells membrane potential. ACh application (10^{-5}M) to the bath was not associated with activation of the K^+ channels in the patch (n=9). Channel activity was increased by application of A23187 (10^{-8}M) to the bath (n=9). NMDG replaced cells were found to have a mean resting membrane potential MP of -43 ± 1.4mV (n=9)

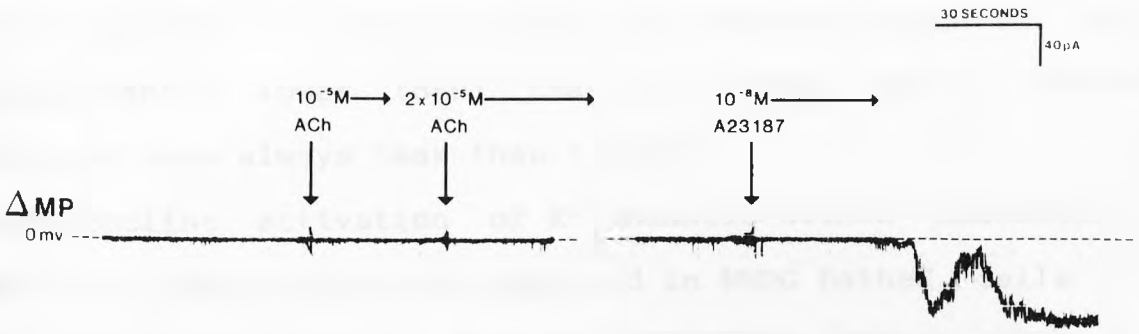


Fig.26. A continous recording of K^+ currents in a patch attached to a cell bathed in Na^+-free, NMDG containing saline with 1.2mM added-Ca^{++}. This solution was non-depolarising to the cell. The patch pipette containing high-KCl HEPES-buffered saline with no added Ca^{++} and 1mM EGTA was clamped at bath potential (Δ MP=0mV). ACh (10^{-5} and $2x10^{-5}$M final bath concentration) was added at the points indicated, as was the ionophore A23187 at 10^{-8}M.

(calculated from the amplitude of currents at the spontaneous membrane potential and the cells reversal potential). This value is similar to that obtained for normal (Na^+-bathed) acinar cells, -46 ± 1.7mV (n=24). The single channel open state probability at the spontaneous membrane potential was significantly lower (n=9) than Na^+-bathed cells, values obtained were always less than 1.0×10^{-4}.

Acetylcholine activation of K^+ channels within basolateral membrane patches could be restored in NMDG bathed cells by the reintroduction of extracellular Na^+, Fig.27. The K^+ channels present within cell-attached patches of Na^+-free bathed cells were voltage sensitive. Depolarisation of a membrane patch attached to a NMDG bathed cell resulted in an increase in the activity of the single channel within the patch (Fig.27A). Outwardly directed K^+ currents were seen as the patch depolarisation passed through the cells reversal potential. ACh addition to the bath (Fig.27B) had no effect on resting K^+ channel activity within the patch. However reintroduction of 20mM-Na^+ to the Na^+-free bathing media in the continued presence of ACh resulted in activation of the channel. This activation was slow to develop but gave a pronounced increase in single channel open state probability and was inhibited by the cholinergic antagonist atropine (10^{-5}M) i.e. it was clearly receptor regulated. Similar results were obtained with other cells (n=3).

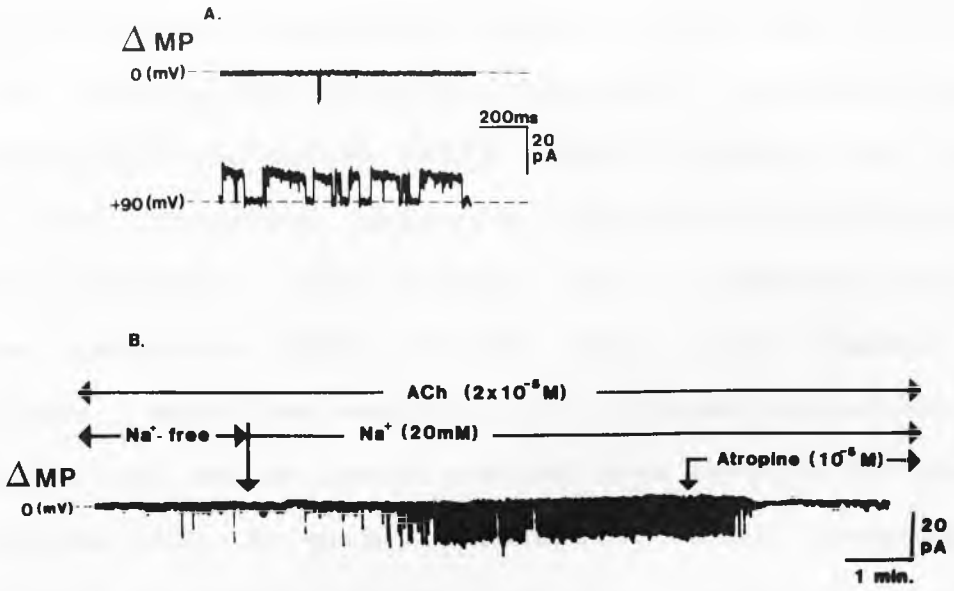


Fig.27. Sections of a continuous recording from a cell-attached patch bathed initially in NMDG (Na^+-free) saline containing 1.2mM free-Ca^{++}. The recording pipette contained high-KCl HEPES-buffered saline with no added Ca^{++} and 1mM EGTA (10^{-9}M-Ca^{++}). A) Signal channel current recorded at the spontaneous membrane potential (Δ MP=0mV) and when depolarising the membrane potential by +90mV (Δ MP=+90mV). B) Continuous current recording from the patch held at the spontaneous membrane potential (Δ MP=0mV) at a slow time scale. The acinar cells were bathed in NMDG containing extracellular saline in the presence of 1.2mM-Ca^{++} and 10^{-5}M ACh. At the point indicated 20mM-Na+ is reintroduced. The cholinergic antagonist atropine was then added at 10^{-5}M final bath concentration.

4ii) THE EFFECTS OF AGONIST ON $[CA^{++}]_i$ MEASURED DIRECTLY WITH INTRACELLULAR CA^{++} INDICATORS.

The Ca^{++}-sensitive fluorescent probes, quin-2 and fura-2 were used to measure the cytosolic free-Ca^{++} concentration in mouse submandibular acinar cells. Small clusters and single cells were isolated using a hyaluronidase/collagenase digestion protocol (see methods) and incubated with the membrane permeable form of the dye. The loaded cell populations were then washed prior to experimentation. The fluorometer was set at predetermined wave lengths for excited and emitted light to give a fluorescent output proportional to the level of bound intracellular dye. Calibration of this signal gave an estimate of intracellular Ca^{++} concentration (see methods).

A) Measurement of Cytosolic Free-Ca^{++} with Quin-2.

Calibration of the fluorescent signal was achieved by taking into account both dye leakage using the Mn^{++}-quench protocol and cell autofluorescence, (see Methods). The resting cytosolic free-Ca^{++} concentration ($[Ca^{++}]_i$) in the submandibular cells was found to be 77 ± 7 nM (n=21) with a range of 38 to 112 nM.

B) Effects of Acetylcholine Stimulation on $[Ca^{++}]_i$ Measured with Quin-2.

ACh was added to the stirred cell suspension bathed in normal (i.e. NaCl; 1.12 mM-Ca^{++}) saline at a concentration of 10^{-5}M. A typical experiment is shown in Fig.28:

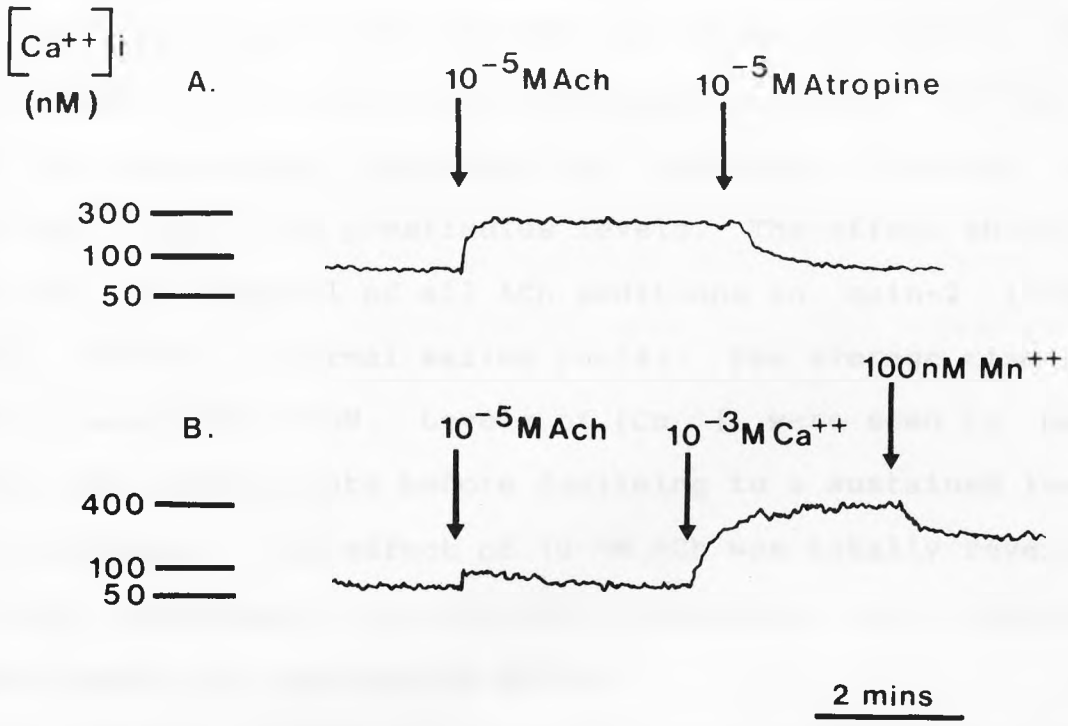


Fig.28. Changes in $[Ca^{++}]_i$ (quin-2 fluorescence) induced by acetylcholine in a suspension of mouse submandibular acinar cells. The cells were suspended in high-NaCl HEPES-buffered (extracellular saline) prewarmed to 37°C and containing: A) 1.2mM-Ca^{++} and B) no added Ca^{++} plus 0.1mM EGTA. At the points indicated ACh was added to the cell suspension to a final concentration of 10^{-5}M. The cholinergic antagonist atropine was sequentially added to suspension (A) at a concentration of 10^{-5}M. Extracellular-Ca^{++} was readmitted to cell suspension (B) at the point indicated at a final bath concentration of 1mM. The quin-2 quenching agent Mn^{++} was added (100nM) to (B) as a control to account for extracellular quin-2 fluorescence.

Addition of ACh to the extracellular Ca^{++}-containing saline resulted in a rapid and sustained elevation in $[Ca^{++}]_i$. The introduction of the muscarinic antagonist atropine ($10^{-5}M$) to the cell-suspension inhibited the sustained response and returned $[Ca^{++}]_i$ to prestimulus levels. The effect shown in Fig.28A was typical of all ACh additions to quin-2 loaded cells bathed in normal saline (n=14); the average rise in $[Ca^{++}]_i$ was 279 ± 27 nM. Levels of $[Ca^{++}]_i$ were seen to peak within the first minute before declining to a sustained level of stimulation. The effect of $10^{-5}M$ ACh was totally reversed in all experiments by addition of atropine at a similar concentration to the bathing saline.

In the absence of extracellular Ca^{++} (0.1mM EGTA) the resting Ca^{++} levels were found to be slightly depressed, 64 ± 10 nM (n=5). Addition of ACh to cells bathed in Ca^{++}-free extracellular saline (Fig.28B) caused only a small transient increase in the fluorescence signal. The reintroduction of extracellular Ca^{++} (1.2mM) to the bathing saline in the continued presence of agonist resulted in a sustained increase in the fluorescence signal i.e. a sustained intracelluar Ca^{++} rise. The divalent cation Mn^{++} (100nM) was added to the extracellular saline to quench extracellular free quin-2 in order to obtain a truer value for the intracellular fluorescence signal.

The elevation in $[Ca^{++}]_i$ upon reintroduction of extracellular Ca^{++} was seen only to occur in the presence of ACh and could be totally inhibited by atropine. It is clear from these results that the change in $[Ca^{++}]_i$ induced by ACh can be separated into two components: an initial transient rise in $[Ca^{++}]_i$ independent of external Ca^{++} and a sustained rise of

$[Ca^{++}]_i$ dependent upon extracellular Ca^{++}.

C) The Effect of Na^+ Removal in Quin-2 Loaded Acinar Cells.

Evidence obtained from the patch clamp data suggest that Na^+-free conditions inhibit the agonist evoked intracellular Ca^{++} rise which is thought to provoke channel opening during stimulation. Quin-2 loaded cells were used to investigate this hypothesis.

K^+ Depolarised Cells.

Quin-2 loaded acinar cells bathed in control saline containing 2.5mM-Ca^{++} were acutely depolarised by addition of 50mM KCl to the extracellular solution. Under conditions of complete extracellular Na^+-depletion (by preincubating the cells in saline containing high-KCl and 2.5mM-Ca^{++} prior to experimentation) the effect of ACh on intracellular Ca^{++} levels within these cells was monitored, Fig.29. Addition of KCl to Na^+-bathed cells had no effect on the fluorescence signal and therefore no effect on $[Ca^{++}]_i$. ACh addition to the K^+-depolarised cells gave a rise in $[Ca^{++}]_i$ that was at best transient. The resting-Ca^{++} concentration in K^+-depolarised cells was 47 ± 8 nM, the maximal intracellular Ca^{++} rise was 73.4 ± 10 nM, (n=7). Both resting Ca^{++} levels and agonist evoked responses were attenuated in K^+-depolarising conditions.

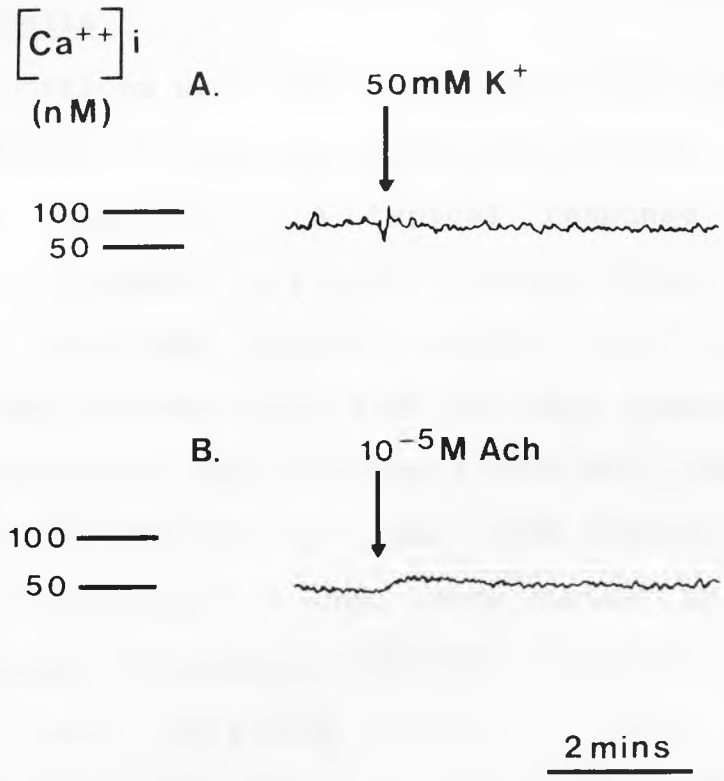


Fig.29. Fluorescence signal recorded from quin-2 loaded submandibular acinar cells. The cells in A) were bathed in a HEPES-buffered saline containing high-NaCl and 1.2mM added Ca^{++}. Acute application of KCl (final concentration 50mM) to the cell suspension occured at the point indicated. The cells in B) were preincubated in a HEPES-buffered high-KCl saline containing 2.5mM-Ca^{++} for 20min before being experimented upon. At the point indicated ACh was added to the cell suspension to a final concentration of 10^{-5}M. Both suspensions in figures A) and B) used solutions prewarmed to 37°C.

NMDG Bathed Cells.

Na^+-free conditions were also achieved by preincubating the cells in extracellular saline containing N-methyl,D-glutamine (NMDG) and 1.2mM-Ca^{++}. A typical response to agonist stimulation is shown in Fig.30. Cells bathed in control saline gave sustained agonist evoked $[Ca^{++}]_i$ responses. Conversl y when acinar cells from the same preparation were bathed in NMDG saline and challenged with ACh, they gave only small and transient $[Ca^{++}]_i$ rises. The resting $[Ca^{++}]_i$ in NMDG treated acini was 78 ± 10nM, very similar to levels seen in the control situation. The peak response of agonist application was 185 ± 23nM (n=10). This result is significantly larger than the K^+-depolarised situation ($p<0.001$).

A summary of the results obtained with quin-2 loaded acinar cells is shown in Fig.31.

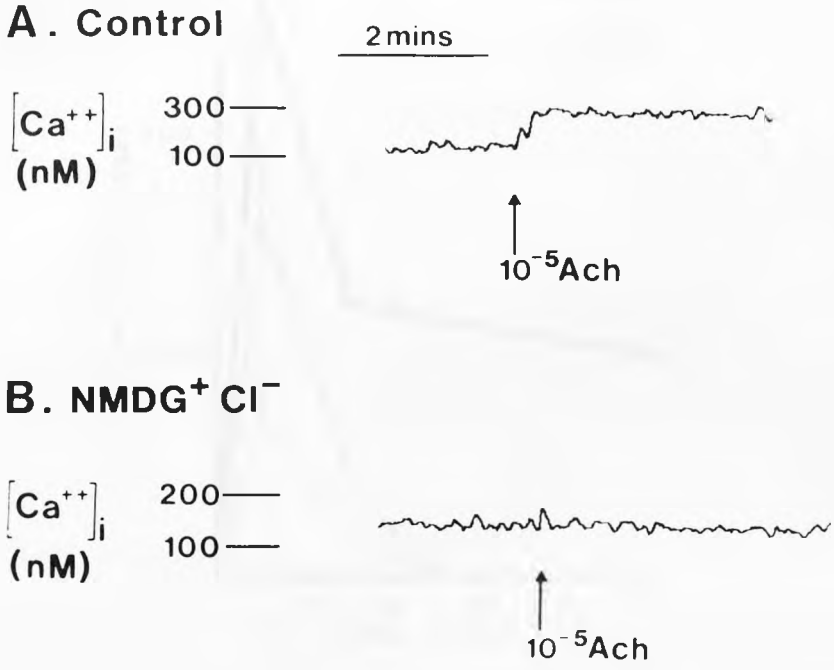


Fig.30. Quin-2 loaded submandibular acinar cells bathed in normal (Na^+-containing) or NMDG (Na^+-free) extracellular saline, with [Ca^{++}] present at 1.2mM. A) Control agonist response, ACh (10^{-5}M) addition to the cell suspension occured at the point indicated. B) Cells from the same preparation, but loaded in NMDG containing saline for 20min prior to experimentation. ACh (10^{-5}M) was likewise added to the cell suspension.

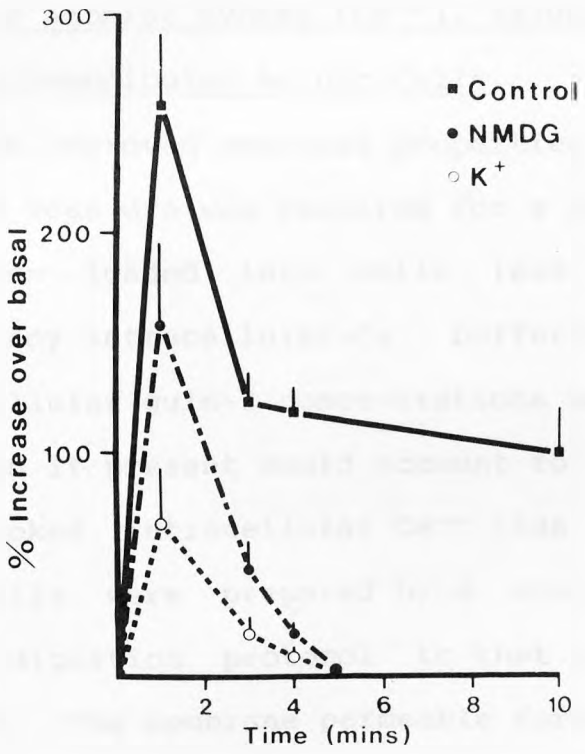
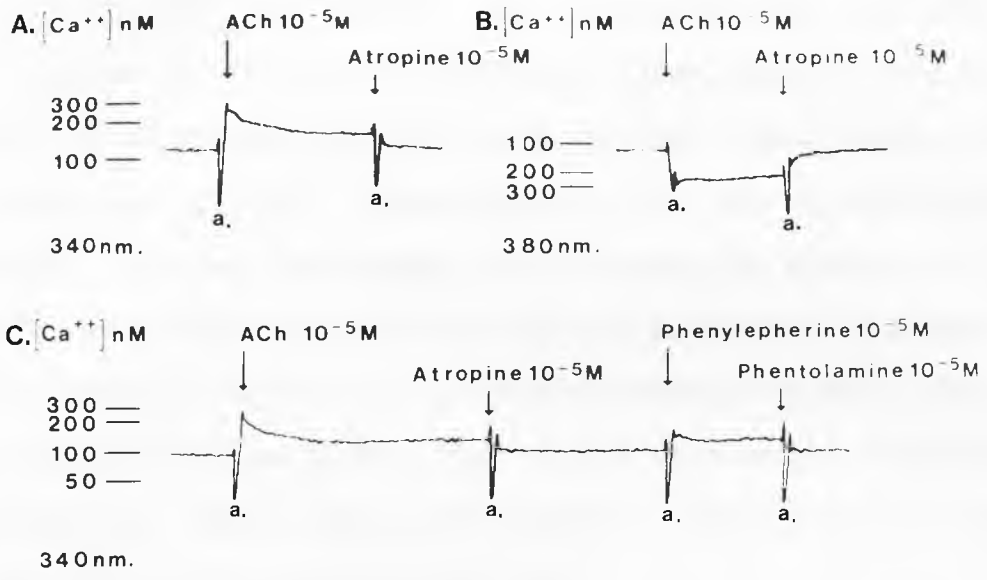


Fig.31. Intracellular-Ca^{++} rise expressed as percentage increase over basal in submandibular acinar cells stimulated with ACh (10^{-5}M) under conditions of control, NMDG and K^+ substitution for external Na^+. The difference between control and Na^+-free conditions was highly significant after the first minute of stimulation ($p<0.0001$) for all points whether expressed as percentage increases or as absolute values (not shown).

D) Resting and Agonist Evoked $[Ca^{++}]_i$ Values Obtained from Fura-2 Loaded Submandibular Acinar Cells.

Fura-2 with its improved spectral properties over quin-2 was used since x30 less dye was required for a similar amount of fluoresence when loaded into cells (see Methods). The possibility of any intracellular-Ca^{++} buffering occurring due to high intracellular quin-2 concentrations was investigated. This phenomenon if present could account for the absence of an agonist evoked intracellular Ca^{++} rise under Na^+-free conditions. Cells were prepared by a similar collagenase /hyluronidase digestion protocol to that used for quin-2 experimentation. The membrane permeable form of this dye was loaded into cells at a lower concentration (4uM compared with 20uM for quin-2). Cells were then incubated at 37°C for 40min in the presence of the dye before being washed and experimented upon. The fluorometer was reset to the relevant wavelengths to excite and then collect emitted light from the trapped fluorophore. A similar $[Ca^{++}]_i$ calibration protocol to that used for quin-2 measurements was then performed on the cells (Methods). A typical trace obtained from fura-2 loaded cells under normal extracellular conditions (high-NaCl, 2.5 mM-Ca^{++}) is shown in Fig.32:



a.– Addition artifact.

Fig.32. Fura-2 loaded submandibular acinar cells bathed in high-NaCl HEPES-buffered saline with 1.2mM added-Ca^{++}. Fura-2 can be used to monitor $[Ca^{++}]$, with the fluorescent signal excited at either A) 340nM or B) 380nM. At the points indicated in the traces, ACh was added to the cell suspension to a final concentration of 10^{-5}M, this was followed by addition of the competitive antagonist atropine at a similar concentration (10^{-5}M). Also shown at the points indicated in C) is the subsequent addition of the alpha-adrenergic agonist phenylephrine (10^{-5}M) and antagonist phentolamine (10^{-5}M).

Introduction of ACh to the cell suspension resulted in a rapid and sustained change of the fluorescent signal excited at either 340 or 380nM. The increase and decrease in fluorescence monitored at 340nM and 380nM resectively is the result of increased Ca^{++} binding to the trapped pool of dye. The ability of Ca^{++} measurement at two wavelengths is utilised in dual wavelength spectroscopy to produce a ratio which is unaffected by dye leakage or bleaching. As the above contaminating effects on the fluorescent signal were not appreciable in this study, the single wavelength fluorescence measurements made were sufficiently adequate to monitor changes in $[Ca^{++}]_i$ with either dye.

The $[Ca^{++}]_i$ response to agonist occurred in two phases; peak values for the $[Ca^{++}]_i$ rise were reached within the first minute of the response which then declined to a lower and more sustained value. The agonist evoked response was abolished upon addition of atropine (10^{-5}M) to the cell preparation. The average value for resting $[Ca^{++}]_i$ in fura-2 loaded cells was 101 ± 30nM (n=6), which varied between 65 and 160nM. The mean values for the ACh evoked rise in intracellular-Ca^{++} were at peak 254 ± 66nM and sustained 155 ± 37nM (n=6). The specific a-adrenergic agonist phenylepherine was shown to evoke similar characteristic $[Ca^{++}]_i$ rises (Fig.32C).

The type of response obtained by ACh stimulation of either quin-2 or fura-2 loaded acinar cells was identical. As the resting values of $[Ca^{++}]_i$ were not significantly different there does not appear to be any appreciable intracellular Ca^{++} buffering with the quin-2 dye loading protocol under normal conditions.

4iii) RODENTS AS MODELS FOR HUMAN SALIVARY FUNCTION.

The patch-clamp studies corroborating the electrophysiologial findings for the K^+ channel found in mouse submandibular and human submandibular gland acinar cells (Chapter Three) are expanded in this chapter. Investigation of the messenger mediated control of the K^+ channel found in human submandibular acinar cells was undertaken using the cell-attached patch recording configuration. The effect of ACh addition to a normally bathed human submandibular acinar cell is seen in Fig.33. Inwardly directed currents were seen due to the cells resting membrane potential. Application of agonist to the bath (extracellular surface of the cell not in contact with the patch) resulted in a pronounced and sustained increase in inward K^+ current. The rise in channel activity within the patch was inhibited completely by atropine. A rise in current amplitude was seen during the initial stages of agonist activation (insert b), this is consistent with the cellular hyperpolarisation seen in rodent submandibular acinar cells under similar conditions. The current amplitude measured at the spontaneous membrane potential indicates this channel to be the large Ca^{++} and voltage sensitive K^+ channnel seen in excised patches (Chapter Three). Similar effects of ACh have been recorded in other human submandibular acinar cells (n=3). The data presented shows a clear similarity in the control of K^+ channel activity between rodent and human acinar cells.

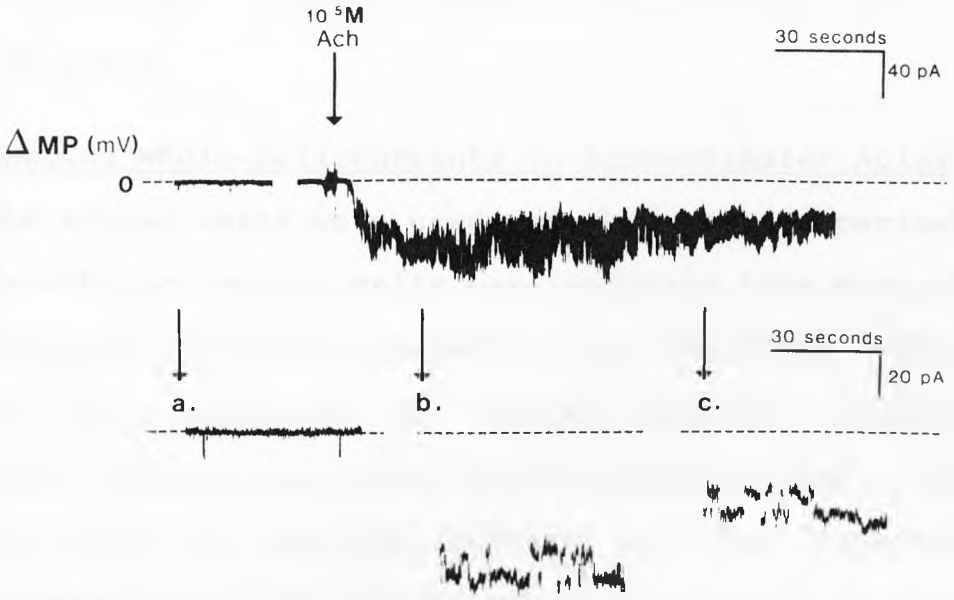


Fig.33. A continous current recording taken from an insitu cell-attached patch of a human submandibular acinar cell. The recording pipette contained high-KCl HEPES-buffered saline with no added Ca^{++} and 1mM EGTA. The cell was bathed in extracellular saline containing 1.2mM-Ca^{++}. The recording pipette was clamped at the spontaneous membrane potential(ΔMP=0mV). ACh (10^{-5}M) and atropine (10^{-5}M) were added to the bath at the points indicated. The inserts a), b) and c) are expanded time scales for the above response.

5i) WHOLE-CELL CURRENT RECORDING IN SALIVARY AND LACRIMAL ACINAR CELLS.

A) Resting Whole-Cell Currents in Submandibular Acinar Cells.

Single acinar cells were used for whole-cell experimentation. Submandibular acinar cells were isolated from mice using the collagenase digestion procedure (see Methods). The single cells were separated by density gradient centrifugation through albumin containing extracellular saline. These were either kept in sterile culture or used immediately for experimentation. The cells were maintained in culture for up to seven days, during this period they gave clear agonist responses.

Upon formaton of a Giga-ohm resistance seal a brief suction pulse was applied to the recording pipette causing the isolated membrane patch to be lost to the pipettes interior. The cells cytoplasm rapidly equilibrates with the ionic milieu of the recording pipette that it is in communication with. (Hamill et al., 1981; Fenwick, Marty & Neher, 1982). When a stable whole cell membrane potential was achieved (monitored in current-clamp as a steadily rising negative potental which reaches a plateau usually within 1min), the cell was clamped at or near to this potential. The average membrane potential obtained under conditions where the recording pipette contained high KCl, low-Ca^{++} (1mM EGTA and no added-Ca^{++}) HEPES buffered saline and the bath normal high NaCl, 1.2mM-Ca^{++} HEPES buffered saline was found to be -37± 4.8mV (n=18). This value is in close correlation with the

resting membrane potential determined in cell-attached patches (Chapter Three). Cells with low resting membrane potentials and large non-rectifed currents were considered as damaged and were discarded. A typical whole cell current recording obtained from a mouse submandibular acinar cell is shown in Fig.34. From the zero current level the interior of the whole-cells were sequentially clamped at positive and negative membrane potentials in order to obtain resting i-v relationships. In Fig.34 it can be seen that depolarising voltage jumps superimposed on the zero current level evoked an outward current. This reached maximal amplitude within 30 to 40ms and was sustained during the timecourse of the pulse. When hyperpolarising voltage potentials were superimposed on the zero current level very small currents were evoked, even for a large hyperpolarisation i.e -100mV of the membrane potential. The resting currents were recorded in the presence of Ca^{++}-free conditions ($10^{-9}M$) in the intracellular saline. The non-linear i-v relationship at depolarising potentials seen for all whole-cells was consistent with voltage activation of an outwardly directed current. All clamped potentials have a current leakage component which is inversely related to seal resistance. However the high resistance (10 to 20) Giga ohm seals used in these studies made correction for this component unnessesary.

The rectified outward current which predominates in the submandibular whole-cell recording under physiological ion gradients was similar to that seen in lacrimal acinar cells. In this tissue it has been demonstrated (using noise analysis tecniques) to be due to the opening of the voltage and Ca^{++} sensitive maxi-K^{+} channel (Trautmann & Marty, 1984).

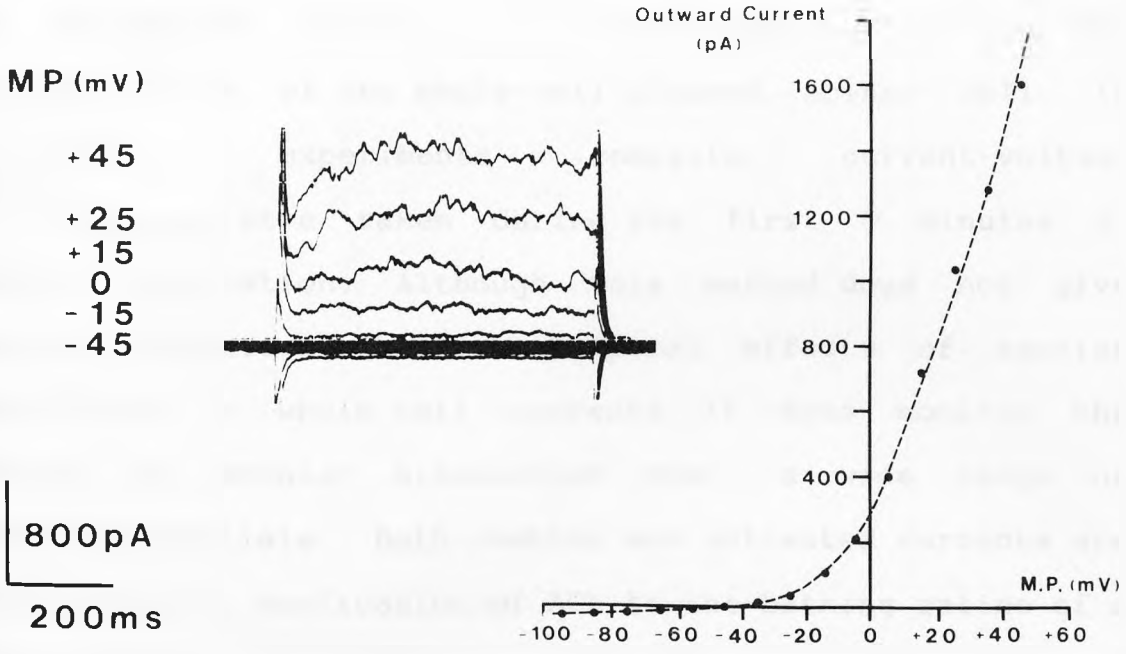


Fig.34. A) Whole-cell currents recorded from a single submandibular acinar cell. The recording pipette contained 140mM KCl, no added Ca^{++} and 1mM EGTA and the bathing saline 140mM NaCl, 1.2mM-Ca^{++}. The cell was clamped at zero current level, corresponding to a cell resting membrane potential of -50mV. Depolarising and hyperpolarising voltage jumps were superimposed upon this potential. Outwardly directed currents are seen as upward deflections, inwardly directed currents as downward deflections. B) The corresponding current-voltage relationship for the recording. Depolarising the cell by 50mV to 0mV MP evoked an outward current of 125pA whilst hyperpolarising the cell by -40mV to -90mV MP evoked a small inward current of approx. 10pA relative to the zero current level.

B) Secretagogue Evoked Whole-Cell Currents in Submandibular Acinar Cells.

The cholinergic agonist acetylcholine was applied to the bathing saline of the whole-cell clamped acinar cell. In preliminary experiments complete current-voltage relationships were taken during the first 5 minutes of agonist application. Although this method does not give precise infomation about the temporal effects of agonist stimulation on whole-cell currents it does monitor the effects of agonist stimulation over a wide range of membrane potentials. Both resting and activated currents are seen in Fig.35. Application of ACh to the bathing saline of a submandibular whole-cell resulted in a large increase in outward current at positive clamped potentials which lasted the time course of the experiment. An increase in inward current was also seen. The magnitude of these current changes are best observed in the corresponding i-v relationship Fig.35C. where a leftward shift at depolarising membrane potentials occured. The increase in outward current was most pronounced at positive holding potentials around the zero current level. In common with the earlier insitu cell-attached patch recordings, the activated current could be completely reversed by application of atropine (10^{-5}M) to the bathing media. The results described in Fig.35 are typical of five such experiments. The insert (Fig.35D) demonstrates a drawback in this protocol since the initial events associated with ACh stimulation of whole-cell currents remain hidden.

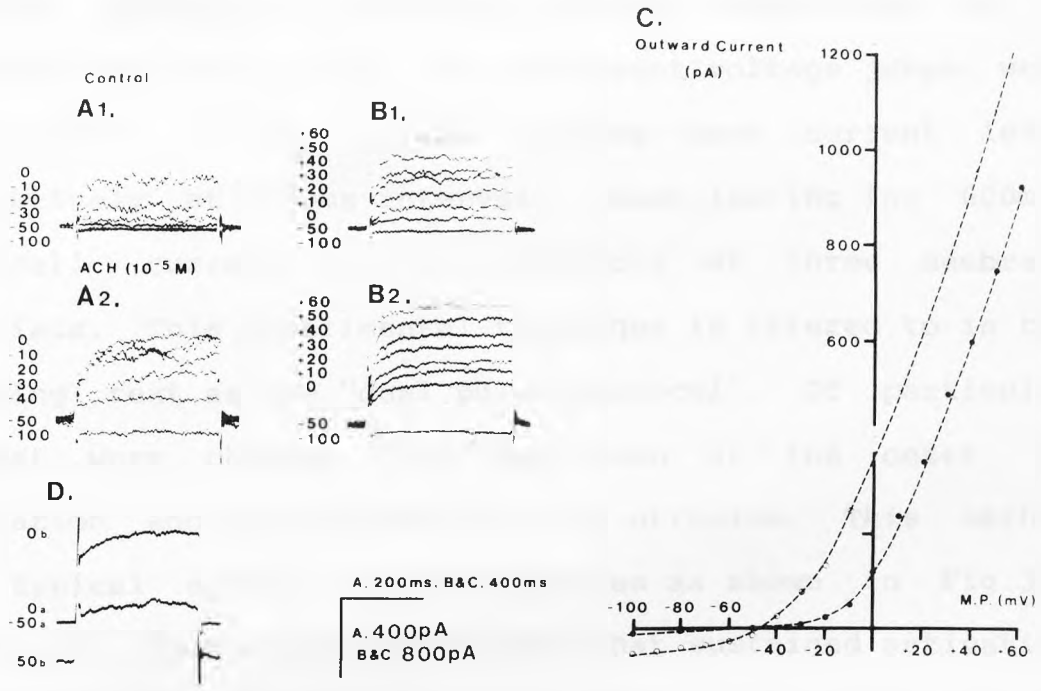


Fig.35. Whole-cell current recordings from a mouse submandibular acinar cell under physiological ion gradients. The recording pipette contained high K+, low-Ca^{++} (1mM EGTA) saline and the cell was bathed in high NaCl; 1.2mM-Ca^{++} saline. The cell was held at the zero current potential i.e. normal resting membrane potential MP=-50mV. A series of depolarising and hyperpolarising voltage jumps were applied at 10mV intervals to a maximum change in clamped potential of +110mV and -50 mV. A1) and B1) resting whole-cell currents (200pA/div. and 400pA/div. respectively). A2) and B2) the same whole-cell currents but in the presence of extracellular saline containing ACh (10^{-5}M) (200pa/div. and 400pA/div. respectively). C) The corresponding current-voltage relationship for both resting and activated currents is shown. Closed circles represent resting conditions, open circles the agonist stimulated situation. D) The insert shows changes in holding and 0mV currents seen during the initial phase of agonist activation.

A new protocol was developed to investigate the time dependent effects of cholinergic agonist stimulation on K^+ currents in whole-cells. Two different voltage jumps were superimposed on top of the resting zero current level consecutively at 600ms intervals, each lasting for 600ms. Whole-cell current could be recorded at three membrane potentials. This experimental technique is refered to in the following text as the "dual pulse protocol". Of particular interest were changes in current seen at the onset of stimulation and during inhibition by atropine. This method gave typical agonist evoked responses as shown in Fig.36. This figure demonstrates once again that sustained activation of currents was seen during agonist application (n=4). At 0mV MP there was a clear increase in outward current whilst at -48mV MP (holding potential) oscillation in the recorded current occurred which after 30s became predominantly inward. Finally at -90mV MP a large increase in an inwardly directed occured.

In order to assess the role of either cationic or anionic conductive pathways in the generation of the whole-cell current the relative contributions made by each ion at the MP was accounted for. The equlibrium potentials for the ions were: E_{Cl}=0mV; E_{Na}=-35mV and E_K=-90mV. The clamped membrane potentials were chosen to reflect as much as possible the movement of a single ion. At the voltage clamped potential of -90mV MP any current seen could be due to either Na^+ or Cl^- movement with no contribution made by K^+ as the membrane potential is equal to E_K. Any possible contribution being made by Na^+ movement could be ignored as Marty and colleagues (1984) have demonstrated little role for a Na^+ permeability

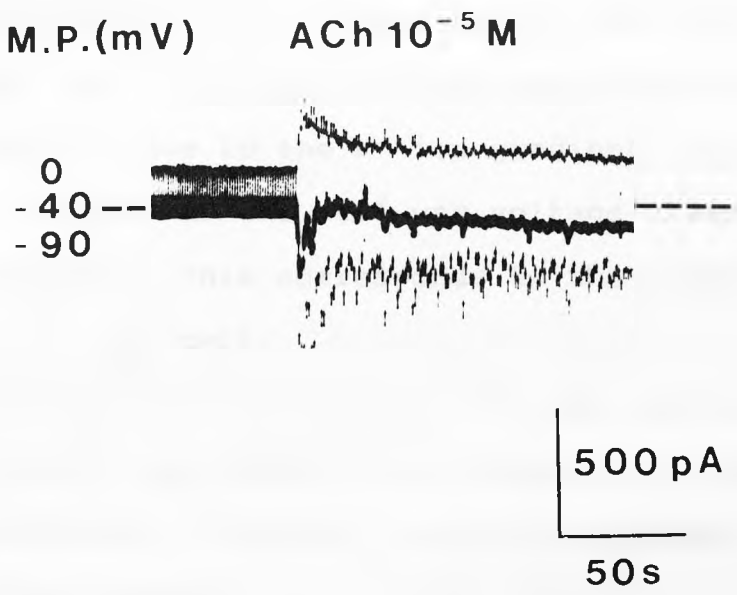


Fig. 36. Whole-cell current recordings taken from a single submandibular acinar cell using the dual pulse protocol. The recording pipette contained intracellular-like saline: high-KCl, low-Ca^{++} (no added-Ca^{++} and 1mM EGTA) and the cell was bathed in normal extracellular saline: high-NaCl, 1.2mM-Ca^{++}. The cell was held at -48mV MP corresponding to the zero current level. Two voltage jumps to potentials of 0mV and -90mV MP were alternately superimposed at 600msec intervals throughout the experiment. At the point indicated ACh (10^{-5}M) was added to the bathing saline.

pathway during cholinergic stimulation of whole-cell currents in lacrimal acinar cells. Conversly at 0mV MP equal to E_{Cl} there would be no movement of this anion and any outward current would be due to the K^+ ion gradient. At the holding potential of -48mV MP the cell was voltage-clamped with no input of current, this corresponds to the resting membrane potential of the cell.

As the holding potential in these cells was found to lie between E_K and E_{Cl} this indicates that both K^+ and Cl^- conductive pathways contribute to the resting current.

During agonist activation (Fig.36) the membrane permeability of both K^+ and Cl^- ions increased as demonstrated by an increase in recorded current at both 0mV and -90mV MP. These permeability changes likewise effect the zero current level and were manifest as oscillations in current at this potential. The -50mV holding potential current oscillations were in phase with those seen at -90mV MP but out of phase with oscillations in current at 0mV MP. This indicates a stronger Cl^- current influence at this membrane potential an effect demonstrated by the more general inward shift in the current level. The net result would be an overall depolarising influence at this potential if the cell was not voltage clamped. The effects of ACh could be reversed by addition of atropine (10^{-5}M) to the bathing saline.

A diagrammatic summary of the change in the cellular reversal potential evoked by agonist stimulation drawn from to the current changes recorded in the preceeding figure is seen in Fig.37:

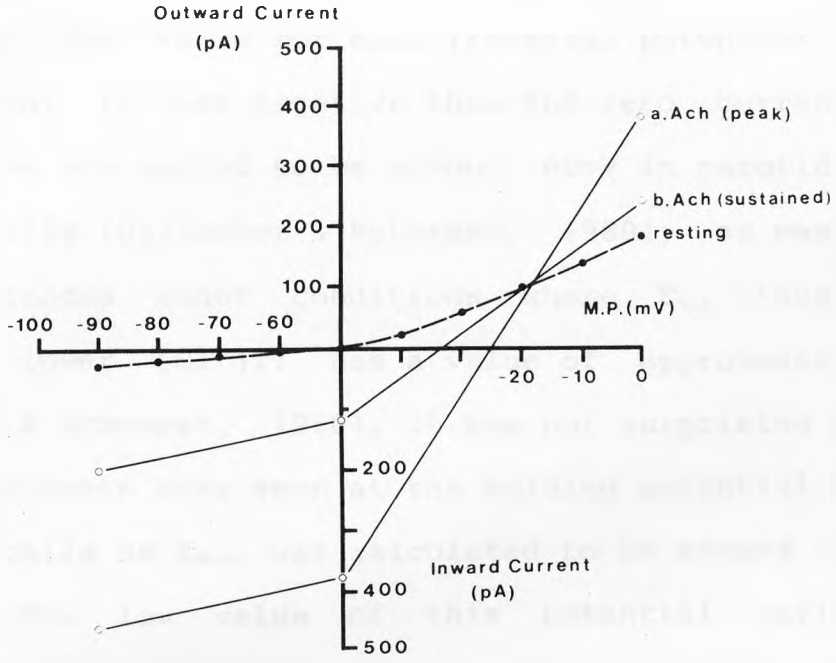


Fig.37. This figure corresponds to the currents measured during agonist stimulation of the submandibular whole-cell shown in Fig.36. Currents were taken at resting, maximal (within 1sec) and sustained phases of stimulation at the three holding potentials indicated. The broken line represents a complete i-v curve obtained under resting conditions prior to the initiation of the dual pulse protocol. a) The maximal inward current recorded during the initial phase of agonist stimulation. b) The current generated during the sustained phase of stimulation.

The initial phase of agonist stimulation had the greatest effect on depolarising the cells reversal potential. In the figure **37** the value for E_{ACh} (reversal potential for ACh stimulation) is less negative than the zero current level. E_{ACh} values are quoted to be around -60mV in parotid salivary acinar cells (Gallacher & Petersen, 1980), as measured by microelectrodes under conditions where E_{Cl} (due to the normally lower $[Cl^-]_i$) has a value of approxmately -25mV (Schneyer & Schneyer, 1960). It was not surprising then that inward currents were seen at the holding potential of -48mV in these cells as E_{ACh} was calculated to be around -18mV (see figure). The low value of this potential reflects the symmetrical Cl^- gradients existing across the cell (E_{Cl}=0mV). The reversal potential for the agonist (Fig.37) was associated with an outward current of approximately 100pA.

C) Evidence for a Cl^- Conductive Pathway in Submandibular Whole-Cells.

From the whole-cell currents seen in the prece ding section there appears to be a significant Cl^- conductive pathway activated by cholinergic receptor stimulation in these cells. Demonstration of a Cl^- current was achieved by investigation of the agonist evoked whole-cell currents during Cl^--free conditions (n=3). A typical whole-cell current recording is seen in Fig.38:

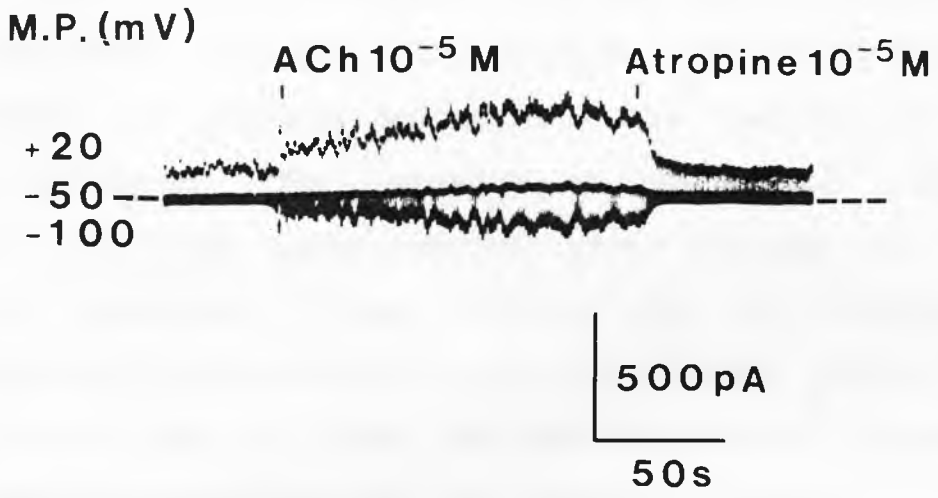


Fig.38. Whole-cell current recording of a submandibular cell using the dual pulse protocol. The cell was dialyzed with HEPES-buffered Cl^--free saline (140mM K-glutamate) containing no added Ca^{++} and 0.5mM EGTA. The bathing saline was an identical Cl^--free saline but contained 1.2mM-Ca^{++}. The whole-cell MP was clamped at: +20mV MP, -50mV MP (zero current level) and -100mV MP. At the points indicated ACh (10^{-5}M) was added to the bathing solution and sequentially followed by the cholinergic antagonist atropine (10^{-5}M).

Addition of ACh to the Cl^--free bathing media evoked an outward current at +20mV and -50mV MP. An inward current at -100mV was also seen. The upward shift seen at the zero current level clearly suggests the cell would undergo a hyperpolarisation if not in voltage clamp. The inhibition of this effect by atropine demonstrated the response to be receptor-regulated. The absence of an inward current component at the zero current level during Cl^--free conditions provides clear evidence for a functional cholinergically controlled Cl^- pathway in these cells. The inward current seen at -100mV was due to inward K^+ movement as the membrane potential was more negative than E_K.

An estimate of E_{ACh} obtained during Cl^--free conditions can be seen in Fig.39. The resting i-v relationships recorded under Cl^--free conditions displayed normal current-voltage relationships but were shifted to the right of normal currents. This indicates that the removal of the resting Cl^- conductance in the continous presence of the resting K^+ conductance shifts the cell towards hyperpolarisation. However the zero current values were not significantly different to those obtained under standard conditions. This anomaly suggests that other mechanisms apart from passive conductance pathways may contribute towards the generation of the resting membrane potential.

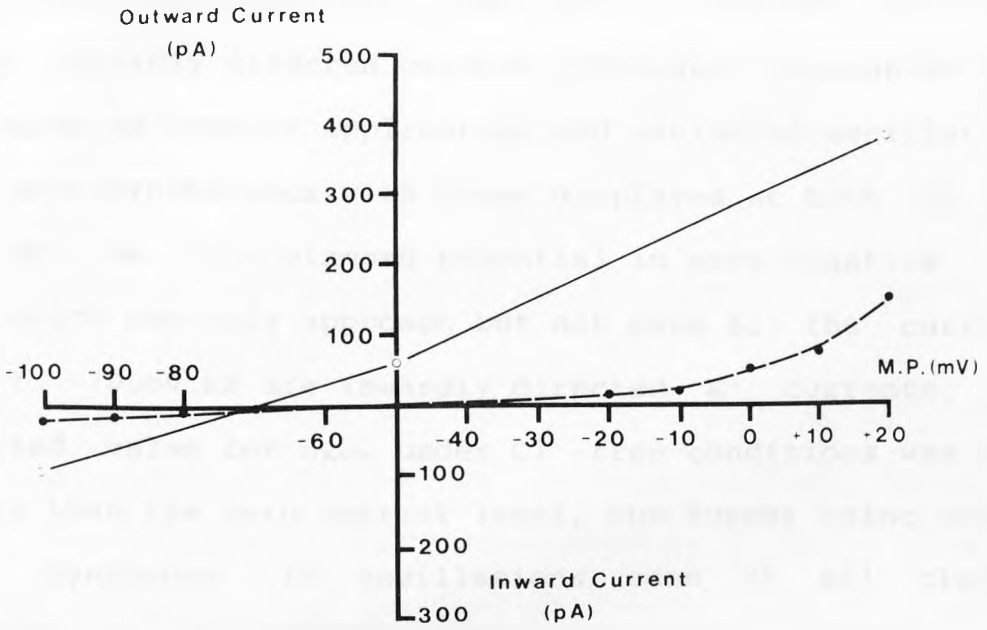


Fig.39. Changes in recorded currents at three clamped membrane potentials in Cl^- depleted acinar cells when challenged with the cholinergic agonist ACh (10^{-5}M). The resting current-voltage relationship is shown as the broken line (closed circles). The line marked a) (open circles) represents current amplitudes taken 2min following ACh addition.

In figure 39, ACh caused an increase in outward current at both the holding potential (-50mV) and at +20mV MP. At -100mV MP the inwardly directed current increased throughout the time course of agonist application and exhibited oscillations which were synchronous with those displayed at both -50 and +20mV MP. As this clamped potential is more negative than E_{ACh} (which can only approach but not pass E_K) the currents seen at -100mV MP are inwardly directed K^+ currents. The calculated value for E_{ACh} under Cl^--free conditions was more negative than the zero current level, the former being around -70mV. Synchrony in oscillations seen at all clamped potentials in the Cl^--depleted cells when compared with Cl^- bathed cells (where the inward currents generated at -48 and -90mV MP were synchronous with each other but not with the outward current at 0mV MP, see Fig.36) further supports the findings that a Cl^- current component was missing under these conditions.

D) Lacrimal Whole-Cell Current Recordings.

Lacrimal acinar cells were used in all the following studies because of their high yield of intact single cells which are required for the whole-cell technique. The acinar cells were isolated using a collagenase digestion protocol (see Methods). The electrophysiological responses to muscarinic stimulation when compared with submandibular cells are very similar (Trautman & Marty, 1984; Petersen, 1980). A typical whole-cell current recording made under control conditions is shown in Fig.40. Resting whole-cell currents displayed a similar outward rectification to those seen in submandibular acinar cells. Depolarising the membrane from the zero current

level activates a voltage dependent outward current whilst very little inward current is seen at hyperpolarising membrane potentials. The pronounced rectification corresponds to the presence of the highly selective, voltage sensitive outward K^+ current (Trautman & Marty, 1984).

E) The Effects of Acetylcholine on Lacrimal Whole-Cell Current.

The dual pulse protocol was used in lacrimal acinar cells which were voltage clamped at the zero current level. A typical agonist evoked current response is shown in Fig.41. The effect of ACh on the lacrimal whole-cell current response (Fig.41) was similar to that seen in submandibular acinar cells. ACh evoked a sustained increase in outward current at 0mV. However, very little movement in current was seen at the zero current level (-50mV MP) and a largely transient increase in inward current occured at -90mV MP. Although this inward current demonstrates the presence of a Cl^- conductive pathway it does not appear not to be as prevalent as that seen in submandibular cells. The stimulated currents were inhibited by the addition of atropine (n=12). The specific alpha adrenergic agonist phenylephrine likewise evoked currents of similar amplitude and duration (n=3). This effect could be reversed by phentolamine (not shown in Fig.41).

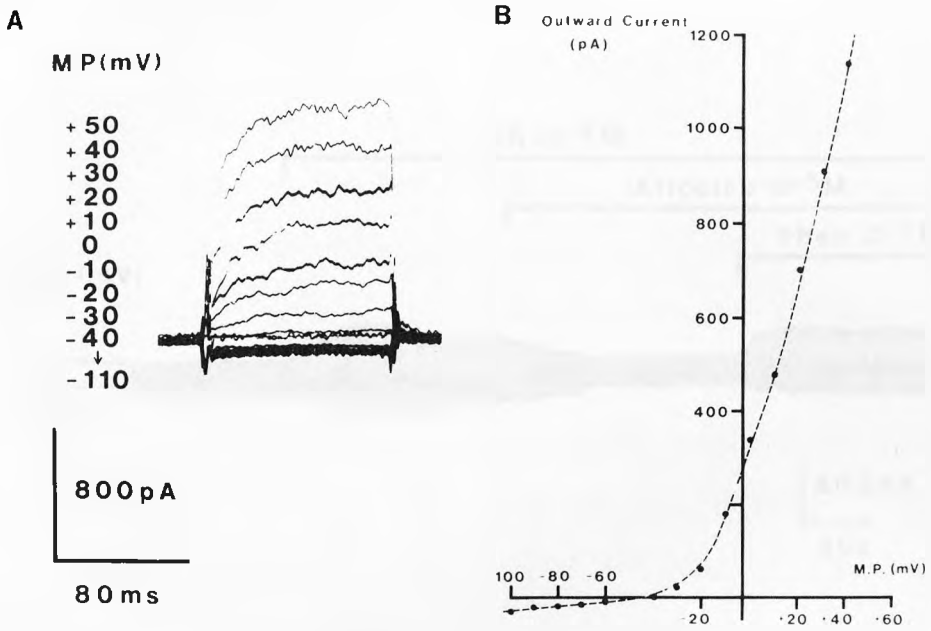


Fig.40. A whole-cell current recording taken from a single isolated lacrimal acinar cell dialysed with high-KCl HEPES-buffered saline containing no added-Ca^{++} and 0.5mM EGTA (10^{-8}M-Ca^{++}). The cell was bathed in normal physiological saline (high-NaCl, 1.2mM-Ca^{++}). A) The currents recorded and B) the corresponding current-voltage relationship.

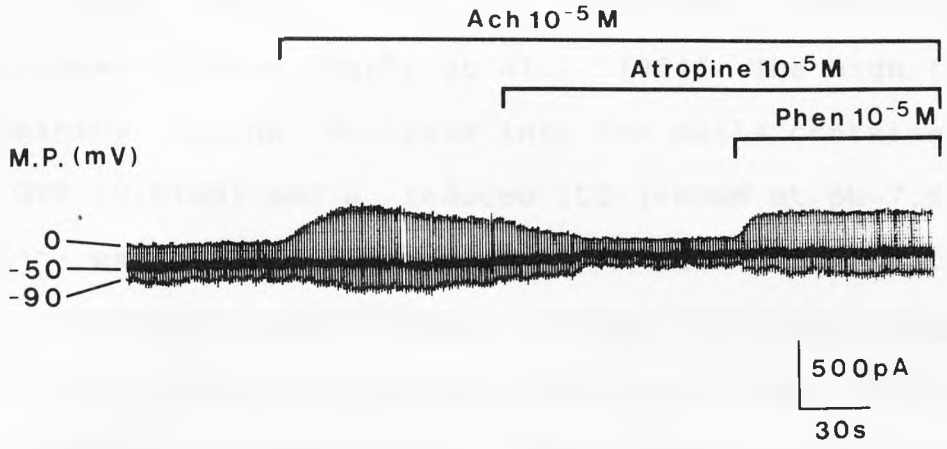


Fig.41. Continuous dual pulse whole-cell current recording from an isolated lacrimal acinar cell bathed in high NaCl, 1.2mM-Ca^{++} extracellular saline. The recording pipette contained high-KCl saline with no added-Ca^{++} and 0.5mM EGTA. The cell was clamped at zero current potential (reversal potential)= -50mV MP, 0mV and -90mV MP. Adition of ACh (10^{-5}M) to the bathing solution was indicated at the point shown. Atropine (10^{-5}M) was then added to the media followed by the specific a-adrenergic agonist phenylephrine (10^{-5}M), as shown.

5ii) THE CONTROL OF MEMBRANE K^+ AND Cl^- PERMEABILITIES MONITORED BY THE WHOLE-CELL TECHNIQUE.

Throughout the following series of experiments the recording pipette was filled with a modified HEPES-buffered intracellular saline (Marty et al., 1984). The high (140mM) K^+-containing saline dialysed into the cells contained ATP (4mM) GTP (0.01mM) and a reduced $[Cl^-]$=60mM at pH=7.6, the free-Ca^{++} was buffered to around 10^{-8}M with 0.5mM EGTA for control conditions (see Methods). Unless otherwise stated the extracellular solution remained as standard high NaCl, 1.2 mM-Ca^{++} HEPES buffered saline. The activation of whole-cell currents by acetylcholine was investigated using a technique developed for internal pipette perfusion (see Methods). ACh and other extracellular saline additions were perfused on to the cell using a multibarrelled superfusion system (see Methods).

Membrane currents were recorded using the dual-pulse protocol and the effects of changing the composition of the perfused intracellular saline monitored in resting and activated lacrimal acinar cells. Decreasing $[Cl^-]_i$ from 140 to 60mM had little effect on the zero current level at low-Ca^{++} pipette conditions as evidenced by the absence of a noticable shift in the zero current potential.

The effect of artificially manipulating $[Ca^{++}]_i$ and $[Ca^{++}]_o$ in isolated lacrimal acinar cells using internal cell perfusion is shown in Fig.42:

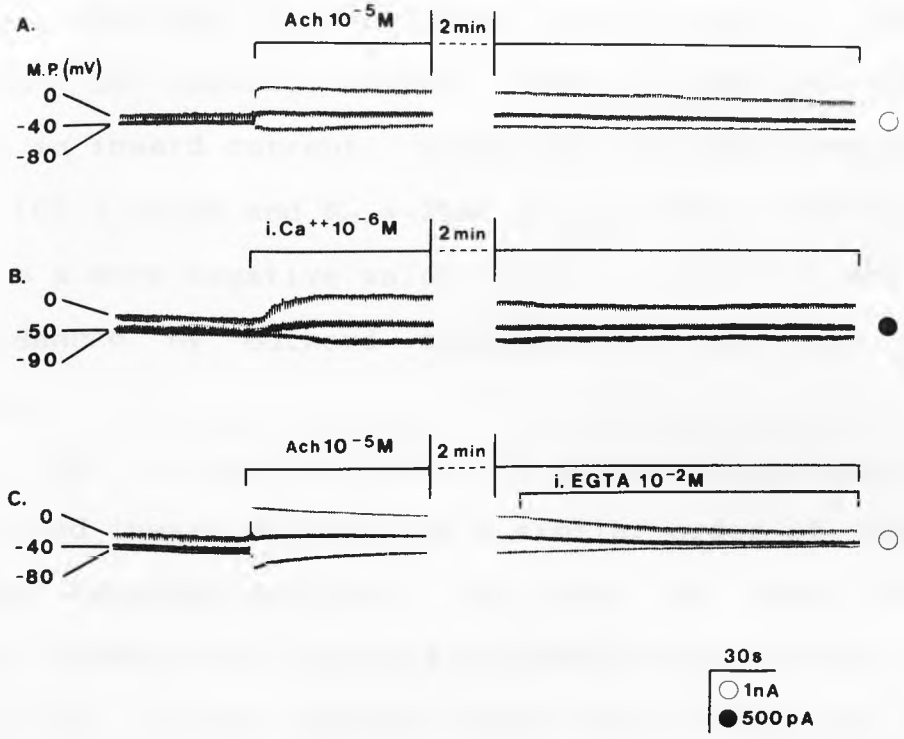


Fig.42. Whole-cell currents recorded from a single lacrimal acinar cell. The recording pipette contained the modified intracellular saline in which the [Cl^-] has been reduced to 60mM with no added-Ca^{++} and 0.5mM EGTA (unless otherwise stated). The cell was bathed in normal extracellular saline. A) Control whole-cell currents, the duration of superfusion of the cell with an identical saline with the addition of ACh (10^{-5}M) is indicated by the continous bar. B) This cell was internally perfused with saline with an ionised-Ca^{++} concentration of 10^{-6}M (see Methods). C) This cell was internally perfused with 10mM EGTA during extracellular ACh (10^{-5}M) perfusion.

Cholinergic stimulation of lacrimal acinar cells dialysed with the modified intracellular saline gave a sustained elevation in outward current with a smaller transient increase in inward current. Using the improved intracellular saline $[Cl^-]_i$=60mM and E_{Cl}=-25mV it was clear that E_{ACh} had moved to a more negative value than the zero current level as evidenced by outward currents at the zero current potential.

Raising $[Ca^{++}]_i$ from 10^{-8} to 10^{-6}M caused activation of both outward and inward currents by a similar order of magnitude to those recorded with ACh. The onset of these currents appeared slower when compared to agonist stimulation and the rise in Cl^- current always lagged behind that of the K^+ current (n=6). Internally perfusing cells with EGTA at concentrations much in excess of those needed to ensure low-Ca^{++} conditions in control cells demonstrated that the ACh stimulated currents were abolished by removing intracellular Ca^{++} (n=5).

The external Ca^{++} dependency was likewise investigated using the whole-cell technique. Typical whole-cell currents recorded during removal and readmission of extracellular Ca^{++} in the presence and absence of ACh are seen in Fig.43:

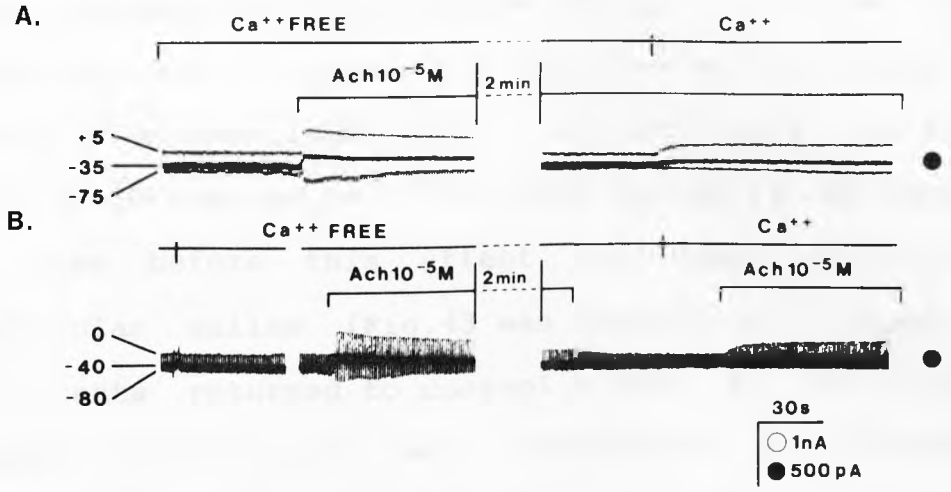


Fig.43. Lacrimal whole-cell currents monitored using the dual pulse protocol. The recording pipette contained the modified intracellular saline together with 0.5mM EGTA and no added-Ca^{++}. A) The cell was bathed in extracellular saline containing 0.1mM EGTA and no added-Ca^{++} (Ca^{++}-free conditions). A similar but ACh (10^{-5}M) containing saline was superfused over the cell at the point indicated. After a period of nearly 5min Ca^{++} was re-introduced (1mM) into the superfused ACh containing saline. B) A similar protocol to A), however after the initial effects of ACh application (10^{-5}M) this agonist was removed from the superfusion and Ca^{++} (1mM) readmitted. The superfusion was then changed for a combination of both Ca^{++} and ACh as indicated.

Bathing the cell in low-Ca^{++} (0.1 mM EGTA) control saline had no effect on resting whole-cell currents (n=7). Addition of ACh to the bath 10^{-5}M caused an initial increase in both outward and inward currents but this was not sustained in the absence of extracellular Ca^{++}. Lacrimal cells were found to require preincubation in a Ca^{++}-free saline (0.1mM EGTA) for some time before this effect was seen. In Ca^{++}-free extracellular saline (Fig.43 was typical of 6 experiments) the currents returned to control levels in the continued presence of ACh within 5min. Readmission of extracellular Ca^{++} in the continued presence of agonist resulted in activation of currents at all potentials. The recording of Fig.43B, shows that readmission of Ca^{++} to a low-Ca^{++} bathing media had no effect by itself on whole-cell currents.

5iii) THE CONTROL OF $[CA^{++}]_i$: THE EFFECTS OF INTERNAL PERFUSION OF INOSITOL POLYPHOSPHATES ON THE VOLTAGE AND CA^{++} SENSITIVE WHOLE-CELL K^+ CURRENT.

A) The Effects of Internally Perfused Inositol (1,4,5) Trisphosphate (IP_3) on Lacrimal Whole-Cell Currents.

The dual pulse protocol was used in conjunction with internal pipette perfusion to investigate the possible role of 1,4,5-IP_3 in mobilising intracellular Ca^{++}. Lacrimal acinar cells were monitored for changes in the amplitude of the Ca^{++}-sensitive whole-cell K^+ current characterised earlier in this chapter. Typical whole-cell current responses recorded before, during and after IP_3 introduction into lacrimal cells are shown in Fig. 44. As demonstrated in this figure, variable responses were recorded. In each case where an activation of K^+ current occured the response was short lived. Three such responses are shown in Fig.44. Transient increases in K^+ current were observed in 50% of cases (10 out of 18 experiments) and showed no correlation with the IP_3 concentration; these levels are supramaximal for Ca^{++} release in other permeabilized epithelial cells (Streb et al.,1983; Burgess, 1984). It was therefore unlikely that the lack of effect is due to enzymatic breakdown. If IP_3 was included in the intracellular saline containing 0.5mM EGTA and no added Ca^{++} ($<10^{-8}$M free-Ca^{++}), no responses were obtained at any of the concentrations tested: 10^{-5}M (n=5); 2×10^{-4}M (n=2) and 10^{-4}M (n=2).

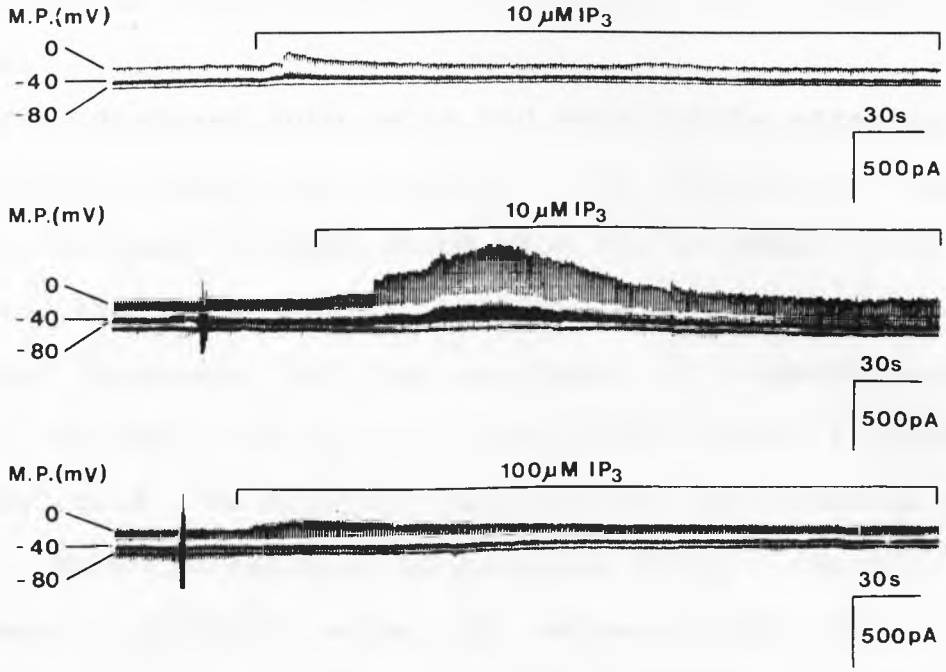


Fig.44. Lacrimal acinar cells bathed in normal extracellular saline containing 1.2mM free-Ca^{++} and dialysed with the modified intracellular saline containing free-Ca^{++} buffered to around 10^{-7}M, (0.1mM EGTA). The dual pulse protocol was used to monitor current amplitude, the top two traces represent an average and the best IP_3 (10^{-5}M) response recorded when this compound was internally perfused into each cell in the control intracellular saline. The bottom trace shows the effect of dialysing a cell with 10^{-4}M IP_3.

B) Inositol (1,3,4,5) tetrakisphosphate (IP_4): Its Effects Alone and in Combination with IP_3 on the Whole-Cell K^+ Current.

IP_4 when dialysed into cells had very little effect on the Ca^{++} sensitive whole-cell current. IP_4 dialysis of lacrimal cells alone and in combination with IP_3 is shown in Fig.45. Only in the presence of both IP_3 and IP_4 was there a sustained increase in the amplitude of outward currents (n=12). Neither IP_3 or IP_4 alone could elicit a sustained response (n=34). This effect was reversed upon removal of IP_3 and IP_4 from the intracellular saline (n=5). Fig.45C shows an almost identical effect of extracellular ACh (10^{-5}M) superfusion i.e. an increase in amplitude of outward currents. The later section of the trace shows the effect of extracellular Ba^{++} on whole-cell currents. $BaCl_2$ (88mM) substituted for NaCl in the extracellular saline markedly reduced currents in the unstimulated cell and abolished the ACh evoked outward current. These effects were reversible (n=4).

C) The Extracellular Ca^{++}-Dependency of the IP_3/IP_4 Evoked Whole-Cell Current.

The previous whole-cell recordings of agonist evoked currents in both salivary and lacrimal acinar cells have exhibited a clear extracellular Ca^{++}-dependency. This effect was looked for in acinar cells dialysed with the combination of inositolpolyphosphates shown to evoke a sustained activation of these Ca^{++}-sensitive currents. The results of this investigation are shown in Fig.46:

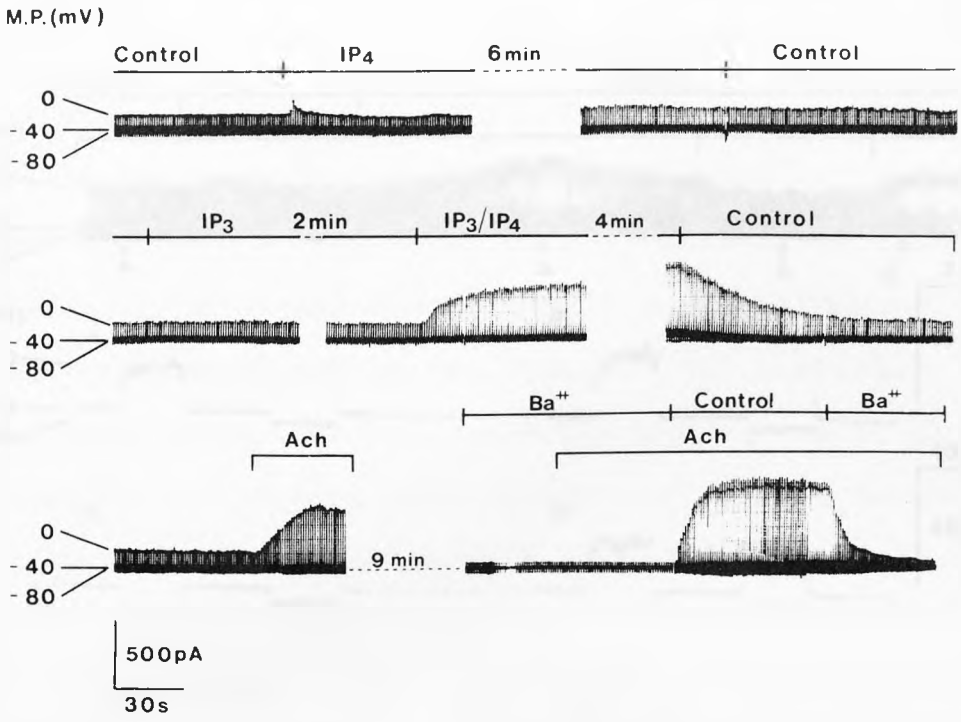


Fig.45. Sections of continuous recording from a lacrimal acinar cell monitored with the dual pulse protocol. The cell was dialyzed with the modified high-K^+ saline containing 0.5mM EGTA with no added Ca^{++} and was bathed in normal extracellular saline. A) The effect of changing control intracellular saline for one containing 10^{-5}M IP_4. B) The effect of an internal saline containing 10^{-5}M IP_3. C) The effect of an internal saline containing both IP_3 and IP_4. D) Removal of the inositol polyphosphates from the dialysate. E) The application of ACh (10^{-5}M) to the bathing saline. F) The effects of externally applied Ba^{++} (88mM) on whole-cell currents.

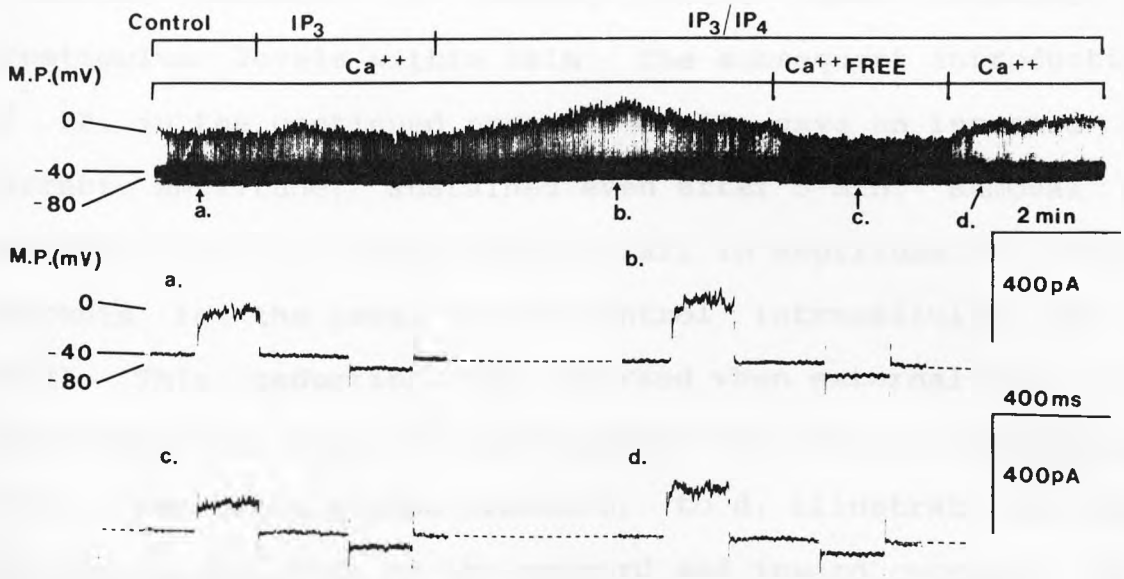


Fig.46: A lacrimal whole-cell dialysed with control modified intracellular saline containing 0.1mM-EGTA and no added Ca^{++} (10^{-7}M free-Ca^{++}) and bathed in normal high NaCl containing saline. A) Introduction of IP_3 (10^{-6}M) into the internal saline B) The switch to an internal saline containing IP_3/IP_4 (both 10^{-6}M) C) Removal of extracellular Ca^{++} and replacement with saline containing 0.1mM EGTA in the continued presence of IP_3/IP_4. D) Readmission of extracellular Ca^{++} (1mM) in the continued presence of IP_3/IP_4. The oscilliscope traces are expanded time scales for the currents at points a) to d).

The results in Fig.46 demonstrate that IP_3 addition to the intracellular saline perfusing the whole-cell gave only a transient increase in outward current which returned to prestimulus levels within 1min. The subsequent introduction of IP_4 in the continued presence of IP_3 gave an increase in current amplitude, sustained even after 5 min. Removal of extracellular Ca^{++} then caused a fall in amplitude of outward currents to the level of the control intracellular saline (n=7). This reduction was reversed when external Ca^{++} was reintroduced in the continued presence of both polyphosphates (n=4). The oscilloscope traces a, to d, illustrate the time course and amplitude of the outward and inward currents. The large noisy outward currents with short (<50mS) relaxation time constants are similar in shape to those seen earlier in the chapter and are a characteristic of currents found in the voltage and Ca^{++} activated maxi-K^+ channel (Trautmann & Marty, 1984).

6i) PATCH-CLAMP EXPERIMENTS IN OTHER EXOCRINE CELL TYPES.

A) Rodent Small-Intestine.

A K^+ conductive pathway is thought to play a role in both secretory and absorbtive pathways found in the basolateral membranes of intestinal epithelial cells (Donowitz & Walsh,1986; Brown, Burton & Sepulveda,1983). This pathway is reportedly sensitive to changes in cytosolic Ca^{++} (Brown & Sepulveda, 1985; Donowitz, 1983 and Smith & McCaba, 1984) and to blockade by Ba^{++} (Dharmsathaphorn et. al., 1985.).

Enterocytes were isolated as small clumps and single cells from the upper two thirds of the rat small intestine using a NaCitrate/Dithiotheritol and EGTA cell-dispersion protocol (see methods). In 10-15% of the cells obtained, clear polarity in single cells was observed (see photograph in methods) and areas distal to the brush border membrane were used for patch-clamp experimentation i.e. basolateral. In all the following experiments the recording pipette contained a HEPES-buffered saline containing high-KCl, no added Ca^{++} and 1mM EGTA (10^{-9}M-Ca^{++}). Membrane patches excised from these cells form predominantly the inside-out patch recording configuration. A patch exposed to symmetrical high KCl gradients is shown in the following Fig.47:

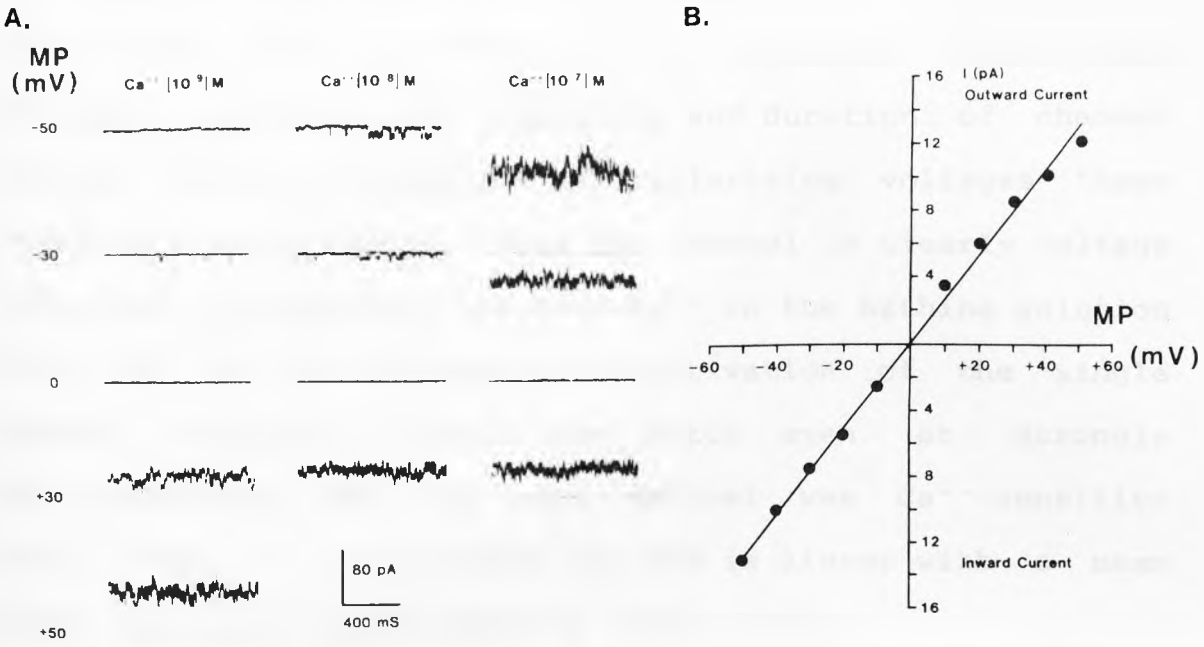


Fig.47. A) Sections of continuous recording from an excised patch of basolateral membrane isolated from rat enterocytes under symmetrical high-KCl saline. A Ca^{++}-free solution was used in the recording pipette. The bath ionised-Ca^{++} concentration was raised from 10^{-9}M to 10^{-7}M. B) The corresponding current-voltage relationship.

Single channel currents were recorded that reversed at zero MP. Under conditions of low-Ca^{++} bathing the intracellular face of the patch (10^{-9}M free-Ca^{++}), membrane depolarising voltages increased the frequency and duration of channel opening whilst at membrane hyperpolarising voltages these parameters were reduced. Thus the channel is clearly voltage sensitive. Increasing the free-Ca^{++} in the bathing solution from 10^{-9} to 10^{-7}M resulted in activation of the single channel currents within the patch even at strongly hyperpolarising MPs i.e. the channel was Ca^{++}-sensitive (n=3). The i-v relationship Fig.47B is linear with a mean slope equal to a conductance of 250pS. (n=6).

The selectivity of this channel for K^+ was demonstrated in experiments on excised patches exposed to asymmetrical cation gradients i.e. substituting bath K^+ for Na^+ in excised, inside-out patches as shown in Fig.48. The ion gradients in this figure are reversed when compared to the physiological membrane. Inward currents were seen at all potentials including 0mV MP. The channel exhibited clear voltage sensitivity (described earlier). The corresponding current-voltage relationship was no longer linear but showed a clear rectification of current. Although the zero crrent level was not reached it was estmated by extrapolation to be around +80mV MP. Since E_{Na}=+35mV and E_{Cl}=0mV MP the i-v relationship indictes that the channel is highly selective towards the K^+ ion.

Insitu cell-attached recordings were made under conditions of high-KCl pipette, low-Ca^{++} (no-added Ca^{++} plus 1mM EGTA) and high-NaCl, 1.2mM-Ca^{++} bath. Examples of recorded currents are shown in Fig.49.

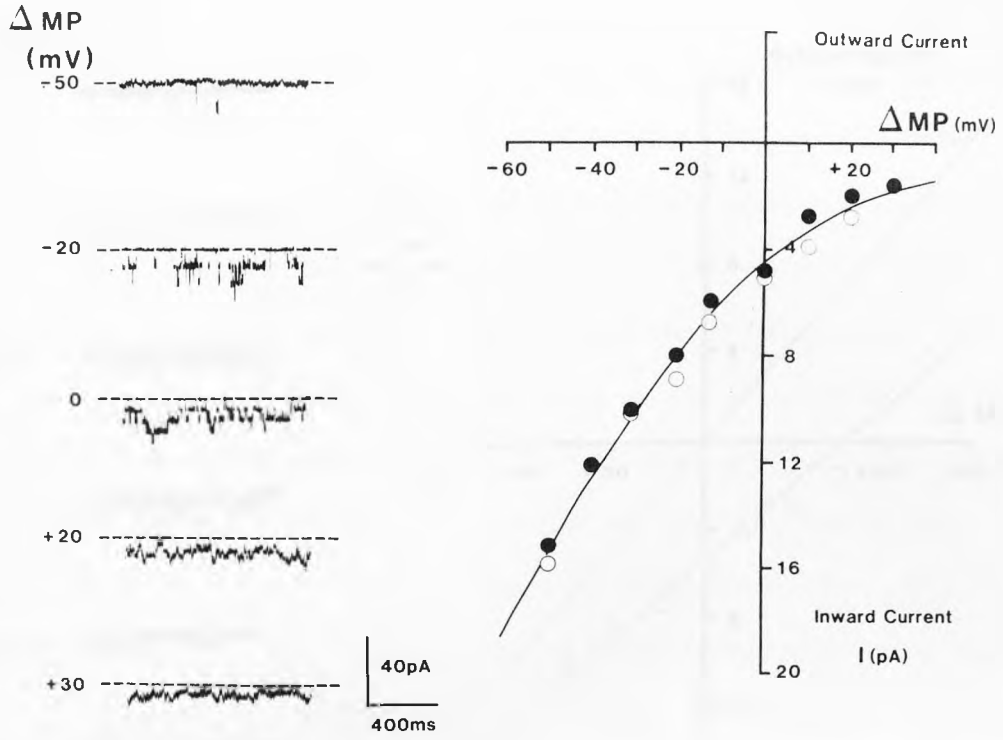


Fig.48. Sections of continuous current recording from excised inside-out patch under asymmetrical gradients. The pipette contained high-KCl, no added Ca^{++} and 1mM EGTA and the bathing solution, high-NaCl also with no added Ca^{++} and 1mM EGTA. To the right can be seen the current-voltage plot.

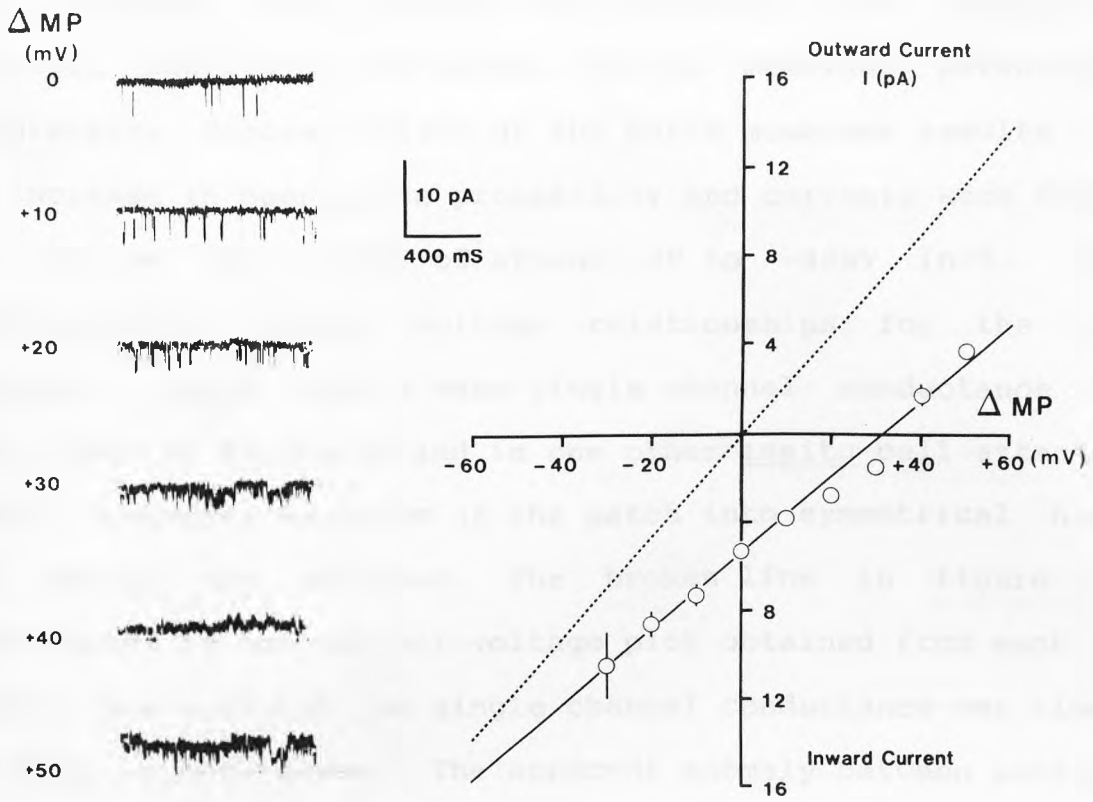


Fig.49. Single channel currents and the corresponding current-voltage relationship for a cell-attatched patch. The recording pipette contained high-KCl; low-Ca^{++} (1mM EGTA and no-added Ca^{++}) and the cells were bathed in normal extracellular saline. The transmembrane potential across the patch was varied from the normal resting potential of the cell i.e. Δ MP=0mV. To the right can be seen the corresponding current-voltage relationship (open circles). Also depicted is the same relationship for the channel obtained from the patch when excised from the cell and under symmetrical high-KCl gradients (broken line).

The frequency and duration of opening of the insitu K^+ channels was low at the normal resting membrane potential. Progressive depolarisation of the patch membrane results in an increase in open-state probability and currents were found to reverse for a ΔMP of around -30 to -40mV (n=5). The corresponding current voltage relationships for the K^+ channels insitu gave a mean single channel conductance of 155+-10pS. In figure 49 and in one other insitu cell-attached patch recording excision of the patch into symmetrical high KCl saline was achieved. The broken line in figure 49 corresponds to the current-voltage plot obtained from such a patch. Upon excision the single channel conductance was close to 250pS in both cases. The apparent anomaly between excised patch and insitu conductance measurements for the maxi-K^+ channel will be discussed later.

The effects of Ba^{++} blockade was tested on two excised, outside-out patches, Fig.50. shows one of these recordings. Application of Ba^{++} to the extracellular surface of the patch resulted in blockade of the large conductance K^+ channels present within the patch.

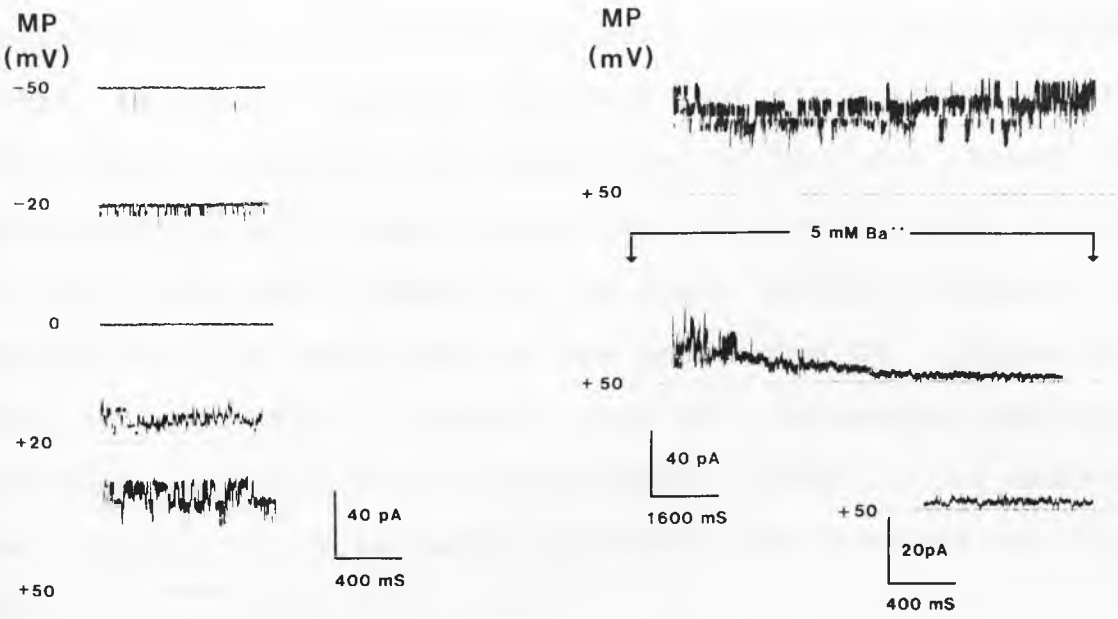


Fig.50. Continous current recording of an excised outside-out patch. The patch was exposed to symmetrical high-KCl HEPES-buffered saline containing no added Ca^{++} and 1mM EGTA present in both bath and recording pipette. To the **left** can be seen currents at various membrane potentials. In this patch five channels were nearly fully active at a membrane potential of +50mV MP. To the **right**, the same patch held at +50mV MP but exposed to a high-KCl bathing solution containing 5mM Ba^{++}. The inset (bottom **right**) shows that in the presence of 5mM Ba^{++} a smaller residual Ba^{++} resistant conductance persists.

B) Human Sweat Gland.

The primary role of the eccrine sweat gland is fluid and electrolyte secretion within the coil region and reabsorption within the ductal region. Evidence that fluid secretion in this gland occurs by mechanisms similar to those found in other exocrine cell types such as salivary or lacrimal is not so well advanced. Secretion in sweat glands induced by methacholine or phenylephrine has been shown Ca^{++} dependent (Sato et. al.,1981). Recently this Ca^{++} dependancy has been confirmed in single cells (Sato & Sato, 1988). It is assumed that similar fluid secretory processes are involved in this gland.

Investigations were carried out using primary cultured human sweat gland cells isolated from secretory coil, ductal and whole gland regions (see methods). The main aim was to characterise conductances from apical and basolateral membranes separately. When the cell growth reached confluence an apical polarity could be ascribed to the upwards facing surface of the cells (Lee et al., 1986). However they were extremely flat (<2uM) and a rounding up protocol (see methods) was needed to fascil itate patch-clamping; thus destroying cellular polarity. Initial experiments were carried out with the recording pipette containing high KCl, low-Ca^{++} (1mM EGTA) to look for possible K^{+} conductances. Preliminary results using the cell-attatched patch recording configuration are shown below in Fig. 51:

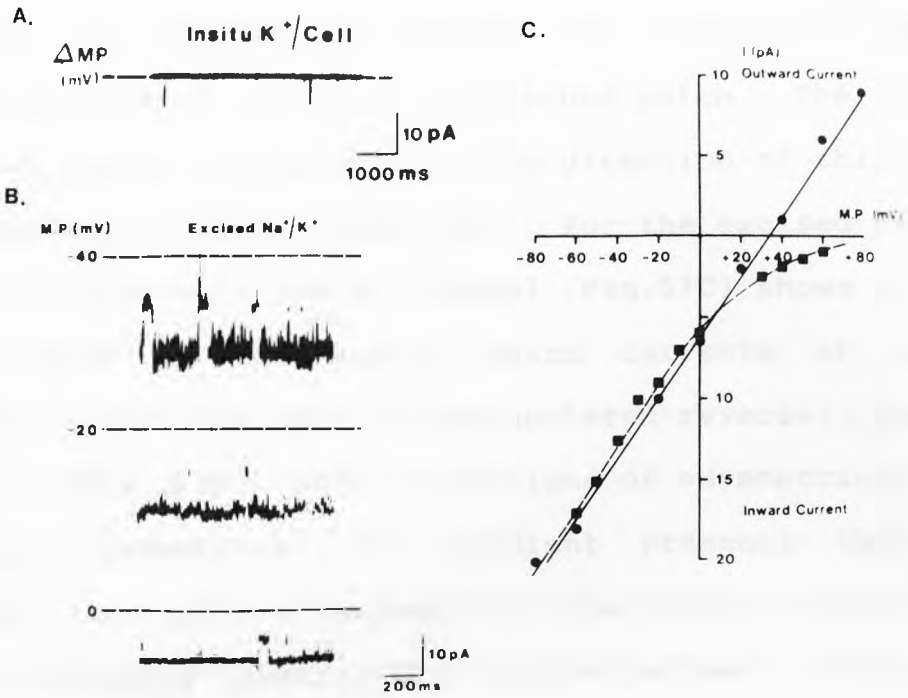


Fig. 51. Single channel currents recorded from an insitu cell-attached patch of a human sweat gland cell grown in coil (secretory) selective media. The recording pipette contained high KCl; low-Ca^{++} (1mM EGTA) saline. The cell (part of a cluster) was bathed in normal high NaCl; 1.2mM-Ca^{++} saline. A) Inward currents were seen at zero change in membrane potential (Δ MP=0 mV) for the cell-attached patch. B) Patch excision into the bathing solution resulted in massive activation of single channel currents (1.2mM-Ca^{++} present). C) The corresponding current-voltage relationship for both conditions.

The large conductance channel had a very low frequency and duration of opening throughout the range of membrane potentials imposed in the cell-attached patch. The reversal potential insitu estimated from the direction of this channel was found to be -30 to -40mV ΔMP. For the excised patch the i-v relationship of the K^+ channel (Fig.51C) shows a clear rectification of the solely inward currents at positive membrane potentials with an extrapolated reversal potential close to +80mv ΔMP. Under conditions of asymmetrical K^+/Na^+ gradients (symmetrical Cl^- gradient present) the channel therefore was highly K^+ selective. The linear single channel i-v relationship seen in the cell-attatched configuration with high-K^+ pipette gave a conductance of 250pS.

6iii) THE CA++ ACTIVATED NON-SELECTIVE CATION CHANNEL FOUND IN CELLS.

A) The Non-Selective Channel found in Salivary and Lacrimal Acinar Cells.

Salivary and lacrimal acinar cells were found to possess another type of cation conducting channel. When using NaCl HEPES-buffered salines within the recording pipette and bath and recording from excised inside-out patches exposed to salines containing very high ($<10^{-6}$M) levels of ionised Ca^{++}, the non-selective channel was seen. Symmetrical high NaCl gradients were best suited to investigate this channel in the excised patch recording configuration as the openings of the large conductance (250pS) K^+ channel were removed. Single-channel currents recorded in this situation are shown in Fig.52. The non-selective channel 52.B has a conductance of 30pS (average conductance=24 4, n=5). The channel was Ca^{++}-sensitive since lowering the free-Ca^{++} concentration bathing the intracellular face of the patch to below 10^{-6}M inhibited channel activity (n=5) whilst increasing Ca^{++} from 10^{-6} to 10^{-4}M increased channel activity (n=2). Channel run down was apparent in the more prolonged recordings (over 5min). The channel was found to be equally selective towards K^+ or Na^+ as seen under asymmetrical Na^+/K^+ gradients at very high (10^{-3}M) free-Ca^{++} bathing the cytosolic face of the patch (n=2). The channel did not conduct Cl^- ions (n=2) and was unable to conduct the large impermeant cation NMDG (n=2) which is normally considered to be membrane impermeant.

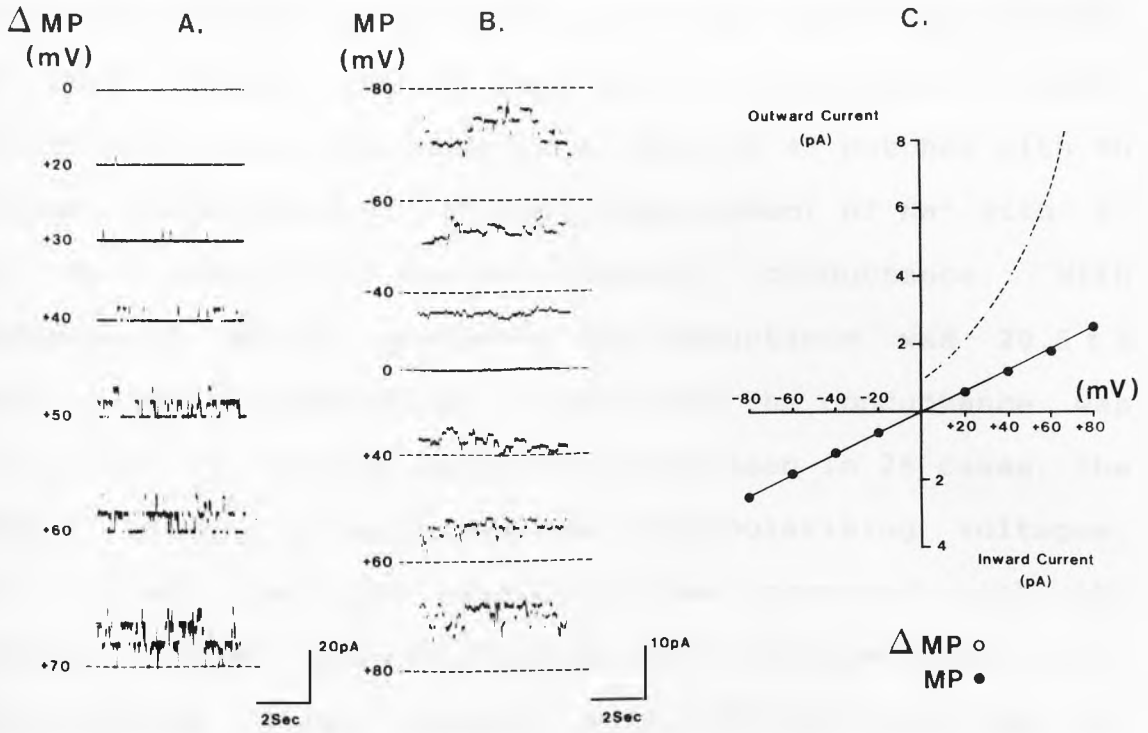


Fig.52. Single channel currents recorded from a salivary acinar cell. The recording pipette contained high-NaCl, no added-Ca^{++} and 1mM EGTA. The bath contained an identical extracellular saline with 2.5mM Ca^{++}. A) In the cell-attached patch outwardly directed K^+ currents were seen, B) upon excision into the bathing media the K^+ selective channels were not observed but a smaller conductive pathway reversing at 0mV MP with slower channel kinetics was seen. C) The accompanying current-voltage relationship for both the K^+ channel seen within the insitu cell-attached patch and the non-selective cation channel found in the same but excised patch. The broken line represents the rectified K^+ current seen insitu (ΔMP across the patch).

B) The Non-Selective Cation Channel of Human Sweat Gland.

Using the rounded up cells obtained from human sweat glands, the most frequent channel seen was a non-selective cation channel.The channel was seen in a total of 47 patches with an average conductance of 21 ± 2ps. Replacement of Na^+ with K^+ had no effect on single channel conductance. With asymmetrical Na^+/K^+ gradients the conductance was 20.5 ± 3 (n=21) and in symmetrical K^+ gradients the conductance was 20.3 ± 2 (n=11). Voltage sensitivity was seen in 26 cases, the channel closing at patch membrane hyperpolarising voltages. In 10 cases clear Ca^{++} sensitivity was observed with the channel closing below 10^{-6}M free bath (intracellular) Ca^{++} concentration. These channels also exhibited run down in excised patches. This phenomenon could in part be reduced by using the permeabilized cell preparation (see methods). In all 9 experiments where Cl^- was replaced on either membrane surface of an excised patch with SO_4^{2-} or gluconate no rectifying effect on current amplitude was observed; the channel is clearly cation specific.

The cation selectivity of this channel was demonstrated by measuring current amplitude whilst varying the NaCl concentration of the bathing media for an excised inside-out patch whilst retaining a fixed NaCl concentration in the recording pipette. The current-voltage relationship produced is shown in Fig.53. The single channel currents reversed at zero mV MP under symmetrical ionic gradients and showed a clear voltage sensitivity. Membrane depolarisation increased the frequency and duration of channel opening whilst membrane hyperpolarsation progressively inhibited the channels within the patch. The channel had very similar

kinetics to that described in saliary acinar cells (Fig.52). A conductance of 20 pS was observed under normal 140mM NaCl gradients as seen in Fig.53. Increasing bath NaCl to 280mM gave a -17mV shift in the zero current reversal potential to negative membrane potentials whilst a reduction of bath NaCl to 70mM gave a +17mV shift towards positive values of current reversal potential. Assuming a Nernst distribution for the ions across the membrane we have for each ion:

If Cl^- selective		If Na^+ selective
$E = 58 \log \frac{[Cl^-]_i}{[Cl^-]_o}$	or	$E = 58 \log \frac{[Na^+]_o}{[Na^+]_i}$
With 280mM NaCl in the bath		
E= -17.4mV	or	E=+17.4mV
With 70mM NaCl in the bath		
E= +17.4mV	or	E=-17.4mV

From the calculated shift in reversal potentials it was clear that only a shift in $[Na^+]$ can explain the changes in current amplitude; the channel was therefore highly cation selective.

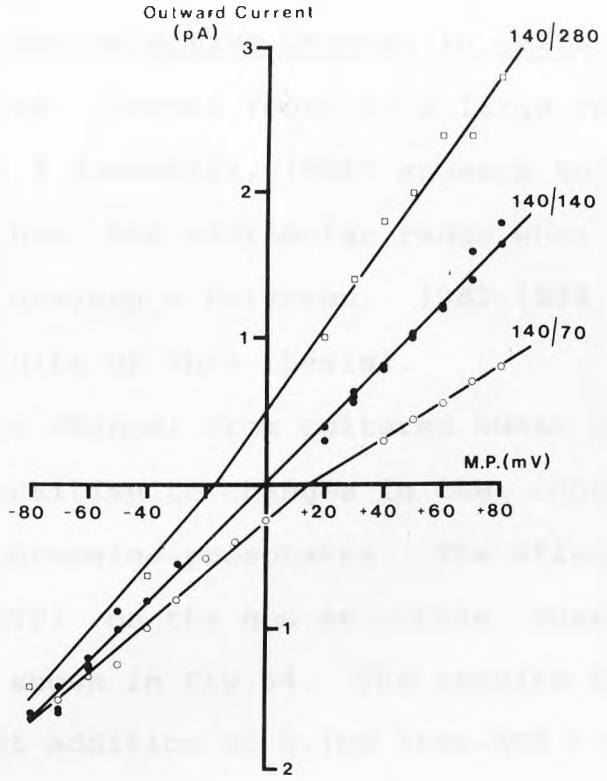


Fig.53. The current-voltage relationship recorded from a patch of membrane excised from a human sweat gland cell obtained from primary culture of the whole-gland (grown in coil selective media). High NaCl containing saline was present on both sides of the membrane; the pipette contained no added-Ca^{++} with 1mM EGTA and the bath 1.2mM-Ca^{++}. The NaCl concentration in the bathing media was adjusted from 140mM to 280mM and 70mM. Osmotic balance was provided by sucrose addition when required to either side of the membrane.

C) A Possible Physiological Control Mechanism for the Activity of the Non-selective Channel in cells.

The non-selective channel found in a large variety of cell types (Partridge & Swandulla, 1988) appears to possess a Ca^{++} sensitivity within the micromolar range when tested in the excised patch (Marayama & Petersen, 1982,1984; Marty et al., 1984 and the results of this thesis).

The non-selective channel from cultured human sweat gland was found to be sensitive to changes in the concentrations of intracellular adenosine phosphates. The effect of adenosine triphosphate (ATP) on the non-selective channels seen in these cells is shown in Fig.54. The results seen in Fig.54 demonstrate that addition of 0.1mM free-ATP caused a partial inhibition of the channel activity; 0.5mM free-ATP caused a more pronounced inhibition whilst 1.0mM free-ATP completely inhibited channel activity. ATP did not have any effect on channel conductance. These effects were freely reversible and seen in four cases. The non-selective channel of rat insulinoma cell line has been reported as being similary ATP sensitive (Sturgess et al., 1986).

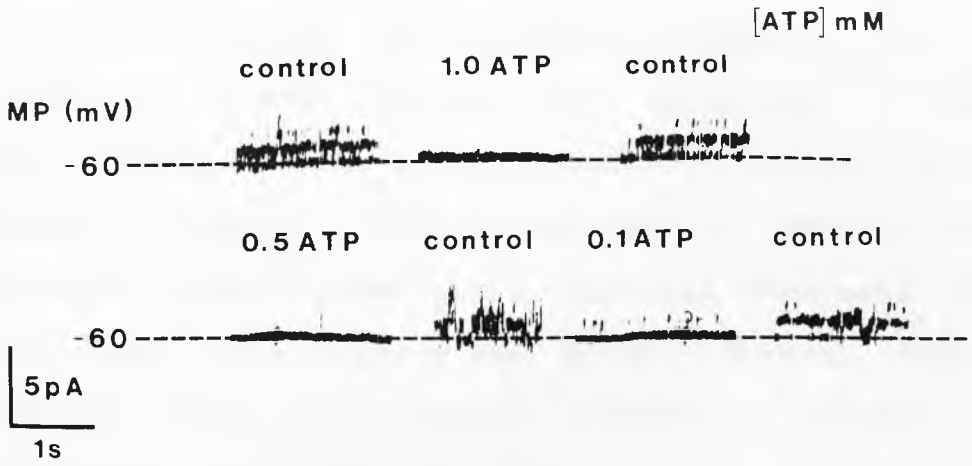


Fig.54. Sections of a continuous current recording taken from a saponin permeabilized (see methods) sweat gland cell grown from primary cultured whole gland (incoil selective media). The recording pipette and bathing media/permeabilized cell contained high-NaCl saline. The bath contained 1.2mM-Ca^{++} and pipette 1.0mM EGTA. Adenosine triphosphate (ATP) was added to the bathing media as indicated at three ionised concentrations (0.1mM, 0.5mM and 1.0mM), determined by an iterative computor analysis proceedure (see Methods Chapter). The effects of ATP were freely reversible at all the concentrations tested.

THE BASIC CHARACTERISTICS OF THE MAXI K^+ CHANNEL.

The data presented in this thesis on excised and cell-attached patches of basolateral membrane from mouse submandibular gland acinar cells provides a detailed characterisation of the large conductance voltage and Ca^{++} activated K^+ channel. The voltage and Ca^{++} sensitivity was investigated under symmetrical high-KCl gradients in the excised patch by measuring the single channel open-state probability. This was markedly affected by changes in the transmembrane potential and similarly by changes in the ionised Ca^{++} concentration bathing the internal surface of the patch membrane. Activation of the channel could be achieved by depolarising membrane potentials or by increasing the internal patch membrane Ca^{++} concentration over a very narrow range between 10^{-8}M and 10^{-7}M. At 10^{-7}M activation of the channel was such that it was predominantly open at all voltages within the range of -70 to +40mV MP. Under symmetrical high-KCl gradients in the excised patch the channel attained a mean single channel conductance close to 250pS. Imposing more physiological gradients across the patch by bathing the intracellular aspect with high-NaCl saline produced a pronounced rectification of single channel conductance. The magnitude of the extrapolated reversal potential for the K^+ channel which approached E_K under these conditions indicated a strong K^+ selectivity for the channel over Na^+ ions. The K^+ channels pharmacological specificity towards Ba^{++} ions and TEA was also investigated using this patch conformation. Ba^{++} was found to inhibit the large

conductance K^+ channel at millimolar concentrations in a manner similar to that reported in other exocrine cells (Iwatsuki & Petersen, 1985). The affect of Ba^{++} on the channel would account for the inhibition of cholinergically evoked fluid secretion in perfused salivary glands reported by Evans and Young (1984). Ba^{++} also inhibited the outwardly rectified whole-cell currents found in both salivary and lacrimal acinar cells. These currents have been demonstrated by Trautmann and Marty (1984) using the ensemble noise analysis technique to be due to the opening of the same large conductance, voltage and Ca^{++} activated K^+ channel. The low Km value for external membrane TEA blockade of the channel was consistent with inhibition of other large unitary conductance K^+ channels found in a variety of cell types (Lattore & Miller, 1983; Ritche, 1985).

The characteristics of the K^+ channel within the excised patch was compared with those obtained from the cell-attached patch. Cells chosen for analysis were part of large acinar clusters and therefore electrically coupled to the rest of the acinus (Roberts et al., 1978). Initially KCl rich physiological saline was used within the pipette whilst maintaining the cells in NaCl rich extracellular saline. The K^+ channel recorded within the electrically isolated cell-membrane patch attained a mean conductance of 264 ± 11pS, a value very similar to that of the K^+ channel in the excised patch under symmetrical 140mM KCl gradients. This confirms earlier K^+-selective microelectrode studies (Poulsen & Oakley, 1978) which indicate that the intracellular K^+ concentration in these cells is close to 145mM. The rectification seen for single channel conductance in the

excised patch under asymmetrical Na^+/K^+ gradients was mirrored when similar gradients were imposed across the cell-attached membrane patch by replacing K^+ for Na^+ as the major cation in the recording pipette. This rectification was successfully modelled using the constant field equation for a highly K^+ selective membrane. By applying the constant field theorem a permeability coefficient for the channel was found. The calculated K^+ permeability of the channel remained constant around $4x10^{-13}cm^3s^{-1}$ under all the combinations of Na^+ and K^+ gradients tested in both excised and cell-attached patches. The conductance of the channel displayed identical characteristics in both the excised and the cell-attached patch.

Retaining high-KCl saline within the recording pipette allowed for the measurement of the cells resting membrane potential. This was calculated from the amplitude of the single channel currents at the spontaneous membrane potential and by the reversal potential for the single channel currents within the patch. The mean reversal potential for normally bathed submandibular acinar cells was -43 ± 1.8mV, this value correlating well with results obtained from microelectrode studies (Nishiyama & Petersen, 1974; Wakui & Nishiyama, 1980). Replacement of the extracellular Na^+ with K^+ depolarised the cell. The channels within the cell attached patch behaved identically to those under symmetrical high-KCl gradients in the excised patch. The loss of a membrane potential was reflected by a leftward shift in both the current-voltage relationship (the channels reversed at 0mV MP) and in the single channel open-state probability curve. As cellular depolarisation had no effect on the activity of

the channels within the patch apart from shifting the o-p curve by a magnitude equal to the resting membrane potential, clear evidence exists for the absence of a voltage dependant Ca^{++} influx pathway in these cells.

The voltage sensitivity of the channel within the cell-attached patch was used to provide an estimate of the resting Ca^{++} concentration within submandibular acinar cells. This was possible because the channel displayed similar voltage characteristics, becoming more active at positive changes in patch membrane potentials and less active at negative changes in patch membrane potentials. By comparing the open-state probability curves for both insitu cell-attached and in the excised patch under symmetrical high-KCl gradients and accounting for the resting membrane potential, the resting intracellular Ca^{++} concentration within these cells approximated to 10^{-8}M. A similar rational has been used in pig pancreatic acinar cells where the intracellular Ca^{++} was found to be between 10^{-8} and 10^{-7}M (Marayama et al., 1983b) and in lacrimal acinar cells to be no more than 10^{-8}M (Marty et al., 1984).

The voltage dependant effects of the resting K^+ channel activity within the cell-attached patch was more easily demonstrated when an estimate of membrane resistance was made. The low values for resting open-state probability measured at the spontaneous membrane potential (Fig.17) appeared to indicate that small changes in membrane potential did not appreciably affect changes in K^+ current. However this interpretation ismisleading in this respect as it is the magnitude of change in o-p which has most effect on increasing membrane K^+ permeability. The greatest changes take place at low

levels of o-p around the resting membrane potential indicating a clear role for the voltage sensitivity of the K^+ channel under resting conditions in regulating the membrane potential.

The large conductance K^+ channel found in other exocrine acinar cells possessed similar characteristics. Both eccrine sweat gland and rodent gut 250pS K^+ channels were voltage and Ca^{++} sensitive. The measurement of single channel conductance in cells isolated from rodent gut was underestimated in the cell-attached mode. This phenomenon was manifest as an unstable reversal potential for the K^+ currents and is seen When cells exhibit a spontaneous membrane potential which does not remain constant during changes in patch potential (Fishmeister et al., 1986). This effect is limited to small cells similar to those obtained from the rodent gut. The ubiquitous nature of the large conductance K^+ channel (Lattore & Miller, 1983) is an important aspect when developing a model for fluid and electrolyte secretion in exocrine acinar cells.

EFFECTS OF NEUROTRANSMITTER ON K^+ CHANNEL ACTIVITY WITHIN CELL-ATTACHED PATCHES.

Parasympathetic nerve stimulation results in copious salivary secretion. The studies outlined in the introductory chapter have shown this secretagogue to act via stimulus-permeability coupled changes towards both K^+ and Cl^- ions. The insitu cell-attached mode was employed to investigate whether the parasympathetic agonist acetylcholine could have an effect on the K^+ channels within basolateral membrane patches. Addition of ACh to Na+-bathed submandibular acinar cells resulted in a

dramatic increase in K^+ channel activity within the patch. This effect was receptor regulated as the cholinergic antagonist atropine inhibited the response. A second messenger was postulated since ACh had no direct access to the membrane within the recording pipette. Evidence for the primary role of Ca^{++} in this response is well documented (see Introduction). An extracellular Ca^{++} dependency for the agonist response was demonstrated. Bathing the cells in Ca^{++} depleted saline and challenging with ACh did not evoke an increase in K^+ channel activity within the patch. However subsequent addition of Ca^{++} to the bath restored the activated currents in 70% of cases. In cells bathed either in Ca^{++} free or Ca^{++} containing saline a transient increase in current amplitude indicating a cellular hyperpolarisation was seen during the initial stages of agonist stimulation. This was likewise inhibited by atropine. The cells which were unresponsive to agonist when bathed in Ca^{++}-free or Ca^{++}-containing conditions also hyperpolarised. The K^+ channels apparent inability to be activated under these conditions may reflect diffusion barriers to Ca^{++} between the membrane trapped within the pipette and the rest of the cell. Changes in cytosolic Ca^{++} concentration during cholinergic agonist stimulation were followed using the intracellular Ca^{++} indicator quin-2. ACh caused an increase in cytosolic Ca^{++} to levels around $3x10^{-7}M$, sufficient to fully activate the channels within the patch. Similar Ca^{++} rises have been documented in parotid acinar cells (Takemura, 1985). The low resting levels of Ca^{++} as measured with this dye (below $10^{-7}M$) was also consistent with a regulatory role for Ca^{++} in this response. In addition the quin-2 studies confirmed the

extracellular Ca^{++} dependency for ACh stimulation seen in the cell-attached patch and demonstrated the intracellular Ca^{++} rise to be dependant upon both intracellular Ca^{++} release and extracellular Ca^{++} influx. These findings corrobotate the two phase Ca^{++} mobilisation model proposed by Putney (1977) based on radioisotope flux measurements. The main effect of agonist stimulation in these cells is to maintain Ca^{++} influx, a point which is discussed later in the context of the control of cellular Ca^{++} levels by inositol polyphosphates.

The cell-attached patch recording configuration when used to monitor the effects of acetylcholine application to the cell clearly implies a role for $[Ca^{++}]_i$ in the control of the basolateral K^+ channel. A similar effect of ACh on the large conductance K^+ channels activity in cell-attached patches of rodent small intestine indicates that these cells also obey a similar model for fluid secretion. Although the effects of ACh were not investigated in cultured eccrine sweat gland cells, this gland is predominately cholinergic (Sato & Sato, 1981) and sweat secretion <u>invitro</u> is dependent upon extracellular Ca^{++} and activated by the Ca^{++} ionophore A23187 (Sato, 1981). Most recently it has been shown that secretion is accompanied by a rise in intracellular Ca^{++} using quin-2 (Sato & Sato, 1988). The similarities here with the intracellular Ca^{++} measurements carried out in submandibular cells likewise indicate a common model for fluid and electrolyte secretion in exocrine acinar cells.

CHANGES IN THE RESTING MEMBRANE POTENTIAL OF ACINAR CELLS DURING SECRETION.

The spontaneous membrane potential recorded with the insitu cell-attached patch falls between the equilibrium potentials for K^+ and Na^+ or Cl^- ions (Lundberg, 1958). Evidence reviewed in the introductory chapter advanced the concept that changes in the membrane conductance underlie the fluid secretory process. The presence of other ions apart from K^+ in the generation of an agonist response in submandibular acinar cells was investigated.

Removal of extracellular Na^+ and replacement with another cation such as N-methyl,D-glucamine has little effect on the membrane potential, indicating a poor contribution made by a Na+ permeability pathway to the resting membrane potential. In contrast removal of extracellular Cl^- and replacement with SO_4^{2-} results in a pronounced hyperpolarisation of the membrane potential towards E_K when monitored with conventional microelectrodes (Nishiyama & Petersen, 1974). This hyperpolarisation was further characterised in a later paper by Roberts, Iwatsuki & Petersen (1978) in mouse parotid which demonstrated a recovery in the membrane potential within 10min following Cl^- removal. Petersen in the same paper with these workers and with Iwatsuki in lacrimal acinar cells (1978) showed that agonist stimulation could evoke multiphasic changes in membrane potential. At an appropriate dose of agonist a hyperpolarisation in the membrane potential followed by a depolarisation was obtained; this latter response was inhibited by removal of extracellular Cl^-. As discussed earlier agonist activation of single channel currents within the cell-attached patch was accompanied by a

transient increase in current amplitude which returned to prestimulus levels (within 1min) during the sustained phase of single channel current activation. The increase in current amplitude indicates a cellular hyperpolarisation over the rest of the cell membrane which is not voltage clamped by the pipette. As this hyperpolarisation does not reach and remain at E_K this is indicative of either a Cl^- or a Na^+ permeability pathway being likewise stimulated. A candidate channel is the Ca^{++} activated non-selective cation channel which has been identified both in salivary and lacrimal acinar patches and also in sweat gland cells and a large variety of other cells (Partridge & Swandulla, 1988). However evidence suggests that it does not play an important role in salivary or lacrimal acinar cell secretion. Marty and colleagues (1984) using NaCl perfused lacrimal whole-cells and manipulated Cl^- gradients demonstrated that during cholinergic stimulation, the resultant inward current was carried largely by Cl- ions with only a very minor contribution made by the non-selective channel.

The presence of a Cl^- conductive pathway was looked for by direct measurement of the total resting and stimulated currents across the plasma membrane of whole-cells. In the resting condition the whole-cell current was dominated by the outwardly directed K^+ current. ACh stimulation was associated with an increase in both outward and inward currents across both salivary and lacrimal cell membranes. These were similar to those observed in salivary (Iwatsuki, Marayama & Nishiyama, 1985) and lacrimal (Trautmann & Marty, 1984; Marty et al., 1984; Findlay, 1984) acinar whole-cells. An activated Cl^- pathway was demonstrated by bathing and dialysing

submandibular whole-cells with Cl^--free salines. Under these conditions the agonist evoked inward current was lost. The reversal potential for agonist stimulation was found to be more negative than the cells reversal potential under resting or stimulated conditions in the presence of Cl^-, approaching E_K. This provides further evidence for a functional Cl^- pathway existing in these cells. The abolition of an inward current component during Cl^--free conditions has been described by Findlay & Petersen (1985) although no estimate of E_{ACh} could be made. The effects of ACh on stimulating a Cl^- transport pathway in lacrimal gland have been confirmed by Cl^--selective microelectrode studies (**Saito** et al., 1987a).

Fluctuations in recorded current were demonstrated in salivary acinar cells. Cells bathed in NaCl rich extracellular saline and dialysed with KCl rich extracellular saline underwent oscillatory changes in current. At potentials similar to or below the cells resting membrane potential the inwardly directed Cl^- current was found to dominate. The E_{ACh} under symmetrical Cl^- gradients was more positive than the spontaneous potential. This would indicate that in the absence of voltage clamp the cell would be seen to undergo an overall depolarisation. Dialysing the acinar cell (in this case lacrimal) with a more physiological Cl^- concentration of 60mM (Marty et al., 1984) gave currents at the reversal potential of the cell which were dominated by outwardly directed K^+ movement. The value for E_{ACh} was now more negative than the cells resting or zero current level. These results indicate an important aspect when describing a role for both K^+ and Cl^- conductive pathways in salivary or

lacrimal cells. The dominance of either ion at a specified membrane potential will depend upon the magnitude of the clamped potential and the ionic gradients present.

In describing a role for both K^+ and Cl^- permeability pathways within these cells, control of these channels by $[Ca^{++}]_i$ has been directly demonstrated using the whole-cell perfusion technique. By manipulating the Ca^{++} concentration of the dialysate a similar activation to that obtained with agonist in the presence of extracellular Ca^{++} was achieved. Interestingly the rise in inward Cl^- current always occurred later than the rise in K^+ current. Marty and colleagues (1984) have demonstrated small 1-2ps Cl^- channels activated in cell-attached patches by the Ca^{++} ionophore A23187 which respond to micromolar concentrations of intracellular Ca^{++}. The finding of a differential Ca^{++} sensitivity between K^+ and Cl^- permeability pathways is capable of adequately describing multiphasic potential changes when measured with conventional microelectrodes. The initial hyperpolarisation is due to activation and dominance of the outwardly directed K^+ current, the subsequent depolarisation being due to the slower activation of the less Ca^{++} sensitive Cl^- current.

Thus there is good evidence that salivary and lacrimal acinar cells may regulate their membrane potential and hence fluid and electrolyte secretion through the Ca^{++} sensitivity of both K^+ and Cl^- channels. This general observation may hold true for exocrine acinar cells containing both of these channels. However other mechanisms are needed where the cell contains a poorly developed K^+ permeability pathway. Petersen and Marayama (1984) have shown that in rodent pancreas the agonist cholecystokinin (CCK) activates the non-selective

channel. This channel is permeable to both Na^+ and K^+ ions but the influx of Na^+ would predominate due to the larger potential difference between E_{Na} and E_K and the cells membrane potential. This additional influx could then be fascillitative to the transcellular movement of Cl^- by recycling through the Na^+-K^+-Cl^- co-transporter and Na^+/K^+ pump (see model for secretion, last section of this chapter). It is difficult to reconcile the apparent lack of Ca^{++} sensitivity of this channel in the excised patch with the activation of this channel by CCK described earlier as the cellular Ca^{++} concentration would have to be maintained at quite unphysiological levels. Another permeability controlling factor appears to be needed. The adenosine triphosphate (ATP) sensitivity of a similar non-selective channel found in sweat gland cells may provide a vital clue for the understanding of the control of the non-selective channel in all cell types. Evidence published (Dunne et al., 1986; Petersen & Findlay, 1987) has found a reciprocal relationship between cellular ADP and ATP concentrations in the control of another type of ATP sensitive channel. A similar relationship may play an important role in regulating the non-selective channel in cells with underexpressed large conductance voltage and Ca^{++} sensitive K^+ channels.

Finally as the Cl^- channel is less Ca^{++} sensitive than the K^+ channel it would be interesting to postulate if a similar controlling factor could be found here. No evidence exists for the direct effect of adenosine nucleotides. Recently it has been demonstrated in normal cultured airay epithelial cells (Frizzell et al., 1987; Li et al., 1987) that the Cl^- channel present in these tissues can be opened by the

catalytic C subunit of Protein Kinase A involving the dephosphorylation of ATP. This channel is of a different size to that described by Marty and colleagues (1984) and has a poorly understood Ca^{++} sensitivity. This demonstrates that exocrine cells may utilise other forms of control for Cl^- channels in addition to or apart from Ca^{++}.

THE THEORY OF SECONDARY MESSENGER OPERATED CHANNELS (SMOC'S).

The whole-cell studies using the voltage and Ca^{++} sensitive outward K^+ current as an indicator of intracellular Ca^{++} confirmed that stimulation of cholinergic muscarinic receptors evokes intracellular Ca^{++} release (independent of external Ca^{++}) and Ca^{++} influx (dependent upon extracellular Ca^{++}). Electrophysiological data exists in excitable cell systems which clearly demonstrates that the neurotransmitter evoked Ca^{++} influx into these cells is carried by a voltage dependent Ca^{++} channel which is activated by membrane depolarisation (Reuter, 1983, 1985). It as been demonstrated that Ca^{++} influx into salivary acinar cells does not occur through this type of channel (eg. K^+ depolarisation of salivary acinar cells does not result in raised intracellular Ca^{++}).

The activation of channels within cells can be divided into direct and indirect effects of receptor activation based on electrophysiological evidence. Channels activated by the binding of a hormone or neurotransmitter to a specific receptor which then increases membrane Ca^{++} permeability are often referred to as receptor operated channels (ROC's Bolton, 1979; Berridge, 1982; Reuter, 1983). It has been proposed (Meldolesi, 1987) that this group should be further

divided into two subsets: a) those where the channel function and receptor are of the same molecule or intimately linked so that both functions coexist together (true ROC's), and b) those probably activated by secondary messengers generated inside the cells as a consequence of receptor occupation (second messenger operated channels or SMOC's). The classical example of a ROC is that of the nicotinic acetylcholine receptor (Sakmann et al., 1985; Boulter et al., 1986), another the brain glutamate receptor or otherwise known as the NMDA-receptor (MacDermott et al., 1986; Jahr & Stevens, 1987; Cull-Candy & Usowicz, 1987). Both have been investigated by electrophysiological techniques and are permeable not only to monovalent cations but also Ca^{++}. The Ca^{++} permeability is not the primary physiological function of these channels and little direct evidence exists to suggest that they may regulate Ca^{++} influx in cells. There appears, at least for the nicotinic receptor where the most characterisation has taken place, a large molecular heterogeneity of the channel in different cells (Meldolesi & Pozzan, 1987). Another ROC described recently is the ATP activated Ca^{++} and Na^{+} permeable channel of smooth muscle (Benham & Tsein, 1987). This is directly activated by the ligand ATP without the involvement of a diffusible second messenger. The channel appears 3:1 selective for Na^{+} over Ca^{++} and shares with the other two ROC's described above a resistance to inhibition by the classical voltage dependant Ca^{++} channel inhibitors cadmium and nifedipine.

The second group of voltage independent channels the SMOC's are as yet poorly understood in their function and are grouped together as uncharacterised Ca^{++} entry into many cell

types. They are thought to be part of an indirect mechanism for increasing the cell membrane permeability towards Ca^{++} and most of the characterisation of these putative channels until recently has been by non-electrophysiological means. In the context of salivary acinar cell function indirect evidence for these channels has been outlined in the introductory chapter and by the Ca^{++} influx reported in this thesis.

There has been within the last two years preliminary descriptions of SMOC's using the patch-clamp technique. Reuter and colleagues (1986) observed a rise in intracellular Ca^{++} following peptide induced activation of neutrophils. This occurred simultaneously with the opening of two different non-selective conductances that appeared permeable to Ca^{++} as well as other cations. These channels were thought to open as a consequence of the elevated $[Ca^{++}]_i$ and were insensitive to inositol(1,4,5)triphosphate (IP_3). Recently Nasmith and Grinstein (1987) have reinvestigated this phenomenon using the intracellular dye indo-2 and the Ca^{++} chelator BAPTA to disprove any direct effect of $[Ca^{++}]_i$ on Ca^{++} influx. They have suggested that the Ca^{++} mobilisation is modulated either by direct receptor activation of the channel by the peptide (thought not to occur in the previous study) or by an as yet unidentified secondary messenger.

Another potential SMOC has been observed in lymphocytes by Kuno & Gardner (1986, 1987). In their earlier paper these workers reported a small Ca^{++}-permeable channel activated after the cells were stimulated with a mitogen. This channel could be inhibited by a rise in intracellular Ca^{++}, negating

control by this messenger. More recently (1987) they have proposed that this channel is controlled by the secondary messenger IP_3. They report to have seen activation of single channel currents within excised patches on addition of IP_3. No one has reported or confirmed these findings in cells and as such this is the only evidence for a direct Ca^{++} influx pathway controlled solely by this secondary messenger.

Apart from Ca^{++} channels per se other regulatory processes for Ca^{++} homoeostasis have historically been studied in detail. The possible involvement of either Na^{+}/Ca^{++} exchange or active Ca^{++} transport in controling Ca^{++} movement in cells is not discussed at great length (for review see Carafoli, 1987). It has been demonstrated in hepatocytes (Joseph & Williams, 1983) that plasma membrane Ca^{++} efflux during agonist stimulation is inhibited during the initial phases of agonist-receptor interaction. This is followed by a build up of $[Ca^{++}]_i$ and activation of the plasma membrane Ca^{++}ATPase and Na^{+}/Ca^{++} exchange mechanisms. Intracellular Ca^{++} however is not the only factor thought to regulate Ca^{++} efflux from cells. Protein Kinase C activation (Rickard & Sheterline, 1985; Rink & Sage, 1987) has also been reported to cause similar effects in neutrophils and platelets. Recently in erythrocytes Smallwood and colleagues (1988) have demonstrated independent Ca^{++} and PKC activation of the plasma membrane Ca^{++} pump indicating that cells may regulate agonist evoked Ca^{++} efflux by a number of mechanisms.

THE INOSITOL LIPIDS AND THEIR ROLE IN THE REGULATION OF CELLULAR Ca^{++}.

Historical Developement.

The biochemical aspects of phosphoinositide metabolism were pioneered by the work of Hokin and Hokin. They demonstrated enhanced incorporation of radiolabelled phosphate (^{32}Pi) into the total phospholipid fraction in slices of pigeon pancreas upon stimulation by cholinergic agonists (1953, 1954) and subsequently identified the individual phospholipids labelled as phosphatidyl,1-D-myo-inositol (PI) and phosphatidic acid (PA) (1955, 1958). Later this group showed incorporation of [^{3H}]inositol into PI in pancreas and brain fractions (1958) and by a series of kinetic studies looking at the synthesis and degradation of these two compounds in avian salt gland slices during the next ten years proposed a "phosphatidylinositol-phosphatidate cycle" (1967).

The model proposed that ACh stimulation resulted in the breakdown of PI to diacylglycerol (DAG) (catalysed by a PI phosphodiesterase Dawson, 1959; Kemp et al., 1961). The DAG was then phosphorylated to form PA (Hokin, 1959) and removal of the signal PA was acheved by its convertion back to PI by the sequential actions of cytosine triphosphate diacylglycerol (CTP-DG) cytidyl transferase (Argonoff, 1958) and PI synthetase (Paulus, 1960). This allows for the recycling of the tail region of the DAG moiety with only the head-group undergoing turn over, thus negating the need for denovo synthesis of PI. Also this requires only a small pool of inositolphospholipid pool for signalling. It has since been shown that biosynthesis of PI from PA via CTP-DG and

from myo-inositol occurs within the endoplasmic reticulum (ER) (Benjamins & Argonoff, 1969; Parri & Hokin-Neaverson, 1984) whereupon it is transported by a specific PI-exchange protein (Somerharju et al., 1983; George & Helman, 1985) to the plasma membrane. Its subsequent phosphorylation at this site to PIP and PIP_2 occurs via the action of two specific kinases which are predominantly localised with the plasma membrane (Abdel-Latif, 1986). Unlike PI which is mainly cytosolic (Hokin & Hokin, 1964; Michell & Hawthorn, 1965; Downes & Michell, 1982), the polyphosphoinositides PIP and PIP_2 are thought to be located within the cytoplasmic face of the plasma membrane (Downes & Michell, 1982; Abdel-Latif, 1983; Berridge, 1984). Thus they are ideally situated to play a role in signal transduction.

The correlation of receptors which mediate their cellular response through Ca^{++} with the stimulation of inositol lipid metabolism was made by Michell (1975). Support for Michells hypothesis came from observations made in blowfly salivary glands stimulated with 5-hydroxytryptamine (5HT) (Fain & Berridge, 1979a/b). A calculated dose of 5HT initially caused PI loss and Ca^{++} uptake, followed by receptor refractoriness and decline of the Ca^{++} uptake. However, incubation of the glands in an myo-inositol containing saline restored receptor sensitivity of both the PI turnover and Ca^{++} uptake. More recently in this tissue it has been shown that the recovery of the Ca^{++}-gating effect is dependent upon both the synthesis of PI and Phosphatidylinositol-4,5-bisphosphate (PIP_2) (Sadler, Litosh & Fain, 1984).

PIP_2 HYDROLYSIS AND THE PRODUCTION OF PHOSPHOINOSITIDES, CONTROLLING FACTORS IN CELL SIGNALLING.

Polyphosphoinositides were isolated in the brain as early as 1949 (Folch) and characterised in detail by Grado & Ballou (1961). Brockerhoff and Ballou (1962) subsequently demonstrated the rapid turnover of PI and PIP_2 in brain fractions and Santiago-Calvo and colleagues in avian salt gland (1964) found depressed steady-state levels of both phosphatidylinositol-4-phosphate (PIP) and (PIP_2) during agonist stimulation. In the late seventies Abdel-Latif demonstrated that cholinergic or muscarinic stimulation of iris smooth muscle was associated with a breakdown of PIP_2 (within 5min) and an increase in inositol-1-phosphate (IP), inositol-4,5-bisphosphate (IP_2) and inositol-1,4,5-triphosphate (IP_3) (1977 and 1980 respectively). But despite this the link between PIP_2 breakdown and Ca^{++} signalling was not made because these workers believed this effect required Ca^{++} and therefore could not be the cause of Ca^{++} mobilisation in these cells. This finding dampened research into the polyphosphoinositide field until PIP_2 hydrolysis by agonist stimulation was investigated in liver. Kirk (1982) together with colleagues (1983) and Seyfield & Wells (1984) demonstrated that PIP_2 breakdown was independent or partially independent upon Ca^{++}. This finding has since been confirmed in other tissues (Sekar & Hokin, 1986). In the parotid acinar gland the rapid breakdown of PIP_2 has been shown to be independent of Ca^{++} (Putney, 1982; Putney et al., 1982a & b). More recent evidence (Irvine & Dawson, 1984a; Kolesnick & Musacchio, 1984) demonstrates that resting levels of $[Ca^{++}]_i$ are not rate limiting for PIP_2 conversion to IP_3 and

diacylglycerol. In blowfly salivary gland Berridge and colleagues (1984) found an increase in cellular IP_3 levels prior to the onset of Ca^{++}-dependant events. Likewise the receptor activated conversion of PIP_2 to IP_3 proceeds (Drummond et al., 1985; Wollheim & Biden, 1986) or coincides with (Bevan et al., 1984; Thomas et al., 1984; Alexander et al., 1985; Ramsdell & Tashjian, 1986) a rise in internal Ca^{++} in a numerous variety of cell types. Hydrolysis of PIP_2 occurs via a membrane bound phosphodiesterase commonly called phospholipase C. This together with receptor and coupling G-protein forms the signal transduction unit described as the phosphoinositidase complex (Downes & Michell, 1985). Existing evidence for the involvement of a G-protein interaction in this complex will be discussed later.

THE ROLE OF IP_3 IN CELL-SIGNALLING.

The link between receptors involved in secondary messenger signal transduction (SMOC) and the hydrolysis of PIP_2 is now readily apparent. The first direct demonstration that the phosphoinositol breakdown product of PIP_2, namely IP_3 could itself effect internal Ca^{++} was made in "permeabilised" pancreatic acinar cells (Streb et al., 1983). These workers used Ca^{++} electrodes to monitor IP_3 evoked Ca^{++} release from endoplasmic reticulum (ER). The permeabilised cell preparation has been favoured because until recently it was the only way of introducing these very polarised molecules into cells. Similar results have now been obtained in a large variety of permeabilised cells and internal membrane fractions (Berridge, 1987). The unifying result from these studies is that Ca^{++} is released from a non-mitochondrial

Ca^{++} pool derived solely from the ER. Further, electron probe X-ray analysis of smooth muscle has revealed preferential Ca^{++} release from ER located at the cytoplasmic boundary of the plasma membrane (Bond et al, 1984; Williams et al, 1985) suggesting that the ER deeper within the cell contributes less to Ca^{++} release. This finding is supported by deep injection of IP_3 into oocyte cells (Busa et al., 1985) and in ER microsome studies (discussed later). The permeabilised cell studies have also shown that IP and IP_2, the other possible products of phospholipase C activation have no or little effect on mobilising intracellular Ca^{++}.

Intracellular injection of IP_3 has also been performed in a variety of tissue with the following effects: activation of a Ca^{++} sensitive Cl^- current in xenopus oocyte and Ca^{++} mobilisation (Nadler et al., 1985), stimulation of limulus photoreceptor (Brown et al., 1984; Fein et al., 1984) and Ca^{++} mobilisation (Brown & Rubin, 1984; Payne et al., 1986), hyperpolarisation in salamander rods (Waloga, 1985), sea urchin egg depolarisation and fertilisation (Slack et al., 1986; Whitaker et al., 1984; Turner et al., 1985) and increased K^+ current in smooth muscle (Klockner, 1985) and lacrimal gland (Evans & Marty, 1986).

All these IP_3 stimulated events in the different cell types have Ca^{++} as a secondary messenger. However very little direct evidence (with the exception of the report by Kuno & Gardner, 1987) exists to confirm a role for IP_3 in mediating Ca^{++} influx (Ueda et al., 1986; Delfert et al., 1986).

The nature of the IP_3 sensitive Ca^{++} store has been under investigation in recent years. Specific IP_3 receptor binding studies in adrenal cortex (Baukal et al., 1985), liver

microsomes (Spat et al., 1986a) and liver cells and neutrophils (Spat et al,. 1986b) demonstrate that the potency of binding reflects the specificity of the inositides in promoting Ca^{++} release i.e. $Ins(1,4,5)IP_3 > Ins(2,4,5)IP_3 > Ins(4,5)IP_2$. This suggests that the phosphates at the 4 and 5 position are essential for this role, the 1-phosphate enhancing receptor affinity. Spat also observed that the receptor density is less than the dissociation constant for the putative binding site, indicating a close coupling between IP_3 formation and Ca^{++} release. Further characterisation of IP_3 binding in brain cerebellar membrane homogenates has been provided by Worley and colleagues (1987a/b) who report enhanced (x250) binding of $Ins(1,4,5)IP_3$ over the recently discovered $Ins(1,3,4,5)IP_4$ (discussed in the following sections) or $Ins(1,4)IP_2$. Submicromolar Ca^{++} concentrations were found to inhibit binding and this is interpretated as a means by which IP_3 induced Ca^{++} release may physiologically regulate itself. Also modest shifts in cell pH were found to alter IP_3 binding with enhancement occurring at an alkaline pH and attenuation at an acidic pH. This correlates with the reported effects of pH on IP_3 evoked Ca^{++} release. An enhancement occurring with increased pH between values of 6.5 and 7.5 (Brass & Joseph, 1985; Clapper & Lee, 1985).

The ER pool sensitive to IP_3 is believed to be composed of other components necessary for its function. Removing ATP or adding vanadate results in the inhibition of Ca^{++} sequestration which demonstrates the presence of an ATP driven Ca^{++} pump. Addition of ATP to permeabilised cells can lead to a decrease in $[Ca^{++}]_i$ confirming this observation.

Addition of Ins(1,4,5)P_3 to ATP depleted or vanadate treated cells results in a enhanced release of Ca^{++} (Prentki et al., 1984), this effect is thought to occur not through inhibition of the Ca^{++}-pump but rather by stimulation of passive Ca^{++} efflux (Henne & Soling, 1986). As the IP_3 sensitive Ca^{++} release is not temperature dependent, a Ca^{++} channel rather than a carrier has been postulated (Smith et al., 1985a).

In support of the binding studies discussed earlier, Ca^{++} release from the ER induced by IP^3 is inhibited by a prior elevation in $[Ca^{++}]_i$ (Delfert et al., 1986; Chueh & Gill, 1986; Jean & Klee, 1986). Ca^{++} release from the ER is also dependent upon high intracellular K^+ concentrations (Joseph & Williamson, 1986). A TEA and furosemide sensitive K^+ conductive pathway has been identified in internal membrane vesicles (Muallem et al., 1985) and stimulation of $^{86}Rb^+$ uptake by IP_3 into ER vesicles has been shown (Shah & Pant, 1988). A plausible explanation for these effects is that IP_3 mediated Ca^{++} release requires K^+ as a counter ion to neutralise charge differences between pool and cytoplasm. This would require the putative K^+ channel to be inhibited and not activated by the internal ER Ca^{++} concentration.

The work of Kemmer and colleagues (1987) has postulated the presence of a charge equalisation system operating during Ca^{++} sequestration. Ca^{++} uptake into the ER of permeabilised pancreatic acinar cells via a Mg^{++} dependent ATPase requires the presence of Cl^- anions. The existence a large Cl^- channel in giant liposomes of rat ER has recently been found (Schmid et al., 1988). Finally it has been reported recently that Ca^{++} uptake into the IP_3 sensitive pool is likewise sensitive to pH (Thevenod et al., 1988). Ca^{++} sequestration via the ATP

sensitive Ca^{++} pump is promoted by a low pH within the ER, generated by active transport of protons into this pool. Thus the IP_3 sensitive Ca^{++} pool has mechanisms present to control both Ca^{++} release and uptake which maintain an unequal Ca^{++} gradient with the cytoplasm.

THE Ins(1,4,5)P_3 RELEASABLE CA^{++} POOL AND THE GTP EFFECT.

Dawson (1985) working on rat liver microsomes found that low concentrations of GTP and the presence of polyethylene glycol (PEG) were needed to promote IP_3 mediated Ca^{++} release. GTP by itself was only capable of causing a slower Ca^{++} release. Since this initial discovery similar effects have been reported in other cell types. Gill and colleagues (1986) using permeabilised N1E 115 neuroblastoma cells described a specific effect of GTP in releasing Ca^{++}. ITP, ATP, CTP, GMP and GTP did not have an effect. The non-hydrolysable analogues of GTP, $GTP_\gamma S$ and GppNHp did not stimulate Ca^{++} release but $GTP_\gamma S$ like GDP could inhibit the GTP effect. This suggests a requirement for the hydrolysis of a terminal phosphate. $GTP_\gamma S$ inhibition of Ca^{++} release from the intracellular Ca^{++} pool distinguishes this response from its effects on G-protein mediated activation of PLC (Cockcroft & Gomperts, 1985; Cockcroft, 1987). The GTP induced Ca^{++} release was also found to be temperature dependent, being reduced four fold by a two fold reduction in temperature from 37°C to 19°C. Other groups working in rat liver microsomes (Ueda et al., 1986; Comerford & Dawson, 1988), guinea pig parotid gland microsomes (Henne & Soling, 1986; Henne et al., 1987; Ueda et al., 1986b) and in permeabilised cells of pancreatic islets (Wolf et al., 1987), macrophages (Kimura et

al., 1988) and hepatocytes (Thomas, 1988) have since investigated this effect in more detail. Henne demonstrated that the GTP Ca^{++} releasing effect was not related to activation of PLC (prior incubation with neomycin had no effect) and was independent of IP_3 evoked Ca^{++} release (1986). Although they demonstrated a requirement for PEG, other colloids such as BSA could substitute with similar effects. Following this the first demonstration of separate compartments for GTP and IP_3 induced Ca^{++} release was made by Henne & Soling (1987) using percoll gradients and enzymatic markers. They concluded that the IP_3 releasable Ca^{++} pool was composed of ER of the same density as the plasma membrane or was tightly associated with it, whilst the GTP releasable Ca^{++} pool was heavier in density and associated with or was part of the rough ER in guinea pig parotid cells. The mitochondrial inhibitor NaN_3 had no effect on the Ca^{++} uptake into these pools which required ATP and Mg^{++}, demonstrating them to be entities discrete from mitochondrial Ca^{++} stores. Cromerford and Dawson (1988) by the use of fluorescent probes inserted into hepatocyte microsomal vesicles made the discovery that GTP promotes fusion of these vesicles; an effect requiring Mg^{++} and PEG but not Ca^{++}. This explains Dawsons original findings that the IP_3 induced Ca^{++} release was potentiated by GTP but does not answer how GTP could evoke a Ca^{++} release in some systems in the absence of IP_3. The physiological significance of the GTP effect was investigated in permeabilised macrophages (Kimura et al., 1988). The chemotactic peptide N-formylmethionyl-leucyl phenyalanine (fMetLeuPhe), known to cause IP_3 formation and cell activation was found to release only approximately 20-

30% of the non-mitochondrial Ca^{++} pool. This figure was comparable to that evoked by exogenous IP_3 in the cell preparation and significantly lower than the 60-70% of total non-mitochondrial Ca^{++} released by GTP in the presence of PEG. It was concluded that receptor activation in this cell preparation did not cause greater Ca^{++} release than that evoked by IP_3. Recently a paper by Thomas (1988) has attempted to rationalise the GTP effect. In freshly permeabilised hepatocytes Ca^{++} release was unresponsive to GTP. However on further washing a GTP mediated release was seen suggesting the loss of a cytosolic or loosely associated membrane factor which normally inhibits this response. PEG was not required for either IP_3 or GTP mediated effects in direct contrast to microsome preparations. Gill (1986) reported that microtubule assembly is promoted by PEG in a GTP dependant manner however colchicine was not found to inhibit GTP's effect on Ca^{++} release. In the light of the evidence so far accumulated the PEG requirement appears to act via stabilising membranes which somehow results in the artificial release of Ca^{++} by GTP.

Thomas reported that $GTP_\gamma S$ inhibition of the potentiating effects of GTP on IP_3 hydrolysis only occurs if the cells were preincubated with $GTP_\gamma S$ prior to permeabilisation. If continuous GTP hydrolysis was occurring then the affects of $GTP_\gamma S$ would also be seen after permeabilisation. Nitchilla and colleagues (1986) have described a PEG dependant GTPase in rat liver microsomes which is inhibited by $GTP_\gamma S$ with a similar K_i as obtained for $GTP_\gamma S$ inhibition of the GTP induced Ca^{++} release.

Thomas has also found that GTP exerts its effects by increasing the proportion of sequestered non-mitochondrial Ca^{++} available to IP_3 without changing the total amount of Ca^{++} sequestered. This supports the findings of Dawson for a role of GTP in the coupling of these two pools. Another and perhaps important link in understanding this phenomenon has been revealed. Cullen and colleagues (1988) have demonstrated that the sulphated polysaccaride heparin, known to compete with the Ins(1,4,5)P_3 binding site (Worley et al., 1987), strongly inhibits IP_3 promoted non-mitochondrial Ca^{++} release from rat liver microsomes at micromolar concentrations similar to those used in the binding studies. It has no effect on the artificial (PEG requiring) GTP Ca^{++} release. A similar result has been reported in pancreatic B cells by Nilsson and colleagues (1988). This now establishes two important links, one between binding studies and the IP_3 releasable Ca^{++} pool and the other in the light of morphological evidence (Henne et al.,1987) on the site of this pool within cells i.e. closely associated with the plasma membrane.

In summary the evidence suggests that the normally GTP insensitive Ca^{++} pool, located in the ER is in some way coupled by a GTP-hydrolytic process (probably a kinase reaction) to the IP_3 sensitive Ca^{++} pool associated with or in close proximity to the plasma membrane. Such a model proposes that GTP hydrolysis functions as a physiological regulator of IP_3 induced Ca^{++} release. Dawson and Colleagues (1986) have identified two proteins phosphorylated by [γ^{32}]GTP of molecular mass 38KDa and 17KDa, for which no

physiological functions have yet been found. It remains to be seen if the factor(s) lost on washing the permeabilised cells which are thought to normally inhibit the Ca^{++} releasing effects of GTP are part of the same mechanism that can couple both Ca^{++} storage pools together.

THE EFFECTS OF IP3 PERFUSION IN LACRIMAL ACINAR CELLS.

A considerable amount of evidence has been discussed for the role of IP_3 in releasing intracellular Ca^{++}. This pool may be of finite size but agonist evoked IP_3 production could possibly effect a sustained physiological response if Ca^{++} was inhibited from being taken back up into storage or extruded across the plasma membrane. This is not the case in salivary or lacrimal acinar cells as the ACh evoked responses are clearly dependant upon extracellular Ca^{++}. To overcome the problems of introducing inositolpolyphosphates into cells internal dialysis is the obvious choice. Evans & Marty (1986) using this technique reported that internal dialysis of $Ins(1,4,5)P_3$ into lacrimal acinar cells caused an activation of Ca^{++} sensitive whole-cell currents. Llano and colleagues (1987) have since demonstrated an external Ca^{++} dependency for the IP_3 evoked response in these cells.

When IP_3 was incorporated into the intracellular dialysate containing Ca^{++} buffered at $10^{-7}M$ and introduced via continuous internal perfusion into control lacrimal whole-cells the activation of the Ca^{++} sensitive currents was small and transient. This effect was similar for a range of concentration of IP_3 between 1 and 100uM demonstrating that intracellular access was not a problem. Lowering the ionised Ca^{++} concentration of the dialysate completely inhibited any

effects of IP_3 in these cells. The responses obtained suggest IP_3 is much less effective at stimulating Ca^{++} sensitive currents than reported by Llano and colleagues. One technical aspect requiring consideration is the different approach used to introduce IP_3 into cells. Continuous internal perfusion differs from the conventional technique of including the test substance within the recording pipette prior to achieving a whole-cell. Multiple and much more thorough exchanges of intracellular milieus occur. Thus the differences may reflect the a more effective removal of possible cytosolic control processes for IP_3 metabolism, therefore creating conditions unfavourable for intracellular Ca^{++} release. The ability to perform effective controls by multiple internal solution changes far outweigh the less difficult experimental protocol of just including IP_3 into the recording pipette.

INOSITOLTETRAKISPHOSPHATE AND ITS FORMATION IN CELLS.

Conclusive evidence now exists for the metabolism of Ins(1,4,5)P_3 via two metabolic pathways. The first of these pathways to be discovered was dephosphorylation to Ins(1,4)P_2 by the actions of a 5'-phosphomonoesterase by Downes and colleagues (1982). The phosphoinositide IP_2 is generally found to have no Ca^{++} mobilisation capabilities (Streb et al., 1983). The presence of a cellular phosphorylated form of IP_3 was first discovered in cholinergically stimulated cortical brain slices with phosphates arranged on the myo-inositol ring at the 1,3,4 and 5 positions, IP_4 (Batty et al, 1985). Prior to this Irvine and colleagues (1984b, 1985) demonstrated in cholinergically stimulated parotid acinar

cells the accumulation of a novel tris phosphate Ins(1,3,4)P_3, for which no physiological role could be attributed. Following these discoveries is was proven that (1,3,4)P_3 was not the product of Ins(3,4)P_2 phosphorylation but likely to be the product of Ins(1,3,4,5)P_4 dephosphorylation (Downes et al., 1986). The specific enzyme responsible for this conversion was identified as Ins(1,4,5)P_3-Kinase in rat brain homogenates (Irvine et al., 1986) and has since been isolated in a large variety of cell types i.e. liver (Hansen et al., 1986; Blackmore et al., 1986), human T lymphocytes (Stewart et al., 1986) and RINm5F cells (Biden & Wollheim, 1986). A recent paper (Tarver & Anderson., 1988) has demonstrated the direct Mg^{++} dependent phosphorylation of IP_3 by this kinase using [γ-^{32}P]ATP as a substrate in retinal tissue photoreceptors. The apparent K_m values for ATP and Ins(1,4,5)P_3 (1.4mM and 1.4uM respectively) were also calculated. The K_m for IP_3 phosphorylation is an order of magnitude lower when compared with the K_m for dephosphorylation via 5'-phosphomonoesterase in a variety of cells (Downes et al., 1982). This would indicate a favourability towards IP_4 production in cells. A similar cellular 5'-phosphomonodiesterase is believed to dephosphorylate IP_4 to Ins(1,3,4)P_3 (Hansen et al., 1986).
A cellular bias towards IP_4 production due to the presence of this enzyme appears to be born out by the sequential accumulation of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and finally Ins(1,3,4)P_3 in a wide variety of cells. The time course of formation and metabolism of these inositolphospholipids upon agonist stimulation has been followed in intact preparations of RINm5F cells (Biden & Wollheim, 1986), rat pancreatic

acinar cells (Trimble et al., 1987a), hepatocytes (Hansen et al., 1986) and thymocytes (Zilberman et al., 1987). The slower build up of $Ins(1,3,4)P_3$ through the generation of $Ins(1,3,4,5)P_4$ would suggest that the latter is ideally placed to take part in some form of cellular regulation. In the following section I will discuss avenues of research and provide evidence for a role of $Ins(1,3,4,5)P_4$ in lacrimal acinar cells.

EVIDENCE FOR A ROLE OF IP4 IN CA++ MOBILISATION.

Suggestive evidence for a role of IP_4 in promoting Ca^{++} influx comes from Hansen, Mah and Williamson (1986). They reported preliminary findings that IP_4 could release Ca^{++} from Ca^{++} loaded hepatocyte plasma membrane vesicles whilst $Ins(1,4,5)P_3$ was without effect.

Slack and colleagues (1986) and Irvine & Moore (1986) have demonstrated that when $1,4,5P_3$ was microinjected into sea urchin eggs it induced the formation of a fertilisation envelope only if external Ca^{++} ions were present. Microinjection of $Ins(2,4,5)P_3$, a less potent analogue of $Ins(1,4,5)P_3$ (Irvine & Moore) did not to evoke such a response, neither could IP_4. However the combination of both inositol phospholipids induced an extracellular Ca^{++} dependant fertilisation response. Since $Ins(2,4,5)P_3$ cannot be metabolised to $Ins(1,3,4,5)P_4$, these workers provided indirect evidence for a role of IP_4 in activating a Ca^{++} influx. This data has recently been contested. Crossley and colleagues (1988) disagree on two points, firstly they have been unable to repeat the synergism with $2,4,5IP_3$ and IP_4, a problem experienced by Irvine & Moore (1987). Secondly they

disagree that the $Ins(1,4,5)P_3$ evoked fertilisation requires extracellular Ca^{++} influx.

Internal perfusion of $Ins(1,3,4,5)P_4$ into lacrimal acinar cells was found to be ineffective in increasing Ca^{++} activated whole-cell currents. However internal perfusion of $Ins(1,,4,5)P_4$ in combination with $Ins(1,4,5)P_3$ resulted in an activation of Ca^{++}-sensitive currents which remained sustained only in the presence of extracellular Ca^{++}. This prerequisite for Ca^{++} which is similar to that seen for the ACh evoked response clearly indicates that in combination, $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ are promoting Ca^{++} influx.

The possible direct effect of IP_3 and IP_4 on excised patches of the plasma membrane was not accomplished in detail and did not provide results. Enough experiments on the effect of $Ins(1,4,5)P_3$ on the plasma membrane Ba^{++} currents in excised patches were performed to rule out a direct effect on plasma membrane Ca^{++} influx by this inositolpolyphosphate. In the presence of extracellular isotonic Ba^{++} gradients some small Ba^{++} currents were seen in Ach stimulated cell-attached patches. Occasionally IP_3 treated excised patches under isotonic Ba^{++} gradients similar channel activity was recorded. These currents however were not dependent upon the presence of phosphoinositide.

The inability of $Ins(1,4,5)P_3$ to open Ca^{++} channels within an excised patch conflicts with the previously reported effects in lymphocytes. However both Kuno & Gardener's work (1987) and the findings of Reuter and colleagues (See Nasworth & Grienstein, 1987) in neutrophils can be explained by the conversion of IP_3 to IP_4. Parker and Miledi (1987a) in xenopus oocytes have demonstrated that IP_3 evokes an inward

Cl^- current together with a much smaller and more sustained inward Ba^{++} current (separable because Cl^- channels like K^+ channels are sensitive to Ba^{++} block). These workers have since then provided evidence that both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ may be involved in the generation of this current (1987b). In agreement with the observation of this thesis prior priming with IP_3 is needed to induce an IP_4 effect. Some indication of the nature of the Ca^{++} influx in xenopus oocytes has been obtained by Parker and Miledi. During the presence of high intracellular concentrations of EGTA, IP_4 microinjection was shown to induce a small sustained inward Ca^{++} current which was not inhibited by any of the recognised voltage dependant Ca^{++} channel blockers or a number of non-specific Ca^{++} channel antagonists. The influx however did require a large hyperpolarising current (conventional microelectrode study). Llano and colleagues (1987) similarly report that lacrimal whole-cells voltage clamped at large negative potentials with the dialysate containing $InsP_3$ sometimes display a progressive increase in activation of Ca^{++} sensitive whole-cell current. Presumably this could be the second messenger operated Ca^{++} influx looked for and provides a stimulating incentive to investigate with whole-cell internal perfusion.

THE EFFECTS OF CA^{++} ON PHOSPHOINOSITIDE FORMATION.

A growing amount of evidence suggests that the $InsP_3$-kinase responsible for the conversion of $Ins(1,4,5)P_3$ to $Ins(1,3,4,5)P_4$ is Ca^{++} sensitive (Tennes et al., 1987; Yamaguchi et al., 1987; Biden & Wollheim, 1986; Zilberman et al., 1987). Biden & Wollheim report a x2.5 increase in

activity occurring in cell homogenates over a range of [Ca^{++}] between 10^{-7}M and 10^{-5}M with little increase in the corresponding Ins-P_5-phospho-monodiesterase activity in the same cell. Zilberman and colleagues (1987) likewise show in cell membrane preparations that the $GTP_\gamma S$ stimulated formation of [^{H3}]IP_4 from [^{H3}]IP_3 increases x7 when the free-Ca^{++} is raised from 10^{-7}M to 10^{-6}M. Further they demonstrate that the pattern of accumulation mirrors that seen during stimulation in intact cells under normal conditions. However when bathed in low Ca^{++} saline IP_4 formation was much reduced and a more sustained increase in Ins(1,4,5)P_3 was seen. This is postulated to demonstrate a reduced cellular $InsP_3$-kinase activity under low Ca^{++} conditions.

The as yet uncharacterised $InsP_3$-kinase present in lacrimal acinar cells may likewise demonstrate a Ca^{++} sensitivity. This could provide part of the explanation for the lack of the IP_3 effect in the dialysis experiments presented in this thesis. Apart from an apparent loss of sensitivity for the IP^3 sensitive Ca^{++} pool caused by cellular washout there would also be reduced conversion of IP_3 to IP_4 by any remaining $InsP_3$-kinase activity due to the low Ca^{++} conditions. Certainly the very fact that in the lower Ca^{++} (10^{-9}M) containing dialysate IP_3 had no effect compared with a small effect at 10^{-7}M Ca^{++} containing dialysate supports this hypothesis.

THE EXTRACELLULAR NA^+ DEPENDENCY PRESENT IN SALIVARY ACINAR CELLS.

The K^+ channels monitored within basolateral membrane patches of cells bathed in Na^+-free conditions were found not be

greatly stimulated by cholinergic muscarinic receptor activation. The channels did not appear refractory with regard to their Ca^{++} sensitivity, as shown by their activation upon application of Ca^{++} ionophore to the cell. The Na^+-dependent effect likewise was not associated with a cellular voltage dependency as similar effects were seen with either K^+ or NMDG substitution of extracellular Na^+. The intracellular dye quin-2 confirmed that the $[Ca^{++}]_i$ response to agonist stimulation under Na^+-free conditions was severely curtailed. The magnitude of the intracellular Ca^{++} release appeared reduced and no sustained Ca^{++} rise generated through the influx of Ca^{++} into the cell was seen.

Cellular acidification is a consequence of prolonged incubation in Na^+-free media which appears potentiated upon agonist stimulation. This acidification occurs though the inhibition of the basolateral Na^+/H^+ antiport mechanism. Cholinergically evoked fluid secretion is accompanied by an increase in intracellular Na^+ in acinar cells (Schneyer & Schneyer, 1963; Landis & Putney, 1979), which is inhibited by amiloride (Saito et al., 1987b; Parnod & Putney, 1980). Salivary secretion also depends upon extracellular pH (Case et al., 1982) and indirect evidence for the involvement of protons and bicarbonate in secretion elicited in perfused rabbit and rat submandibular glands has been proposed (Martinez & Cassity, 1985; Novak & Young, 1986; Case et al, 1987). The presence of a Na^+/H^+ exchange mechanism has been demonstrated in submandibular cells of the rat (Pirani et al., 1987) and rabbit (Case et al 1988) and in mouse lacrimal acinar cells (Saito & Nishiyama, 1987a,b&c). The results of these studies indicate that cholinergic agonist activation is

associated with transient acidification. The recovery in cellular pH being due to activation of the Na^+/H^+ exchange mechanism.

More recently Saito & Nishiyama (1988) by direct intracellular pH measurement using a pH sensitive electrode have confirmed earlier findings. They have been able to demonstrate that a sustained acidification in pH_i occurs upon Na^+ removal or by addition of amiloride when the cholinergic agonist is present. These effects were reversible upon the readmittance of extracellular Na^+ or removal of the amiloride. In addition they proposed that acid extrusion in mouse lacrimal acinar cells must be controlled by other mechanisms apart from the cellular pH gradient (Grinstein & Rothstein, 1986) as the pH_i increases above the normal resting level during prolonged agonist stimulation.

Two possible sites of action of extracellular Na^+ depletion in submandibular cells may exist. Either the Ca^{++} influx pathway is blocked as a direct consequence of a reduced intracellular Na^+ concentration or by the build up of protons within the cell.

THE EFFECTS OF pH_i ON PHOSPHOINOSITIDE FORMATION.

Another form of modulation which appears to play an important role in the control of both Ca^{++} release from the intracellular pool and Ca^{++} influx is cellular pH. Evidence for a pH sensitivity for the IP_3 sensitive Ca^{++} pool comes from Brass and Joseph (1985) who reported an increased efficacy in mobilising Ca^{++} from intracellular stores by IP_3 at alkaline pH. It has been demonstrated from binding studies in the brain that the IP_3 receptor is pH dependent (Worley et

al., 1987). However this approach has not yet proven the receptor to be linked to the Ca^{++} pool although good indirect evidence now exists (discussed in previous section on the IP_3 sensitive Ca^{++} pool and the GTP effect).

Another form of pH regulation of importance to the findings of this thesis is the possible pH sensitivity of the $InsP_3$-kinase. Investigations currently under way by Irvine (personal communication) suggest that the $InsP_3$-kinase of sea urchin eggs is pH dependent. A decrease of pH by some 0.5 units from resting pH is believed to inhibit substantially the activity of this enzyme. Phosphatidylinositol generation has been shown to be independent of extracellular Na^+ (Jones & Michell,1976) as is the IP_3 evoked Ca^{++} release (Brass & Joseph, 1985).

As discussed the consequence of removal of external Na^+ (Saito et al., 1988) is inhibition of the Na^+/H^+ co-transporter in exocrine acinar cells. The resulting build up of protons during agonist stimulation leads to sustained cellular acidification. This effect probably reflects the true nature of the extracellular Na^+ dependency reported in this thesis. The inhibition of the conversion of $Ins(1,4,5)P_3$ to $Ins(1,3,4,5)P_4$ by $InsP_3$-kinase under Na^+-free conditions during ACh stimulation would explain the lack of a sustained intracellular Ca^{++} rise.

In a more general context it would be particularly appropriate to demonstrate whether the transient acidification in these cells parallels the IP_3 promoted intracellular Ca^{++} rise. In the light of recent evidence that the IP_3 sensitive Ca^{++} pool itself contains a proton gradient (Thevenod et al., 1988) and that IP_3 receptor binding is

inhibited by an acidic pH change (Worley et al., 1987) then an important means of regulation may reside here.

DAG AND PROTEIN KINASE C ACTIVATION, EVIDENCE FOR PHYSIOLOGICAL REGULATION OF THE Na^+/H^+ EXCHANGE IN ACINAR CELLS.

The other by-product of PIP_2 hydrolysis due to phosphoinositidase activation is diacylglycerol (DAG). DAG is known to activate Protein Kinase C (PKC), an effect mimicked by the tumor promoting phorbol esters which can substitute for this compound both *invitro* and *invivo* (Castagna et al., 1982; Nishizuka, 1984). Activation of PKC also depends upon presence of phosphtidylserine (PS) and Ca^{++}. The action of DAG or phorbol ester is thought to increase the affinity of this kinase for $[Ca^{++}]_i$ so that resting levels of this cation are sufficient to cause activation. In physiological situations the initial rise in Ca^{++} may act synergistically with DAG in stimulating PKC (Dougherty & Neidel, 1986). A role for PKC regulation of the G-protein coupling between receptor and phospholipase C has been suggested in various tissues (Blackmore & Exton, 1986; Muldoon et al., 1987; Osugi et al., 1987). Recently in Flow 9000 cells Lo and Hughes (1988) have demonstrated that pretreatment of permeabilised cells with the phorbol ester 12-O-tetra decanoylphorbol-13-acetate (TPA) inhibited the muscarinic receptor stimulated phosphoinositide formation whilst having little effect on that elicited by $GTP_\gamma S$. However TPA as well as OAG did significantly inhibit $GTP_\gamma S$ from potentiating the muscarinic stimulation. These results suggest that muscarinic receptor stimulated hydrolysis of

PIP_2 in these cells is subject to rapid feedback inhibition by the production of DAG and consequent stimulation of the Protein Kinase C pathway. The inhibitory effects of TPA are best explained at the level of coupling between receptor and G-protein and not at the G-protein phospholipase C linkage. However pretreatment of lacrimal acinar cells with TPA has been suggested to inhibit both ACh and $GTP_\gamma S$ evoked activation of whole-cell currents (Llano & Marty, 1986). In contrast Putney and colleagues (1984) have reported that phorbol esters do not affect basal $^{86}Rb^+$ efflux or cholinergic agonist evoked $^{86}Rb^+$ efflux in rat parotid gland. However a different and less potent phorbol ester was used in these studies. Therefore the exact interactions of PKC with G-protein in the phosphoinositidase complex still remains unclear in salivary glands.

An aspect of Protein Kinase C activation of particular relevance is the evidence that this enzyme may causally modulate the inositolphospholipid pathway through stimulation of the basolateral Na^+/H^+ antiporter. This would lead to a cellular alkalinisation and enhanced Ca^{++} mobilisation both through the IP_3 sensitive Ca^{++} pool and more importantly through a sustained Ca^{++} influx via the increased sensitivity of the $InsP_3$-kinase. PKC stimulation of the Na^+/H^+ exchange leading to the facilitation of Ca^{++} mobilisation has been documented for thrombin stimulation of platelets (Siffert & Acumen, 1988) and also in Chinese hamster lung fibroblasts (Paris & Poutssegur 1986; 1987).

Evidence for a direct effect of PKC on the Ca^{++} activated currents in lacrimal acinar cells has been discussed. Llano & Marty (1986) report that phorbol esters which activate PKC do

not affect resting Ca^{++} sensitive currents but inhibit the effects of agonists, although direct stimulation of the Na^+/H^+ exchange has not been documented. Here lies a problem since stimulation of the Na^+/H^+ exchange leading to cellular alkalisation occurs secondary to inhibition of PI turnover by PKC.

To summarise there is now evidence suggesting a duality of function for PKC in both stimulating Ca^{++} mobilisation via cellular alkalisation and in inhibiting the phospho-inositidase C complex resulting in reduced IP_3 formation. These effects are not mutually exclusive and probably exist together in order to curtail intracellular Ca^{++} release in favour of extracellular Ca^{++} influx and can be explained by phosphorylation of different target proteins. Recent support for this hypothesis has been demonstrated by Valone and Johnson (1987) who find that the phorbol ester phorbol-myristate acetate (PMA) acts differently on agonist induced intracellular Ca^{++} release and extracellular Ca^{++} influx in a concentration dependent manner. The actions of PKC are still far from classified, as exemplified by the lack of functional information on the small number of proteins known to be phosphorylated invivo by this enzyme (Berridge, 1987).

RECEPTOR-PHOSPHOLIPASE C COUPLING.

Agonists that mobilise intracellular Ca^{++} by virtue of their ability to initiate plasma membrane bound PIP_2 hydrolysis are influenced by guanine nucleotides. There is evidence obtained from permeabilised parotid gland (Taylor et al., 1986) that a G-protein modulates signal transduction between the plasma membrane receptor and phospholipase C. In

contrast to the cytosolic non-mitochondrial Ca^{++} pool discussed earlier, the affects of $GTP_{\gamma}S$ are found to be stimulatory on this system resulting in the hydrolysis of PIP_2 to IP_3 and DAG. This effect has been demonstrated in intact lacrimal acinar cells where $GTP_{\gamma}S$ substituted for direct muscarinic agonist stimulation causing activation of whole-cell Ca^{++} sensitive K^+ and Cl^- currents (Evans & Marty, 1986). A similar report has been made by Young and colleagues where $GTP_{\gamma}S$ was found to induce the slow activation of an inward Cl^- current in rat submandibular acinar whole-cells (1988). In both cases $GTP_{\gamma}S$ potentiated the normal agonist evoked currents although in the latter example no effect was seen on the Ca^{++} sensitive K^+ current.

$GTP_{\gamma}S$ stimulated hydrolysis of PIP2 has been demonstrated in many other tissues such as neutrophil membranes (Cockcroft & Gomperts, 1985), blowfly salivary gland membranes (Litosh et al., 1985), cloned rat pituitary GH3 cells (Lucas et al., 1985; Straub et al., 1986), rat hepatocytes (Uhing et al., 1986) and in human leukocytes (Smith et al., 1985b, 1986). The combination of both agonist and guanine nucleotide in the latter two examples directly lowers the Ca^{++} sensitivity of PLC sufficiently to promote PIP_2 hydrolysis at physiological Ca^{++} concentrations. This is in contrast to the supraphysiological conditions necessary to activate this enzyme <u>invitro</u> (Cockcroft et al., 1984; Seyfield & Wills, 1984). Other observations to support the role of a G protein come from receptor binding studies which demonstrate a reduced agonist affinity in the presence of guanine nucleotides (Lynch et al., 1985; Evans et al., 1985) and from the capacity of F^- and Al^{3+} to stimulate hydrolysis of PIP_2

(Blackmore et al., 1985; Taylor & Merritt, 1986). These ions are known to modulate the activity of the G proteins Ns, Ni and transducin from other systems (Stein et al., 1985; Gilman, 1987).

The identity of the protein responsible for signal-transduction coupling in the phosphoinositidase complex appears to be more difficult to reveal. Using the bacterial toxins as a way of classifying the type of G-protein interaction present has yielded at least three possible types (Lo & Hughes, 1987c; Casey & Gilman, 1988). In systems where pertussis toxin inhibits PIP_2 breakdown (in mast cells and neutrophils) Ca^{++} influx into the tissue as well as IP_3 formation is inhibited (Nakamur & Ui, 1985; Bokoch & Gilman, 1984). This G-protein is thought to be similar to or indeed the same as the G-protein responsible in these cells for inhibiting the adenylate cyclase system (Worley et al., 1986). Of more relevance to this thesis is the novel G-protein found in pancreatic acinar cells whose receptor induced IP_3 formation is inhibited by ADP-ribosylation via cholera toxin and is unresponsive to cAMP-generating agents responsible for adenylate cyclase activation (Trimble et al., 1987b). This G-protein can be identified in other cell types i.e. T-cells, where cholera toxin inhibits both agonist induced IP_3 production and rise in $[Ca^{++}]_i$ (Imboden et al., 1986) and the human embrionic pituitary cell line Flow 9000 (Lo & Hughes, 1987a & b). Cholera toxin treatment of Flow 9000 cells likewise caused a dose dependent inhibition of muscarinic stimulated IP_3 formation. However it did not inhibit $GTP_\gamma S$ induced IP_3 formation although $GTP_\gamma S$ potentiation of the muscarinic response was lost.

In summary G-protein's appear to be cell specific and although to date there has been no report in any cell system of a G-protein directly modulating activity of phospholipase C, strong indirect evidence implicates their role in the phosphoinositide C complex.

OSCILLATORY CHANGES IN MEMBRANE POTENTIAL AND THEIR RELATIONSHIP TO $[CA^{++}]_i$.

An effect associated with agonist evoked whole-cell currents which has not been discussed is the phenomenon of membrane current oscillation. Submandibular acinar cells dialysed using the conventional pipette technique were found to display oscillations in current particularly during the initial stages of cholinergic agonist stimulation. These oscillations are the manifestations of changes in the membrane permeability towards both K^+ and Cl^- ions. It is highly probable that these oscillations reflect similar changes in intracellular Ca^{++} concentration.

Interestingly it is known that the Ca^{++} sensitivity of these ionic conductances differ, the Cl^- being least sensitive and requiring micromolar concentrations of Ca^{++} for activation (Evans & Marty, 1984). In addition the largest oscillations were seen to occur at membrane potentials close to E_K and were therefore predominately Cl^- in nature. These two points taken together suggests that the Ca^{++} released by the intracellular stores must undergo rapid, cyclic reuptake or extrusion across the plasma membrane. The Cl^- current could also be affected by some additional control mechanism. Similar cyclic oscillations in Ca++ sensitive whole-cell currents have been reported by Marty and colleagues (1986).

These workers were able to reproduce ACh evoked oscillations with internal dialysis of either $GTP_{\gamma}S$ or $Ins(1,4,5)P_3$. They also find oscillations to be more prevalent at membrane potentials dominated by Cl^- movement.

A decrease in the oscillatory pattern seen during the sustained phase may be the result of two factors; a) the partial recovery of E_{ACh} from its initial more hyperpolarising value (due to K^+ movement) to less negative potentials in the sustained phase so limiting the electrochemical contribution of the Cl^- current and b) the increasing presence of Ca^{++} influx. Oscillatory currents were not seen in lacrimal whole-cells however the saline composition and method of whole-cell dialysis were different (see methods).

Also worthy of mention is the appearance of oscillations in the membrane potential occasionally seen during agonist stimulation whilst recording from cell-attached patches. These did not appear dependent upon external Ca^{++}, an equal number (n=4) were recorded in its absence.

The presence of oscillatory changes in intracellular Ca^{++} within salivary acinar cells is not a new phenomenon. O'Doherty and colleagues demonstrated them in 1980 using Ca^{++}-ion selective microelectrodes. Similar oscillatory phenomena has been reported in insect salivary glands (Rapp & Berridge, 1981) and through direct $[Ca^{++}]_i$ measurement or through Ca^{++} sensitive currents in mouse oocytes (Cuthbertson & Cobbold, 1985), ferret papillary muscle (Orchard et al., 1983), mouse fibroblast L cells (Ueda et al., 1986a), lymphocytes (Tsien & Poenie, 1986), rat hepatocytes (Wood et al., 1986), GH3B6 pituitary cells (Schegal et al., 1987) and

BC3H-1 cultured muscle cells (Amber et al., 1988). Amber and colleagues find that in hepatocytes the frequency of oscillations induced by agonists does not depend upon external Ca^{++}. In its absence the amplitude of the response gradually attenuates due to the loss of released intracellular Ca^{++}, this effect can be reversed by the readdition of external Ca^{++}. In mouse oocytes Protein Kinase C induces oscillations in $[Ca^{++}]_i$ (Cuthbertson & Cobbold, 1985). Mast cells also have been induced to oscillate by injection of the catalytic subunit of Protein Kinase C (Neher & Almer, 1986). These effects can be linked by postulating a negative feedback mechanism occurring at the level of Ca^{++} mobilisation and perhaps therefore IP_3 production. However it must be noted that possible models for oscillatory phenomena may be cell specific and determined by how the cell mobilises Ca^{++} i.e. in cells possessing voltage gated Ca^{++} channels, Ca^{++} influx may play a role. Generally it would be difficult to attribute these effects to PKC inhibition at the level of PIP_2 hydrolysis for two reasons: one, as just described PKC induces oscillations in some cells alone and two, oscillatory changes occur in the presence of phosphoinositides i.e IP_3 or IP_4 injection in oocytes (Parker & Miledi, 1986, 1987) or IP_3 or $GTP_\gamma S$ injection into lacrimal acinar cells (Marty et al., 1986).

Interestingly the ability of IP_4 alone to induce these effects in oocytes was only one twentieth of that induced by IP_3. This figure is an order of magnitude greater than that calculated for IP_4 binding to the IP_3 receptor quoted by Worley and Colleagues (1987). In oocytes, cultured muscle cells and salivary acinar cells the oscillations occurred in

the absence of extracellular Ca^{++} indicating Ca^{++} influx was not required.

The fact that oscillations can occur during EGTA chelation with no effect on their frequency clearly rules out a direct feedback effect of Ca^{++} or pH on Ca^{++} release from the intracellular pool. Also Ca^{++} extrusion across the plasma membrane would not contribute to the oscillatory phenomenon due to immediate chelation of released Ca^{++}. An important observation is that the experiments outlined above are all carried out at room temperature. As oscillations appear to be an intracellular Ca^{++} release phenomenon and as outlined earlier the GTP dependent coupling of non-IP_3 sensitive Ca^{++} pool to the IP_3 sensitive pool is temperature dependent, a possible explanation of these effects is provided. Investigations as to whether the oscillations are merely an artifact of reduced intracellular Ca^{++} pool coupling could be pursued by looking for GTP and temperature dependencies for this effect.

Alternatively the phosphorylating actions of PKC would be another area of pursuit.

Finally investigators have tried to determine whether the oscillations could fullfill a physiological role. As discussed earlier for insect salivary gland (Rap & Berridge, 1981) the oscillatory pattern was first found to be dependent upon the concentration of agonist used (5HT) and also the rate of secretion. This has led to the concept that oscillatory behaviour is a transputor mechanism which promotes finer control by second messenger signalling (Berridge, 1987). In this appealing hypothesis, as cells are all tightly coupled by gap junctions, if the frequency and

not the amplitude of agonist signal is relied upon when communicating between cells then the response would be more finely controlled.

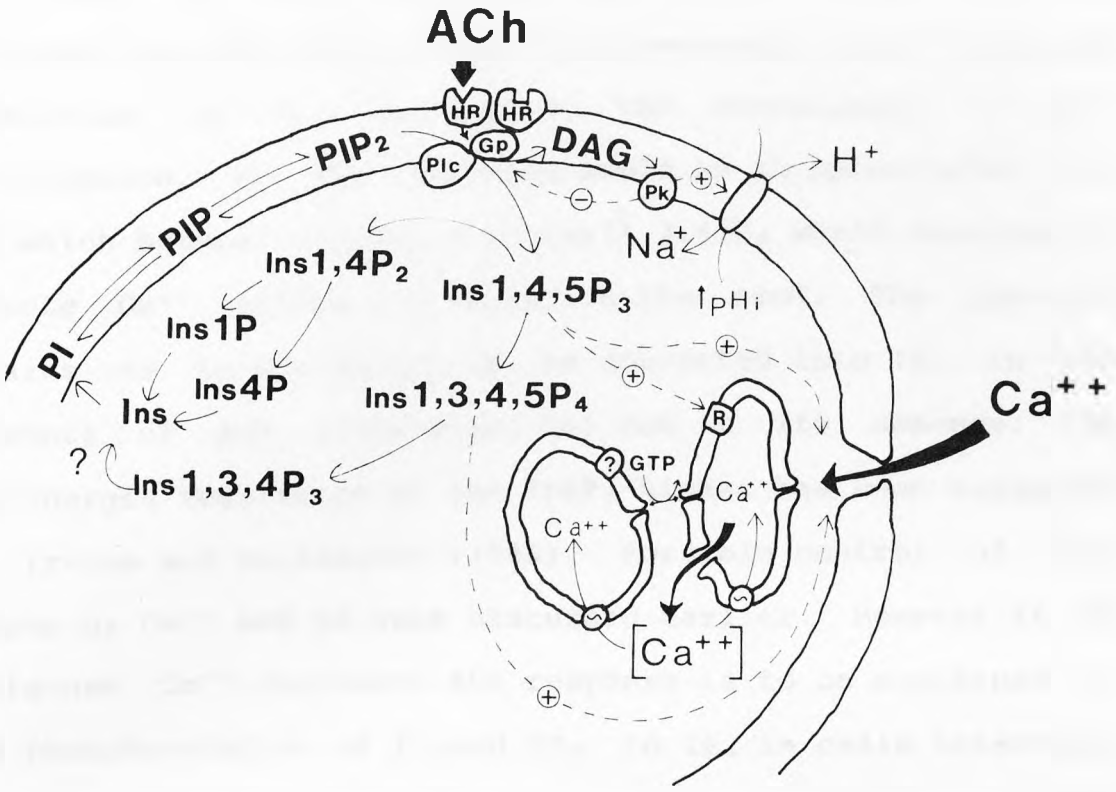
A MODEL FOR SMOC ACTIVATION IN SALIVARY OR LACRIMAL ACINAR CELLS.

Activation of cholinergic receptors in salivary or lacrimal acinar cells results in an elevation of the cytosolic Ca^{++} concentration which activates both K^+ and Cl^- conductances within the plasma membrane. These permeability changes are an integral part of the transport processes which underlie fluid secretion. Cytosolic Ca^{++} is mobilised from two sources: a) internal release and b) influx across the plasma membrane. Evidence exists for the role of $1,4,5IP_3$ in promoting internal Ca^{++} release, however Ca^{++} influx is essential for a sustained physiological response. The evidence presented suggests that this influx is regulated by two inositolpolyphosphates, $1,4,5IP_3$ and $1,3,4,5IP_4$.

The question remains as to how Ca^{++} enters the cell. Putney (1986) observed that the intracellular Ca^{++} pool is resistant to depletion by extracellular Ca^{++} chelators and when empty is rapidly replenished by extracellular Ca^{++} even in the absence of agonist. Ca^{++} restoration to the IP_3 sensitive pool occurs in the absence of a rise in cytosolic Ca^{++}, suggesting a direct link to the exterior of the cell. Based on these results Putney (1986) formulated a capacitive model and proposed IP_3 to be the controlling factor. However in light of the IP_3/IP_4 synergism demonstrated a new aspect can now be incorporated. Before describing my own interpretation of this model it should be noted that the possibility of fine

coupling between Ca^{++} influx into the cytosol and Ca^{++} reuptake into the non-mitochondrial pool or extrusion from the cell cannot be ruled out.

The fact that the IP_3 sensitive Ca^{++} pool appears to be closely associated with the plasma membrane corresponds well with the second messenger function of Ca^{++} release. The demonstration of differential modulation by phorbolester inhibition of agonist evoked Ca^{++} release and influx into platelet cells (Valone & Johnson, 1987) if reproducible would argue against Ca^{++} influx being controlled exclusively by the actions of IP_3 on the internal Ca^{++} pool. The differential sensitivity of IP_3 and IP_4 binding to the Ca^{++} release receptor of the non-mitochondrial Ca^{++} pool (Worley et al., 1987a/b) raises the concept of quite different sites of action for each phophoinositide. The following figure contains the recognised signal-transduction pathway thought to promote Ca^{++} mobilisation in cells and contains possible sites of action for the phosphoinositides in the control of Ca^{++} influx. The proposed secondary modulation of the phophoinositidase C complex and $InsP_3$-kinase by PKC is also included.



The most critical aspect of this figure concerns the coupling of Ca^{++} influx to the IP_3 sensitive pool. The presence of an IP_4 receptor on the cytosolic surface of the plasma membrane has not yet been conclusively proven. Because of this the possible direct coupling of the IP_3 sensitive pool with an IP_4 sensitive Ca^{++} influx mechanism in the plasma membrane has been omitted.

However this hypothesis is appealing as it could explain how the IP_3 sensitive pool is refilled. Thus following IP_3 production and the consequent depletion of the Ca^{++} pool, because of the cellular bias towards IP_4 formation, build up

of this phosphoinositide would activate coupling and influx of Ca^{++} from the exterior. Provided that generation of IP_3 and hence IP_4 was sustained, Ca^{++} influx would continue unabated. Interruption of agonist stimulation would cease the generation of IP_3 and lead to the curtailment of Ca^{++} mobilisation. Any IP_3 remaining would be phosphorylated into IP_4 which before metabolism to $Ins(1,3,4)P_3$ would continue to promote Ca^{++} influx and replenish the pool. The question remains as to why should IP_3 be converted into IP_4 in the presence of ACh stimulation but not in its absence. The cholinergic regulation of the $InsP_3$-kinase has been suggested by Irvine and colleagues (1986). Possible control of this enzyme by Ca^{++} and pH were discussed earlier. However if the sustained Ca^{++} dependent ACh response is to be explained as the phosphorylation of formed IP_3 to IP_4 in cells internally perfused with a Ca^{++}-free saline then membrane bound controlling factors must exist. The control of this enzyme and the refilling phenomenon may occur by discrete mechanisms. Obvious choices for control factors are receptor coupled G proteins and PKC.

An important and as yet uncharacterised response seen during inositolpolyphosphate stimulation of Ca^{++} sensitive currents as monitored with the whole-cell technique may reflect the theme of this hypothesis. Addition of ACh to IP_3/IP_4 stimulated Ca^{++} sensitive currents has a potentiating effect, the magnitude of evoked currents exceed that obtained either by direct agonist stimulation or perfusion of IP_3/IP_4 into the cells.

This potentiation would appear to be involved with Ca^{++} influx and a possible explanation may be found at the level

of secondary modulation. It is known that PIP_2 hydrolysis stimulated by ACh results in the formation of IP_3 and DAG. Since we already have supramaximal concentrations of inositolpolyphosphates present (Streb et al., 1983; Burgess, 1984), DAG formation could be the missing control factor. This would then implicate secondary modulation by PKC via a direct synergistic effect through protein phosphorylation of some unidentified factor. The possibility of cellular alkalisation can be ruled out in these whole-cell experiments due to the controlled intracellular buffering of Ca^{++} and pHi. This hypothesis could be simply tested by adding DAG to the cells in the presence of IP_3 and IP_4. Secondary modulation by PKC could include enhancing the coupling between the influx of Ca^{++} through the plasma membrane and IP_3 sensitive pool or stimulating the level of release from the already coupled pool.

Alternatively this phenomenon could also reflect a direct muscarinic receptor controlled effect of enhancing the flow of Ca^{++} through the IP_3 sensitive Ca^{++} pool. Apart from cholinergic receptor contol of $InsP_3$-kinase discssed earlier other possible links could be provided by muscarinic receptor stimulated G-protein interactions within the plasma membrane or similar interactions linked to the cytoskeleton or internal Ca^{++} pools.

These schemes can be accommodated within the structural model for Ca^{++} mobilisation described earlier. ACh stimulation of IP_3 perfused whole-cells would be expected to show a similar potentiation, although if the affects of IP_3 are entirely cytosolic and no conversion to IP_4 occurs due to the conditions imposed by the internal perfusion, the

potentiating effects may not be seen. This area of research was not investigated.

Evidence to strengthen the coupled Ca^{++} pool hypothesis may come from metabolic studies investigating the temperature dependency of Ca^{++} influx coupled to $[Ca^{++}]_i$ rise during IP_3/IP_4 dialysis of whole-cells.

Already there is evidence that IP_3 promoted intracellular Ca^{++} release is not temperature dependent (Smith et al., 1985). Therefore if the activity of the IP_3/IP_4 evoked Ca^{++} sensitive currents are temperature dependent (a decrease causing inhibition) this would indicate an underlying metabolic process promoting Ca^{++} influx. Temperature dependant changes in channel gating kinetics if present could be identified and accounted for. Alternatively direct measurement of $[Ca^{++}]_i$ with intracellular dyes could be employed. The presence of the already characterised temperature and GTP dependent coupling of intracellular Ca^{++} pools if controlled by the IP_3/IP_4 combination would also be affected. This consideration can be removed by omitting GTP from the dialysate and could prove useful in determining whether differential temperature sensitivities exist for intracellular Ca^{++} release from Ca^{++} influx.

Heparin binds and inhibits the IP_3 receptor located on the intracellular Ca^{++} pool at equivalent physiological concentrations of IP_3 (Cullen et al., 1988; Nilsson et al., 1988). This interaction could be employed to distinguish a separate pathway for Ca^{++} influx other than through a direct linkage between plasma membrane and intracellular pool. If in the presence of both inositides and heparin, Ca^{++} influx still occurs then the model described above would be

unfounded. Synergism between IP_3 and IP_4 at a site unconnected with the IP_3 receptor controlled Ca^{++} pool would then be more likely.

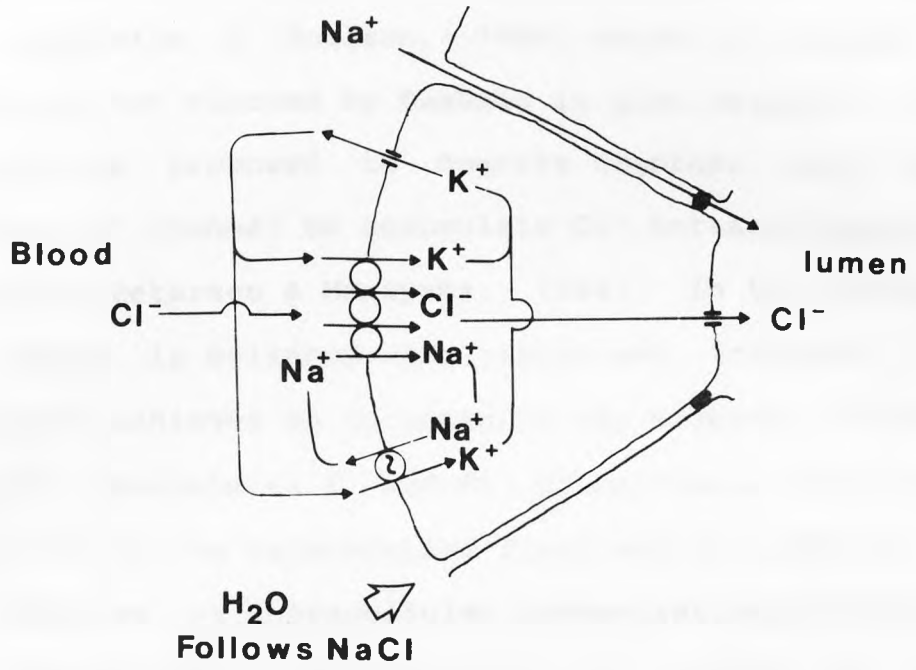
Evidence (Parker & Miledi, 1987) has been presented for a novel IP_4 regulated Ca^{++} influx pathway in xenopus oocytes. As patch-clamping measures the electrical phenomenon of ion transport across membranes it would not differentiate between the opening of a Ca^{++} channel coupled directly to either an open IP_3 sensitive pool or the cytoplasm. Thus electrophysiological characterisation of a similar influx in exocrine acinar cells would not compromise the model above. If the massive Ca^{++} gradient across the plasma membrane (10,000-100,000) is taken into account, the actual size of the conductance needed to carry enough Ca^{++} across the membrane to cause an effect by either route may be below the limits of resolution for excised patches but not for the whole-cell configuration.

The proposed effect of the Na^+/H^+ exchange mechanism in modulating PIP_2 breakdown and IP_4 formation has previously been discussed in this chapter.

A MODEL FOR FLUID SECRETION.

As addressed in the introduction the main electrolytes secreted as primary fluid into the lumen of a salivary acinus are Na^+ and Cl^- with small amounts of HCO_3^- and K^+ (Young et al., 1987). Apart from the passive permeability changes which occur within the basolateral membrane, other transport processes necessary for fluid secretion have now been characterised in these cells. A current model for fluid and electrolyte secretion which includes both passive and active

transport processes thought to be involved in the generation of primary saliva is shown below.



The Na^+-K^+-Cl^- co-transport mechanism is believed to be located in the basolateral membranes of exocrine acinar cells (Exley & Gallacher, 1984) and can be blocked by loop diuretics (Exley, Fuller & Gallacher, 1986; Turner et al., 1986). This carrier is passive and electrically silent, capable of being operated in either direction with no energy input. The movement of any one ion depends upon the presence of the other two and the ratio of the fluxes does not vary in accordance with the concentration of ions present. It is unaffected by membrane potential and transports Na^+, K^+ and Cl^- ions in the ratio of 1:1:2 respectively. Since its first discovery and documentation in 1980 in mouse acinar tumor cells (Geck et al., 1980, 1981) and avian erythrocytes (Kregenow et al., 1979; Haas, Schmidt & McManus, 1982) it has

been reported in many tissues as epithelial absorption of NaCl, cellular accumulation of Cl^- and secretion (see reviews by Geck & Heinz, 1986; Chipperfield, 1986).

The energy requiring Na^+/K^+ pump (Bundgaard et al., 1977, 1981; Nautofte & Poulsen, 1986) which is driven by ATP hydrolysis and blocked by Ouabain is also present. These two systems are proposed to operate together with the Ca^{++} sensitive K^+ channel to accumulate Cl^- intracellularly during secretion (Petersen & Marayama, 1984). In the resting cell the system is balanced with little net transport. Agonist activation achieves an increase in the membrane permeability to both basolateral K^+ and Cl^- conductances resulting in a loss of K^+ to the interstitial fluid and Cl^- into the lumen. The decrease in intracellular concentrations of both these ions dramatically favours Na^+-K^+-Cl^- uptake by secondary active transport as the K^+ recirculates via the Na^+/K^+ pump. The end result is a net uptake of Cl^- which is transcellularly secreted into the lumen, Na^+ following via a paracellular route and water to maintain osmotic balance. The role of the basolateral K^+ channel is thus to initiate the secretory events, the Cl^- channel is likewise a physiological control point. This thesis has shown an apparent Ca^{++} dependant Cl^- conductance to be present in whole-cells (see also Marty,1984). Although the Cl^- channel is not well characterised in terms of regulation or biophysics within these cells, evidence for its location on the luminal membrane comes from the increased luminal negativity seen during secretion (Lundberg, 1958).

The Na^+/H^+ exchange mechanism located in the basolateral membranes of salivary and lacrimal acinar cells may have an

important fascillitative role in promoting secretion. It maintains the intracellular pH at an optimum for many cellular functions implicated directly from the activity of the Na^+/K^+ pump (Eaton et al., 1984) or indirectly in the control of intracellular Ca^{++} release, influx and re-equilibration (see this chapter) when acidification occurs. Additionally it could also enhance the recycling of Na^+ for use in the Na^+/K^+ pump and so indirectly help maintain the paracellular movement of Cl^-.

The Na^+/H^+ exchanger has been postulated to facilitate HCO_3^- transport by increasing the cytosolic HCO_3^- concentration. The presence of a Cl^-/HCO_3^- antiport has been demonstrated in rabbit submandibular (Case et al., 1988) and mouse lacrimal acinar cells (Saito et al., 1988). It has been suggested that this exchange mechanism present on the basolateral membrane could transport HCO_3^- across the lumen via an unidentified conductance (Case et al., 1988). Alternatively it could maintain (as the result of an increased cytosolic HCO_3^- concentration) the intracellular Cl^- concentration above its equilibrium level thus supporting Cl^- transport during maintained secretion (Saito et al., 1988). The importance of either mechanism has yet to be qualified in more detail.

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APPENDIX

The majority of results presented within this thesis have been published by myself in the following papers:

Gallacher, D. V. & Morris, A. P. (1986)
A Patch-clamp study of Pottasium Currents in resting and isolated mouse submandibular acinar cells.
J. Physiology. 373, 379-395.

Morris, A. P. & Gallacher, D. V. & Lee, J. A. C. (1986)
A large conductance , voltage and Calcium activated K^+ channel in the basolateral membrane of rat enterocytes.
FEBS Lett. 206, (1), 87-92.

Morris, A. P., Fuller, C. M. & Gallacher, D. V. (1987)
Cholinergic receptors regulate a voltage-insensitive but Na^+-dependant Calcium influx in salivary acinar cells.
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Morris, A. P., Gallacher, D. V., Irvine, R. F. & Petersen, O. H. (1987)

Synergism of $Ins(1,4,5)P_3$ with $Ins(1,3,4,5)P_4$ in activating Ca^{++}-dependant channels. Nature. 330, 653-655 (1987).

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