VOLTAGE AND PATCH CLAMP STUDIES OF THE IONIC CURRENTS IN SNAIL NEURONES AND FROG SKELETAL MUSCLE

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The work in this thesis can be split into two distinct areas. The first experiments used a conventional voltage clamp technique to look at the calcium permeability of snail neurones and the controlling influence of the intracellular environment. Some of these experiments were performed in collaboration with my supervisor Dr. N.B. Standen.

The second area of study used the relatively new patch clamp technique to investigate the membrane permeabilities in frog muscle and snail neurones. Much of the work on frog muscle was performed in conjunction with Dr. P.R. Stanfield.

All the experiments were carried out in the Department of Physiology of the University of Leicester between October 1981 and September 1984.

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1. INTRODUCTION

Excitable cells play an important part in the functioning of all but the very simplest of animal life forms. In the higher animals their function ranges from the processing of information within the central nervous system, including the sensory and motor mechanisms, to the control of many of the endocrine secretory systems.

Membrane excitability

It has been known for a long time that the excitability of biological membranes is affected by the distribution of ions across the cell membrane (Bernstein, 1902). Hodgkin and Huxley (1952a,b,c,d) showed that it was changes in the relative permeability of the membrane to sodium and potassium ions that underlie the action potentials observed in squid axon. By considering the membrane to consist of a number of simple electronic components it is possible to demonstrate the mechanism of the action potential.

\[
\begin{align*}
\text{OUT} & \quad \text{IN} \\
E_{Na} & \quad E_{K} \\
R_{Na} & \quad R_{K} \\
C_{m} & 
\end{align*}
\]

In the diagram above the batteries represent the electrical potential created by the distribution of the sodium and potassium
ions across the membrane. The potentials of these, $E_K$ and $E_{Na}$, can be calculated from the external and internal ion concentrations using the Nernst equation. For example, the potassium equilibrium potential is given by

$$E_K = \frac{RT}{F} \ln \frac{[K]_o}{[K]_i}$$

where $R$ = the gas constant, $T$ = temperature (°K), $F$ = the Faraday constant and $[K]_o$ and $[K]_i$ are the external and internal potassium concentrations.

$R_{Na}$ and $R_K$ account for the permeability of the membrane that is specific to the sodium and potassium ions and in reality are dependent on the number of channels in a state in which they will pass these ions. Under normal resting conditions $R_K << R_{Na}$ and, as a consequence, the membrane capacitance $C_m$ becomes charged to a potential approaching $E_K$. If $R_{Na}$ is now reduced with respect to $R_K$ this potential shifts towards the value of $E_{Na}$. During the rising phase of an action potential the resistance of the membrane to $Na^+$ is reduced by the activation of a large number of sodium channels, causing an influx of $Na^+$ into the cell. The activation of the sodium channels is voltage dependent, depolarisation of the membrane from the resting potential causing an increase in the number of open channels. A small depolarisation will reduce $R_{Na}$ and cause further depolarisation. This in turn activates more sodium channels forming a positive feedback loop that rapidly changes the membrane potential from the resting value to a value approaching $E_{Na}$.

Repolarisation of the membrane is then brought about by a decrease in $R_K$ and an increase in $R_{Na}$. The decrease in $R_K$ is caused by the activation of a large number of potassium channels as a direct consequence of the membrane depolarisation. The potassium channels are activated in a similar fashion to the
sodium channels, except that the rate of activation is much slower. The increase in \( R_{Na} \), after its initial decrease, is caused by inactivation of the sodium channels; this is also a voltage dependent mechanism that is slower than the original activation process.

Since the original work on the squid axon this set of changes in membrane permeability has been found to describe the basic mechanisms of membrane excitability in a large number of different tissues.

Voltage clamp

The advance in our understanding of the mechanisms of membrane excitability, referred to above, was largely the result of the use of the voltage clamp technique (Cole, 1949; Marmont, 1949). The technique relies on the precise control of the membrane potential accompanied by the measurement of the trans-membrane current and it permits the measurement of the potential dependence of membrane ionic current in isolation from the complicating factors such as membrane capacity currents and the dependence of membrane potential on membrane current. The voltage clamp system provides a controlled current source which prevents membrane currents altering the membrane potential and also, since the potential is held constant, no capacitative currents are induced. Stepping the potential at which the membrane is held can then be used to activate the channels. The membrane capacitance is charged very rapidly to the new potential by the voltage clamp system and thus time and voltage dependent changes in membrane permeability can easily be isolated.
Calcium permeability

The influx of sodium ions is responsible for the rising phase of the action potential in a number of tissues, for example squid axon and vertebrate myelinated nerve. However, there are also many excitable cells which rely either entirely on calcium entry or on a combination of calcium and sodium entry for the initiation of the action potential. Molluscan neurones (Oomura, Ozaki and Maeno, 1961), invertebrate muscle (Fatt and Katz, 1953), Aplysia axon (Horn, 1978) and Paramecium (Brehm and Eckert, 1978), for example, can all produce regenerative calcium-based action potentials under certain conditions (see review by Hagiwara and Byerly, 1981).

Since calcium has an important role as a messenger within cells, the entry of calcium during the action potential offers the possibility of a link between the excitation of the cell membrane and the activation or control of other systems in the cell. Some examples of the cellular processes that are affected by influx of calcium ions are excitation-contraction coupling, transmitter release and ciliary movement (see reviews by Baker, 1976; Erulkar and Fine, 1980; Campbell, 1983).

The control of the influx of calcium through the membrane must, therefore, be of great importance in the overall control of these calcium sensitive systems in order to prevent the entry of excess quantities of calcium. Part of the work in this thesis is aimed at elucidating the mechanisms of control of the calcium permeability in Helix aspersa neurones. These neurones have both calcium and sodium inward currents, both being of large enough amplitude to initiate action potentials in the absence of the
other ion (Chamberlain and Kerkut, 1967; Kerkut and Gardner 1967). It is known that calcium entry and increases in intracellular calcium cause an increase in the potassium permeability of the neurone (Meech, 1974) and that this helps to return the membrane potential to the resting level, thus halting the influx of calcium ions. However, there is uncertainty as to the mechanism of inactivation of the calcium permeability when the cell remains depolarised. Chapter 3 of this thesis is based on experiments designed to investigate the mechanisms of the inactivation of the calcium current in snail neurones. In Chapter 4, a slightly different form of control of the current is investigated. In view of the large number of cellular processes regulated by intracellular calcium and the many cited roles of cyclic adenosine monophosphate (cAMP) in the mediation of cellular processes, the possible metabolic control of the calcium permeability by such a system was investigated.

Patch clamp

Chapters 5 and 6 of this thesis describe work performed using the patch clamp technique to study certain channel characteristics in the neurones of Helix aspersa and in an adult frog muscle preparation. The technique was developed by Neher and Sakmann (1976) for the measurement of the currents present in small electrically isolated patches of cell membrane. It can be used to measure the currents passing through the individual membrane channels that are collectively responsible for macroscopic currents measured using conventional voltage clamp techniques. Although patch clamping works on the same principles as the voltage clamp described above, it can be used to yield a lot of different information about the mechanisms of control of
membrane permeability.

Using patch clamp recording it is possible to measure the elementary currents underlying the macroscopic currents. These currents represent the flow of ions through the individual channels present in the membrane and are normally seen as square steps in current, caused by the opening and closing of the channel. This opening and closing of the channels is thought to be the result of conformational changes in the large molecules forming the channel and, therefore, the technique offers a most direct means of studying the rates of the individual transitions.

As a means of describing the uses of the patch clamp technique, I have outlined below some of the major differences in the uses and problems associated with this technique, compared with more conventional voltage clamp methods. The first and most obvious advantage of the new method is the direct analysis of the potential dependence of the unitary current amplitude in isolation from the potential dependence of the kinetics of channel opening. Previously it has only been possible to estimate unitary current amplitude from noise analysis of small macroscopic membrane currents. The current-voltage relationship normally obtained from macroscopic currents gives a combined result for the change in current due to changes in unitary current amplitude and changes in the average number of channels open. Only under special conditions when the probability of channels being open is the same at the different potentials does the current-voltage relationship for macroscopic currents give a picture of the channel conductance. Another advantage of being able to study the individual unitary currents is in the separation of the different types of current. In macroscopic
recording it is often difficult to isolate one particular current from all the others present in the membrane. However, with single channel recording, channel openings which lead to current amplitudes different from the amplitude of the channel being studied can be identified and omitted from the results. Having said this, it must also be noted that identification of the unitary currents in terms of the macroscopic currents already described in many preparations can initially prove quite difficult. This stems from a major disadvantage of the patch clamp technique, since only the behaviour of a few channels can be studied during each experiment. Thus many of the kinetic properties of the channel activity must be studied over long periods of time in order to gain an overall picture of channel behaviour. This requires the membrane patch to remain electrically stable over long periods of time during which the relevant external factors affecting the channel behaviour must be kept constant so that many seconds of record can be obtained to produce an average view of the channel activity. Finally, the patch clamp technique has made it possible to study the behaviour of many types of channels from very small cells not usually accessible using conventional micro-electrode based methods because of the damaging effects of penetrating the membrane of the cell. The experiments described in chapters 5 and 6 were preliminary experiments used to develop the use of this technique and to characterise a few of the unitary current types observed in terms of the macroscopic currents. The currents in both the frog skeletal muscle and Helix neurones have been studied quite extensively using conventional electrophysiological methods and are therefore ideal for the initial experiments using the new technique.
2. METHODS

Preparation

Giant neurones of the pulmonate snail *Helix aspersa* were used in the voltage clamp experiments described in this thesis. The work was confined to four identifiable neurones on the periphery of the sub-oesophageal ganglia of the snail brain. Using their position and size as criteria for identification, the cells used were numbers 5, 20, 3, and 4 on the left and right parietal ganglia, as described by Walker, Lambert, Woodruff and Kerkut (1970; see fig. 2.1).

These particular cells are well suited to voltage clamp experiments. They are large cells with diameters in the region of 150µm and are situated on the surface of the parietal ganglia thus making the insertion of a number of electrodes into a single cell a relatively simple operation. During the injection experiments, four electrodes were used on a regular basis. All four of the cells used are generally silent and have resting potentials between -62 and -64 mV in normal saline solution (Walker et al, 1970).

Bath

The experimental chamber consisted of a 1.5 cm deep, 2 cm square chamber milled out of a Perspex block. This was partially filled with an elastomeric silicone material (Sylgard 184, Dow Corning Corp.), giving a final bath volume of approximately 2ml. Fig. 2.2 represents the cross-section of the block showing both the bath and the hollowed surround used to pass cooling solution.
Figure 2.1

A sketch of the supra- and sub-oesophageal ganglia of *Helix aspersa* after mounting in the bath. It shows the extent to which the connective tissue was removed during the initial stages of the dissection and the position of the four large cells normally used. Four platinum or nickel-chrome staples were used to hold the preparation on the Sylgard surface. After removal of the surface layers of connective tissue the sub-oesophageal ganglia were partially separated by using two pins to stretch the preparation.

LP left parietal ganglia
RP right parietal ganglia
V visceral ganglia
A cross-section of the experimental chamber viewed from the side. The preparation was pinned out so that the sub-oesophageal ganglia were situated towards the front of the bath. They were viewed from the top and illuminated at an oblique angle from the rear. This tended to highlight the neurones and was found to be the most satisfactory means of lighting. The electrodes were brought in from the sides of the bath at an angle of about 45°, to prevent obscuring the view of the cells.
from a circulator (Churchill).

The bath and preparation were illuminated from the rear using a fibre optic light source (Barr and Stroud) to overcome problems of heat production and electrical noise associated with more direct lighting systems. The preparation was viewed through a binocular microscope (Wild) with a maximum magnification of x100.

Electrical connections to the bath for the voltage follower and the virtual earth were made through agar bridges which spanned between pots, containing 3 molar KCl, and the bath solution (see fig.2.3). Normal Ringer solution formed the basis of the agar gel in these bridges. For accurate recording it was important to have a balanced, stable system connecting both the bath solution and the electrodes to the recording set-up. For this reason Ag-AgCl pellets formed around Ag wire were used to connect to the 3 Molar KCl in both the pots and the voltage electrode holder, thus forming stable Cl- based electrical connections on both sides of the voltage follower, in addition to the virtual earth.

Electrodes

The voltage electrodes were made on a horizontal puller using borosilicate glass capillaries (Clarke E.I.) of 1.2mm external and 0.69 mm internal diameters containing a fine glass fibre which assisted, by capillary action, in the filling of the pulled electrodes. They were back-filled using drawn out nylon tubing to deliver the 3M KCl as close to the tip as possible. Any air bubbles trapped in the electrode were then removed by tapping the electrode or gently heating the bubbles against a microforge filament. The resistance of each electrode was measured and only those with values in the range of 5 to 10 Megohms and with a tip
Figure 2.3

The electrical layout of the voltage clamp recording system. The clamping circuit consisted of the headstage, voltage follower and clamping amplifier which formed the negative feedback loop used to control the membrane potential. The membrane current was recorded using the separate virtual earth circuit and was passed through a leak subtraction system and a Butterworth filter before being recorded. The measured membrane voltage and current were both displayed on a Tektronix oscilloscope and permanent records made by photographing the display or digitising the data and storing it on computer disc.
potential of less than ±2mV, were actually selected for use.

To maintain the speed of the voltage follower it was necessary to shield these electrodes using aluminium foil, which was wrapped around the lower half of the electrode to within 2mm of the tip. The shield was then isolated using a wrapping of paraffin wax film (Nescofilm) and a coating of Vaseline so that no electrical connection with the bath solution could be made during the experiments.

The current electrodes were drawn on a vertical Narashige puller using thin-walled borosilicate capillaries, with 1.0mm external and 0.78mm internal diameters, containing a thin glass filament. The thin-walled glass was used because it was possible to pull electrodes with a tip diameter of less than 2µm that also had a low resistance of 1 to 2 Megohms when filled with 3 molar KCl electrolyte solution. It is important to use electrodes of low resistance since it constitutes a major part of the access resistance into the cell through which any holding current, for control of the cell membrane potential, must be passed.

All the electrodes were supported in separate Perspex holders that were clamped onto Prior manipulators. With the exception of the voltage electrode, all electrodes were connected to the relevant circuitry by means of silver wire either positioned in the barrel of the electrode or bathed in a small reservoir of solution continuous with that of the electrode (see fig.2.3). The voltage electrode was mounted in a similar holder containing 3 Molar KCl but was electrically connected using an Ag-AgCl pellet, for the reason described above. This holder was plugged directly into the headstage of the voltage follower thus keeping the input wiring to the minimum length possible.
Dissection

The snails used were either collected locally or purchased from Philip Harris Ltd. Prior to dissection they were allowed to fall into a dormant state in a dry aquarium kept at room temperature and were then transferred to a refrigerator for several days. This procedure was designed to put all the snails into a similar physiological state since it has been shown that certain of the membrane characteristics may change depending on the state of the snail (Kerkut and Meech, 1967).

The brain of the snail, consisting of the supra and sub-oesophageal ganglia, was removed from the snail and pinned out onto the Sylgard base of the experimental chamber, using staples formed from platinum or nickel-chrome alloy wire, with the anterior surface uppermost. The thick layers of connective tissue that cover and protect the sub-oesophageal ganglia were then teased apart and stripped away using specially sharpened watchmakers forceps. This procedure was continued until the surfaces of the sub-oesophageal ganglia, still enclosed in the thin innermost sheath of connective tissue, were completely exposed. The preparation was then treated with protease (10mg/ml Sigma type XIV) in normal saline for about 15 to 20 minutes. This aided the final stage of the dissection by weakening both the sheath and the connective tissue holding it to the neurones. It was then possible to tear the sheath and gently remove it without causing significant damage to the underlying neurones. Standen (1975a) and Plant (1982) have both found that this treatment during the dissection has no observable effect on the physiological properties of the neuronal membrane.
Solutions

The normal snail Ringer used during the dissection was a variation of that described by Meng (1960), and contained 75mM NaCl, 5mM KCl, 10mM CaCl₂, 15mM MgCl₂ and 5mM Tris/HCl buffered to pH 7.5. In the majority of the voltage clamp experiments, which were aimed specifically at the calcium permeability of the membrane, it was necessary to isolate the calcium current from the sodium and potassium currents. Therefore, the normal saline was exchanged for a sodium-free solution containing the potassium channel blockers tetraethylammonium chloride (TEA, Aldrich or BDH.) and 4-aminopyridine (4-AP, Fluka). The total solution composition was normally 25mM CaCl₂, 10mM KCl, 5mM MgCl₂, 75mM TEA, 2mM 4-AP and 10mM Tris/HCl buffered to pH 7.5. Variations of this solution commonly used contained 25mM SrCl₂ or BaCl₂ in exchange for the CaCl₂. 25mM Ca²⁺ was chosen for the standard experimental solution because this approached the saturating concentration of the macroscopic calcium permeability, any further increases producing only minimal increases in current amplitudes. Plant (1982) evaluated the Km for the calcium channel to be 5.31mM for a depolarisation to 10mV.

Experimental solutions were generally made up on the day of use. The 4-AP was always dissolved in the solution just prior to its introduction into the experimental chamber.

Voltage follower

The cell membrane potential was measured differentially between the cell cytoplasm and the bath solution; the cell potential being measured by the voltage electrode inserted through the membrane, and the bath potential via one of the agar
bridge systems (see fig. 2.3). This type of recording helped to remove noise and any potential shifts associated with the absolute bath potential, which was referenced to earth via the virtual earth current monitoring system. Changes in the absolute bath potential were measured by both inputs of the voltage follower and were thus removed from the output. The cell potential was also measured directly with respect to earth as a means of checking the functioning of the recording circuitry.

In order to maintain a fast response time for the whole voltage clamp system it was important to ensure a fast rise time for the voltage follower. The major limiting factor was the charging time of the capacitance of the voltage electrode and headstage input through the electrode resistance in response to potential changes in the cell. This input capacitance was kept to a minimum in two ways. Initially, as much of the input system as possible was shielded, including the electrode, and was driven to the output potential of the first amplifier of the headstage, thus reducing the capacitative coupling between the electrode and earth. Secondly the input system was fed by a negative capacitance circuit via a 5.6pF capacitor. This circuit was simply a variable gain amplifier following the output of the first amplifier. Since the negative capacitance circuit supplied the voltage follower input through a capacitor it delivered current to the input in response to a change in the measured input potential. Consequently it helped to reduce the charging time of the electrode-input system in response to any step in cell potential. The gain of the negative capacitance amplifier was adjusted for each particular electrode to give the fastest response to a voltage step without oscillation.
Current measurement

The virtual earth was a simple current to voltage converter, which measured the current required to hold the bath at earth. It consisted of an operational amplifier whose negative input was connected to the bath, as well as to its own output via a 1 Megohm resistor. As a result, the amplifier attempted to hold the negative input and the bath at earth by passing a current through the feedback resistor. This current was equal to and opposite of that which flowed from the bath. Therefore, the voltage of the amplifier output was in direct proportion to the current being passed from the bath, 1mV representing 1nA. Under experimental conditions the only sources of current within the bath were the current and injection electrodes. Since they should all deliver current within one particular cell, the current being monitored by the virtual earth gives a direct estimate of the transmembrane current of the cell.

Voltage clamp

In the excitable cell the membrane potential and permeability, with respect to different ions, are highly interdependent. Using the voltage clamp technique the membrane potential can be regulated and its effect on transmembrane current studied. The method was first used on biological systems by Cole (1949) and was then improved by Marmont (1949) and Hodgkin, Huxley & Katz (1949) during work on squid axon. The basic circuit structure used for voltage clamp now has changed little from that used originally, except that modern electronic components have superseded the valve based amplifiers (see review by Moore and Cole, 1963).
The basis of the voltage clamp circuit used was as follows. The voltage follower, which measured the cell potential, was used to control the clamping amplifier, a high gain current source, which passed current into the cell via the current electrode. This formed a negative feedback loop whereby a change in the measured potential caused the clamping amplifier to change the current supplied to the cell, resulting in the potential being brought back to the original value. The potential at which the system attempted to hold the cell could be controlled by applying a voltage to one of the summing inputs of the clamping amplifier.

Both the holding potential and the pulses were controlled in this way. The magnitude of a step in membrane potential was directly proportional to the magnitude of a voltage pulse applied to the clamping amplifier input.

Pulses

The required pulse protocol for each experiment was produced by a digital pulse generator (Neale and Standen, 1977), capable of producing three pulses from 1 to 999 msec long, with a delay of between 0 and 999 msec. The amplitude of each pulse was reduced to an appropriate size for the clamping amplifier input by an active pulse divider.

Clamp speed

The speed of the clamp can be defined as the rate at which it can step the potential of the cell from the holding potential to a new fixed potential. The delay in the changing of the membrane potential is due to the slow charging of the cell membrane capacitance and is governed by three main factors:-
The voltage follower was set up for each electrode, by changing the negative capacitance, to give the fastest possible response time without excessive increases in noise and oscillation. The gain setting on the clamping amplifier was then used to adjust the gain of the negative feedback loop. It was set for each experiment to give the squarest possible membrane voltage pulse without signs of oscillation in either the current or the voltage trace. It was necessary to make this adjustment to compensate for differences in access resistance (i.e. current electrode resistance) and differences in capacitance associated with each particular cell and electrode set-up.

Intracellular injection

Two methods of injection were used during the experiments, iontophoretic and pressure injection.

Iontophoretic injection is injection by means of an electrical current which carries ions from an electrode into a cell. Although it is generally restricted to the injection of single types of ions it has the benefit over pressure injection that repeatable, and to a certain extent measurable, quantities can be delivered to the cell interior. The total number of ions injected is related to the amplitude and duration of the injection current by the equation:
\[
q = \frac{nI t}{zF}
\]

Where
- \( n \) = transport number
- \( I \) = injection current
- \( z \) = valency of the ion
- \( F \) = Faraday constant
- \( t \) = time

The transport number is the proportion of the total injection current that is carried by the ion under consideration and it is generally the greatest source of error in the calculation. Its value depends on both the mobility of the ion being injected and the other ions present in the system. Gorman and Hermann (1979) estimated the value for calcium ions being injected from an electrode containing 100mM calcium chloride to be approximately 0.3.

The injection electrodes were the same as the current electrodes used for the voltage clamp and they were filled with solution containing the ions to be injected at a concentration in the order of 100mM. For calcium injections it was found necessary to break the tips of the electrode to give tip diameters of 2 to 3 \( \mu \)m since the calcium had a tendency to block the electrodes. The current was delivered to the injection electrodes by an iontophoresis unit (W.P.I. model 160 or 161). The iontophoretic injection system also has a number of other benefits over pressure injection. Since the injected ions are carried by a current, any blocking of the electrodes can be seen as a reduction of the injection current. Also injection of ions into the bath solution rather than the voltage clamped cell, as a result of incomplete insertion of the electrode, is picked up by the virtual earth circuit producing shifts in the measured holding current during the injection.

Pressure injection was most often used to verify the effects
demonstrated by iontophoretic injection and to inject uncharged molecules and solutions containing a variety of ions. For these injections double-pulled electrodes with a steep taper were required. The pressure for injection was applied in pulses by a Picospritzer (General Valve Corporation) typically for 10 to 100 msec at pressures of 69 to 690 kPa, using a nitrogen gas cylinder as the source. The success of the pressure injection could only be evaluated by either visible increases in cell volume or the physiological changes resulting from the injection. The only estimates made of injected volume were from changes in the cell diameter measured under the microscope.

Ion sensitive calcium electrodes

Calcium electrodes were based on the sensor compound described by Oehme, Kessler and Simon (1976) containing the neutral carrier N,N',di(11-ethoxycarbonyl)undecyl-N,N'
4,5-tetramethyl-3,6-dioxaoctane diacid diamide as the calcium selective component. In these experiments a gelled form of the sensor compound in a poly(vinylchloride) (PVC) matrix (Tsien and Rink, 1981) was used to form electrodes which were highly selective for calcium over other common ions and gave accurate measurement of calcium concentrations down to below 10^{-7} Molar. The electrodes used to hold the sensor were prepared using a technique similar to that described by Tsien and Rink (1980).

The electrodes were pulled from capillary tubing with 1.5mm external and 1.05mm internal diameters (Clarke.E.I.). The glass was first cleaned in nitric acid heated by addition of ethanol, rinsed in distilled water and thoroughly dried in an oven. It was then pulled on a vertical Narashige puller giving electrodes with a tip diameter of about 1.0μm. This was increased to
approximately 2μm by gently breaking the electrode against a piece of flat ground glass, using a manipulator to advance the electrode to the glass. To improve the properties of the final electrodes (Tsien and Rink, 1930) the glass was silanised using Tri-N-butylchloro-silane (Pfaltz and Bauer). They were held vertically in an aluminium block in a deep glass dish and dried in an oven at 200°C for 15 minutes. 10μl of the tri-N-butylchloro-silane was then dripped into the dish which was covered with a lid. After a further 15 minutes the lid was removed to allow excess vapour to escape and then the electrodes were removed from the oven and left to cool.

Before the electrodes were loaded with the calcium-sensitive resin they were back filled with a pCa 7 (where pCa=-log[Ca]) solution (see table 1) using fine nylon tubing to deliver the solution as close to the tip as possible. It was then forced into the tip by exerting pressure with a syringe connected to the back of the electrode.

Preparation of the resin

Initially all the ungelled resin was mixed with approximately 14% w/w PVC. Prior to use a small portion was diluted using tetrahydrofuran (thf) to give a less viscous fluid and this was then drawn into the end of a fire polished capillary which was clamped to a microscope stage. The tip of a filled electrode was then inserted into the resin through the open mouth of the capillary and a column of sensor about 200μm long drawn into the tip using a 10ml syringe connected to the butt of the electrode. The resulting electrodes were then left to stabilise with the tips in pCa 7 solution, normally for approximately an hour, but occasionally over-night. It was found that the
electrodes tended to become less sensitive with time but that they also became less prone to potential drift.

Measurement and Calibration

The calcium electrodes produce a potential between the solution being monitored and the electrode solution that is approximately proportional to the pCa of the test solution (see fig. 2.4). During calibration the potential is measured between the calibration solution and the electrode. However, during intracellular recording the potential has to be measured with respect to the cell potential. In these experiments the potential was measured differentially using the outputs of the voltage follower and ion sensitive electrode amplifier. The voltage electrode was therefore used to measure the potential of the test solution during calibration and the cell potential during experiments, in order to minimise possible errors associated with potential offsets in the recording system. Due to the high resistance of the calcium electrodes their potential had to be monitored using a high impedance amplifier (Analog Devices 311J) which was connected to the electrode solution using chlorided silver wire. The system had a rather slow response time and high sensitivity to external noise and the signal was therefore heavily filtered before being monitored on a pen recorder.

Each electrode was calibrated before and after each experiment against 5 standard solutions made to the specifications given by Tsien and Rink (1980: see table 1), each containing 100mM KCl, 10mM pH buffer, 10mM calcium ligand and 5mM CaCl$_2$. The pH of the solutions was chosen to give a ratio of bound to unbound calcium ligand of about 1:1 so that the maximum
Figure 2.4

A typical calibration curve for the calcium ion sensitive electrodes. The dashed line is the theoretical Nernstian response (29mV/pCa) which gives an almost exact fit to the calibration curve down to pCa 6. Although the response of the electrodes tended to tail off below this value, they were still generally capable of giving quite accurate estimates of calcium concentration at values as low as 5 \times 10^{-8} M.
TABLE 2.1

The composition of the standard solutions used for the calibration of the calcium sensitive electrodes. The total calcium and calcium chelator concentrations are constant for all the solutions. The pCa of each solution was set by choosing pH values that give the desired apparent dissociation constant for the chelator with respect to calcium. This method ensured that there was always the maximum possible buffering capacity in each solution.

<table>
<thead>
<tr>
<th>pCa</th>
<th>CaCl₂</th>
<th>Chelator</th>
<th>KCl</th>
<th>Buffer</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>5</td>
<td>NTA</td>
<td>90</td>
<td>Hepes</td>
<td>7.39</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>NTA</td>
<td>90</td>
<td>Taps</td>
<td>8.42</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>HEEDTA</td>
<td>90</td>
<td>Hepes</td>
<td>7.70</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>EGTA</td>
<td>90</td>
<td>Mops</td>
<td>7.29</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>EGTA</td>
<td>90</td>
<td>Hepes</td>
<td>7.80</td>
</tr>
</tbody>
</table>

NTA      Nitrilo-tri-acetic acid  
HEEDTA   N-hydroxy ethyl ethylene diamine triacetic acid  
EGTA     Ethyleneglycol-bis-(beta-amino ethyl ether)N,N' tetra acetic acid  
Hepes    (N-2Hydroxyethylpiperazine-N' 2-ethanesulphonic acid  
Taps     (tris[hydroxymethyl] methylamino-propane sulphonic acid)  
Mops     3-(N-morpholino) propanesulphonic acid
buffering was available. The pCa for each solution was checked by recalculating the apparent stability constant for each ligand at the relevant pH values (O'Sullivan, 1969; Martell and Smith, 1974; Plant, 1982), taking into account the correction for 0.1M ionic strength solution (Tsien and Rink, 1980). Table 2.1 gives the exact composition of each standard solution used. Fresh electrodes gave a near Nernstian response for concentrations down towards pCa 7 with a smaller response between pCa 7 and pCa 8. Fig. 2.4 is a typical calibration curve for the electrodes.
PATCH CLAMP METHODS

Introduction

The patch clamp technique was initially developed by Neher and Sakmann (1976) to study single channel currents in biological membranes. They first used it to measure the unitary conductance of acetylcholine-activated channels in frog skeletal muscle. The basic technique relies on the electrical isolation of very small areas of membrane, in the region of 1\(\mu\text{m}^2\) or less, and the recording of the currents which pass across this membrane. The major problems which had to be overcome were the design of a recording equipment with high enough sensitivity but low noise, and the development of the seal forming techniques that produce high resistance seals in the gigohm range between a glass pipette and the cell membrane. Hamill, Marty, Neher, Sakmann and Sigworth (1981) described an improved version of the method which was capable of resolving changes in membrane current below 0.5\(\text{pA}\).

It was this technique that was used as a basis for the single channel recording set-up described here. Recordings were made from two different tissues, the snail neurone preparation already described and membrane vesicles formed from adult frog muscle fibres.

FROG PREPARATION

Apparatus

The experimental bath for the frog muscle preparation consisted of a Perspex block with a 2 by 2 cm hole milled from the centre. The bath base was formed from a large glass coverslip
which was glued in place and permitted the viewing of the preparation from the under side. Around the perimeter of the milled area a magnetic strip was inlayed into the upper surface of the Perspex giving firm but very flexible anchorage for the mounting pins used to hold the muscle, and the small pipes used to change the bath solution. Finally, a glass hook was mounted in a small manipulator, itself attached to the perspex block. This was used to support the centre portion of the muscle and lift it away from the base of the bath.

During experiments the bath was held onto the moveable stage of an inverting Nikon (DIAPHOT-TMD) microscope with Nomarski and phase contrast optics and up to x400 magnification. The headstage of the recording system was also mounted on the microscope stage, using a mechanical manipulator (Prior) to provide coarse adjustment and a Narashige hydraulic manipulator for the finer movements. The microscope was placed on an air table and enclosed in a Faraday cage to keep mechanical and electrical interference to a minimum. The hydraulic manipulator was found to be necessary to provide a means of moving the headstage without any direct contact with the instruments inside the cage. A curtain of brass chain at the front of the cage provided access to the microscope and bath.

Dissection

The cutaneous pectoris muscle was removed from the frog, Rana temporaria, with the small patches of connecting skin and cartilage left intact. The isolated muscle was then pinned out on a Sylgard base in a Petri dish and bathed in frog Ringer solution. The loose layers of connective tissue surrounding the muscle were gently teased apart and cut away leaving the muscle
as clean as possible without inflicting damage to the fibres themselves. Whilst the muscle was still pinned out in this dish it was put through contracture using a high, 120mM, potassium chloride solution. Once relaxed the muscle was transferred to the bath, being draped over the glass hook and fixed using magnetic mounting pins to hold the skin and cartilage, at either end of the fibres, to the glass bottom of the bath. The glass hook was then gently raised, lifting the centre portion of the muscle off the base and placing the fibres under slight tension.

Vesicle formation

The treatment used for the formation of the muscle membrane vesicles was an extension of that described by Neher, Sakmann and Steinbach (1978) for the cleaning of the muscle membrane.

The muscle was first treated with a solution containing collagenase (Sigma type 1A, 400 units/ml) for between 90 and 180 minutes, depending on the room temperature and the original state of the muscle. The solution was still based on the 120mM KCl, 1.8mM CaCl₂ and 5mM Tris maleate, pH 7.2, solution, used to put the muscle through contracture. This treatment had the effect of loosening the connective tissue surrounding the fibres but was not allowed to continue long enough to break the connections between the fibres and the supporting skin and cartilage. The second enzyme treatment was with a calcium-free protease-containing (Sigma type XIV, 0.2mg/ml) solution, consisting of 120mM KCl, 1mM K₂EGTA, 2mM MgCl₂, 5mM Tris maleate, pH 7.2. This enzyme was restricted to the centre portion of the muscle in order to preserve the connections at the fibre ends by means of a loosely fitting Perspex barrier placed over the muscle. The enzyme solution was dripped into the restricted area.
and enzyme free solution flowed through the rest of the bath, thus flushing away excess enzyme. About 20 minutes was found to be a sufficient time to completely free the centre portions of the fibres, producing clean areas of membrane. The whole preparation was finally washed with the enzyme-free, calcium-free solution.

Membrane vesicles formed both during the protease treatment and after the subsequent wash out. The sarcolemma could be seen to swell and bud off from the muscle fibres forming spherical vesicles of differing sizes, the largest reaching diameters of approximately 300µm. Visual observation of vesicle formation suggested that all the vesicles were outside out, no inversion of the membrane ever being seen.

Solutions

The solution for the frog preparation was based on that described by Adrian (1956), consisting of 115mM NaCl, 2.5mM KCl, 1.8mM CaCl$_2$ and 5mM tris maleate, pH 7.2. The high potassium solution used during the first part of the enzyme treatment had K$^+$ exchanged for Na$^+$ with a final concentration of 120mM KCl. The bath solution used during the experiments and for the final enzyme treatment was a calcium-free variation of this solution containing 120mM KCl, 1mM K$_2$EGTA, 2mM MgCl$_2$ and 5mM Tris maleate, pH 7.2.

Sodium channel recordings were made with normal saline solution, see above, in the pipette and potassium channel recordings with a solution having a combined concentration of KCl and NaCl of 120mM, 1.8mM CaCl$_2$ and 5mM tris maleate, pH 7.2. Where necessary, sodium channels were blocked with 3x10$^{-6}$ Molar Tetrodotoxin (TTX) in the bath and pipette solutions. All the
experiments were performed at room temperature, between 18° and 22°C.

Recording set-up

The recordings of single channel currents from the membrane vesicles were obtained using a LIST EPC-5 patch clamp amplifier and headstage. The experiments were performed on-line with a PDP 11/23 Digital computer using a 12 bit analogue to digital (A to D) and D to A converter (502, Cambridge Electronic Design Ltd.) for data recording and trigger pulse generation. Digital records were written direct to an RLO2 disc (see fig.2.5).Outlined below are the roles of the separate units and the differences between this system and the one used for recording from the snail neurones, (see later text).

1. The headstage acts as a current to voltage converter that produces a signal of 10mV per picoamp of current passing in the patch pipette. The input of the headstage, and thus the pipette, is held at a voltage specified by the command signal from the patch clamp amplifier. A small air capacitor surrounding the input also allows for analogue capacity subtraction. The headstage used for the recording from the frog preparation was provided with the LIST patch clamp. The headstage used in the snail neurone recording system was constructed by myself following the circuit diagram given in the appendix.

2. The patch clamp amplifier performs a number of separate roles. It sums the command voltages for the holding potential and any pulses, passing the resulting command to the headstage. This command voltage is also used to produce the capacity compensation which is also output to the headstage. A LIST EPC-5 patch clamp amplifier was used for the frog system and another amplifier
Figure 2.5

The layout of the frog vesicle recording system. Details of the set-up are given in the text.
based on a circuit by David Corey for the snail neurone recordings (see appendix). On maximum gain the LIST EPC-5 produced an output of 100mV per picoamp of current passed in the patch pipette and the Corey design amplifier 1000mV/pA.

3. A small amplifier used to adjust the amplitude of the voltage record before it was digitised.

4. An analogue leak subtraction system which when used with the EPC-5 subtracted a linear function of the voltage record from the current record. The Corey amplifier required the function of the voltage trace to be fed back into the output stage of the amplifier to perform the subtraction.

5. An eight pole Bessel filter with cutoff frequencies of 10, 5, 2, 1 or 0.5kHz. For the snail preparation a Frequencies Devices 8 pole Bessel filter with cutoff frequencies between 0 and 29.9kHz was used.

6. Tektronix dual beam oscilloscope for monitoring the voltage and current.

7. Cambridge Electronic Design (CED) 502 analogue to digital and digital to analogue converter, used to produce the digitised representations of the current and voltage recordings. In the case of the frog preparation it also produced the trigger pulses used to initiate each event. During recordings from the snail preparation the computer was not run on-line. The data were temporarily stored on a Racal (Store 4) tape recorder. By recording the trigger pulses it was possible to synchronise the digitising with the played back data.

8. PDP 11/23 computer running with the RT11 system. All programs for data capture and analysis were written in Fortran and incorporated a large number of CED subroutines. The majority of the original analysis programs were written by me or my supervisor Dr. N. B. Standen.
SNAIL PREPARATION

Dissection

The removal of the ganglia from the snail and their dissection was identical to that described for the voltage clamp experiments except that the enzyme treatment was extended, as described by Lux and Nagy (1981). The neurones were treated for 25 minutes with snail saline solution containing the enzyme protease (Sigma, type XIV) at room temperature. The whole preparation was then warmed to 35°C and treated with trypsin (10mg/ml, Sigma type III) in the normal snail saline solution for 1 hour before being returned to enzyme-free solution and room temperature.

Apparatus

The mechanical apparatus used for the patch clamp recording was essentially identical to that used for the snail neurone voltage clamp experiments with a few basic improvements and alterations. The electrical screening of the Faraday cage was improved and a chain curtain added to the front to reduce electrical interference. A single manipulator (Microinstruments) was used to hold the headstage. This manipulator was motorised in one direction, allowing the final movements of the headstage and pipette to be made from outside the cage. The original baseplate was also placed on an air table to reduce vibration picked up from the floor.
Recording set-up

The single channel recordings from the snail neurones were made using a patch clamp amplifier and headstage constructed at Leicester to the design by David Corey. A full account of the circuit is given in the appendix. These experiments were not carried out directly on-line with the PDP 11/23 computer, but the results were first recorded on a Racal(Store 4) tape recorder before being digitised and stored on disc. The triggering was performed manually using a Farnell stimulator, and the trigger pulses were recorded along with the current and voltage measurements so they could be used to synchronise the computer sampling with the data during the digitising of records, (see earlier text).

Patch pipettes.

The consistent production of good pipettes was found to be important for the formation of high resistance seals with low background noise and, therefore, care was taken with their fabrication. There were three basic tasks involved; the pipettes were first pulled, then coated with Sylgard, and finally fire polished. They were made from soft soda glass capillary tubing (Dade Capilets, microhematocrit tubes, nonheparinised) in preference to the harder borosilicate glass, normally considered to have better electrical properties, because soft glass is pulled more easily and is said to form seals more readily than the harder glasses (Corey and Stevens, 1983).
Pulling

The pipettes were double pulled on a Narashige or a Palmer vertical puller. The first pull used a high current setting and was stopped after a drop of approximately 5mm producing an hourglass shape in the capillary. The second and final pull used a low current setting thus producing a steeply tapered pipette with a relatively blunt tip. An estimate of the relative tip diameters was made by using a 10ml syringe to force air out of the pipette into methanol. The ml measurement to which the plunger of the syringe had to be depressed in order to blow bubbles was then taken as the bubble number measurement. This value became smaller with smaller tip diameters; bubble numbers between 4.0 and 6.0 were normally considered acceptable.

Sylgard coating

The second phase of fabrication was the Sylgard coating of the shoulders of the pipettes. Since the reduction of the pipette capacitance was of such importance, the hydrophobic elastomer was used to reduce the capacitative link between the pipette solution and the bath solution toward the electrode tip, where it is at its greatest. The Sylgard coat increases the distance between the two solutions and inhibits the formation of an aqueous film up the outside of the pipette above the bath solution level.

Semi-cured Sylgard was spread up to the tip of the pipette using a mounted needle and then cured by drawing this area of the pipette into the centre of a heated coil. Several layers were applied in order to obtain a well shaped final coating, since large quantities applied at the same time tended to form a large.
Fire polishing

Fire polishing the pipettes reduced the pore diameter at the tip and polished the glass surface, burning off any dirt and smoothing out irregularities. The pipette was advanced towards a glass bead on a red-hot platinum wire (Hamill, Marty, Neher, Sakmann and Sigworth, 1981). A gentle jet of air was directed at the surface of the bead to produce a steep temperature gradient, so making sure only the very tip of the pipette was softened by the heat from the bead. The degree of fire polishing was measured by re-evaluating the bubble number. Normally a reduction to a value between 2.0 and 3.0 was aimed at and often required more than one approach with the pipette.

Filling and mounting

The pipettes were filled by first drawing solution into the tip and then back filling with a very fine plastic tube. The filled pipettes were mounted in a holder which apart from being unshielded was of a virtually identical design to that shown by Hamill et al. (1981). The holder plugged directly into the headstage and electrical connection was made with the pipette solution by a fine chlorided silver wire.
Seal forming procedures

Once the pipette had been filled and mounted, positive pressure was applied to the barrel of the pipette via the holder. The pressure was controlled and monitored by means of a simple manometer. It was found to be important to maintain the positive pressure from before the time that the tip passed into the bath solution up to the point of seal formation. Failure to do so generally resulted in the pipette not forming a high resistance seal. This was believed to be a result of the pipette tip becoming dirty before the final approach to the membrane.

The resistance of the pipette was measured during the course of the seal forming procedure, using small (0.5mV) pulses of the pipette potential at a frequency of about 4 Hz. The consequent current deflection was then used to estimate the resistance.

Offset potential

Once the pipette was in the solution the recording system was set to zero. The auto-zero circuit in the patch clamp amplifier was used to bring the measured pipette current to zero by changing the command potential. With the pipette current at zero the pipette potential was also assumed to be zero and therefore an offset was applied to the voltage meter to bring its value to zero. It was discovered using a model that the majority of the offset potential was required to compensate for the offset of the head stage amplifier and the potential set up on the chlorided wire used to connect with the pipette solution.
Seal formation

The pipette was initially lined up with the area of membrane to be sealed to by using the coarse controls of the manipulator. The final approach was then made using the remote control of the hydraulic or motorised manipulator drives. The moment at which the tip came into contact with membrane could be detected by an increased pipette resistance, seen as a decrease in the current deflection. The pipette was pushed harder against the membrane until the resistance had at least doubled. At this point the positive pressure being applied to the pipette solution was changed to a suction of about 30 to 40 cm of water. It was at this stage that a seal would form during a successful approach. The electrode resistance could be seen to increase enormously with a simultaneous decrease in noise. The patch resistance was tested with a larger voltage pulse (up to 50mv). Seals with resistances between 10 and 30 gigohms could normally be formed, but on occasions values as high as 80 gigohms were obtained. After sealing, the suction was completely removed. Each attempt required the use of a fresh electrode since, once dirty, they rarely formed a seal.

The overall success of experiments appeared to vary considerably from day to day. For unknown reasons on some days seals would form readily, whilst on rather too many other days the pipettes would not produce any usable seals. On the whole, this great variation was felt to reflect differences in the preparation rather than differences in any other one thing. However, cleanliness was felt to be extremely important.
Analogue leak and capacity current subtraction

Leak subtraction

Leak subtraction before the digitising of the data was necessary in order to keep the variation in current, apart from that caused by channel opening, to a minimum, allowing the maximum possible range of the A to D converters to be used, thus maintaining their 12 bit accuracy. The leak was subtracted from the current trace by means of a simple amplifier that added an inverted form of the voltage trace to the current trace. By varying the amplitude of the voltage trace fed back to this system, it was possible to compensate for seal resistances as low as 2 gigohms. Any inaccuracies in this analogue subtraction were compensated for by the digital leak subtraction performed later (see below).

Capacity subtraction

The subtraction of the capacity current was important for two reasons. It reduced the peak currents, which would saturate the A-D converters, and more importantly, since the system worked by means of feeding current directly into the input, it also helped to reduce the rise time of the pipette voltage in response to a shift in the command potential.

In practice, adjustments to the capacity subtraction were a case of trial and error; the time constants and amplitude of the compensation being set for each separate seal to give the flattest possible current trace in response to a pulse.
Data capture and analysis

After the initial filtering and analogue leak and capacity subtraction, the data was transferred directly, or via a tape recorder, to the computer system. There were two separate forms of storage used for the different types of data.

The current data from the voltage activated channels was recorded as discrete events in arrays of digital values normally 256 or 512 elements long. The digitising rate could be varied, depending upon the time resolution and the total duration of sampling required.

The data from the potassium channels of the snail neurones had to be handled differently as the channel behaviour could be studied over long periods of time, at constant patch potentials. The current trace was digitised continuously, the resultant data points being put into large blocks 1000 elements long for ease of handling.

The majority of the data obtained from the frog vesicles was sampled at a rate of one point per 0.25, 0.5 or 1.0 msec with the current record being filtered at the respective Nyquist frequencies, that is at half the sampling frequency. It was later realised that the single channel data did not hold to the requirements for the Nyquist sampling frequencies (Colquhoun and Sigworth, 1983). Consequently, the later recordings made from the snail neurones were sampled at higher rates with respect to the filter frequency than the Nyquist relationship suggests.

Digital leak and capacity subtraction

The distinct open and closed levels of individual channel activity were extremely useful for the cleaning of the pulsed
records. By selecting events in which no openings occurred, it was possible to produce an array that represented the average current record when no openings occurred. The subtraction of this averaged array from the original records completely removed leak and capacity artifacts leaving traces that could be more readily analysed. Fig. 2.6 shows a series of records before and after this cleaning operation. The figure gives a few examples of the results of the digital leak and capacity subtraction performed on the digitised records. Fig. 2.6 A shows three typical digitised records in which the leak and capacity currents have been largely removed by analogue means. The remaining capacity currents can be clearly seen in the top trace in which the plotted data points were joined together with straight lines to emphasise the transients. Part B of this figure is a plot of the same three traces after the subtraction of an averaged trace. This averaged trace, which is shown in part C of the figure was formed from 16 traces in which no openings occurred.

The conversion of the records to a digital form results in a loss of the data between the sample points. This can be partially corrected using a computer routine which fits the data points to a cubic function (Colquhoun and Sigworth, 1983) and then calculates values for a number of intermediate points. The traces shown in part D of fig. 2.6 have been interpolated in this way to give four times the original number of data points.

Analysis

The records were primarily analysed in order to establish two basic types of channel behaviour, the unitary current amplitude and the kinetics behind the opening and closing of the channels.

Unitary current amplitude was measured using a histogram
Figure 2.6

Digital leak and capacity subtraction

A. A series of three records obtained from the vesicle preparation formed from frog muscle fibres. Leakage and capacity currents have been removed as far as possible by analogue compensation. In the top record the sampled data points have been joined together by straight lines to make it easier to see the remaining capacity current. Filter 1kHz, Sampled at 4kHz.

B. The same records as in A after digital subtraction of an averaged trace in which no channel openings occurred. As for A, the data points have been joined by straight lines in the top record.

C. The averaged record which was subtracted from the record in A to give those in B. The trace was formed by averaging 16 records in which no channel openings occurred.

D. Interpolation of data points using a cubic function as described in the text. The records are those in B, above, with the number of data points increased by a factor of 4.

*The records shown here were obtained by Dr. A. Spruce from the frog preparation with a 120mM Rb+ solution in the vesicles and bath. Unfortunately no records of my own were left uncleaned when this figure was drawn.*
forming technique in which the data points from large sets of records or sections of records were sorted into "bins" depending on the current amplitude at each point. This operation could be performed for both types of data, those sampled as discrete events and those sampled continuously. As can be seen in fig.2.7 the resulting histograms usually showed obvious peaks for the closed and open levels. The presence of more than one channel in a patch resulted in the formation of a series of open level peaks. Where possible the peaks of the histogram were fitted to a Gaussian distribution using an algorithm for least squares estimation of non linear parameters (Marquardt, 1963) in order to obtain estimates of the distribution means, standard deviations and, where applicable, the relative areas under the curves. The difference between the means of the closed and open distributions gave an accurate estimate of the unitary current amplitude. When the channel openings were especially brief and infrequent, the current amplitudes were estimated by eye from the original records.

Open and closed time measurements

The major difficulty encountered with this form of analysis was the recognition of openings and closings and the formulation of criteria that could be used for their identification. The basic criteria had to be the level of the current trace. Initially two cursors were used, one to recognise the openings, the other the closures. Obviously the amplitude of the unitary current with respect to the current noise of the open and closed levels seriously affects the final distributions of times. In an attempt to reduce this problem, for the analysis of the delayed rectifier and sodium currents recorded from the frog vesicles, a
Figure 2.7
A histogram of current amplitudes.

This histogram was formed by sorting the digital current values for 60 seconds of continuously sampled data from a membrane patch on a snail neurone. The patch was depolarised by 100mv and contained at least two channels. The peak to the left represents the distribution of points when all the channels were closed, the spread being caused by the background noise associated with the patch and the recording equipment. The peaks to the right represent the different open levels when 1 and 2 channels were open. The continuous lines are the three Gaussian curves fitted to the histogram using a least-squares method (Marquardt, 1963). The parameters were: (mean, standard deviation, area) 0 (0.00,0.11,5292), 1 (1.59,0.22,7860), 2 (3.11,0.31,1221). The estimated single channel current was 1.59 pA. The recording pipette contained snail Ringer. Filter 1kHz, sampled at 4kHz. Temp 18°C.
single cursor was used to identify the majority of the changes. The analysis of each record was checked by eye and omitted or incorrect openings and/or closings were then manually inserted or deleted as appropriate.
3. CALCIUM CURRENT INACTIVATION

Introduction

The primary aim of these experiments was to investigate the hypothesis that a raised intracellular free calcium concentration causes a reduction in the voltage activated calcium permeability of the snail neurone by a similar mechanism to that responsible for inactivation during a depolarisation. Fig 3.1 A shows a series of calcium currents activated by depolarising pulses ranging in amplitude from 10 to 120 mV. At membrane potentials up to +10 mV Plant and Standen (1981) have shown that the calcium currents are relatively free from contaminating outward potassium currents as a result of the presence of the potassium channel blockers tetraethylammonium chloride (TEA, 75 mM) and 4-aminopyridine (4-AP, 2 mM) which replace Na ions in the external solution. The inactivation of the calcium current can be seen as the decline of the current after its initial activation. At the higher depolarising potentials above about +10 mV the decline due to inactivation combines with the activation of outward currents to give an apparently very rapid decline in the inward current. In the experiments described here the membrane permeability responsible for producing this voltage-activated calcium current in snail neurones was studied using strontium as the current carrying ion. Plant (1982) has shown that 25 mM strontium saline solution, similar to that used here, produces larger currents with a slower rate of inactivation than those found in an equivalent calcium solution. Fig 3.1 B is a plot of the current-voltage relationship for one cell bathed first in a 25 mM Ca containing solution and then a 25 mM Sr
Figure 3.1
Calcium current-voltage relationship.

A. A series of records showing the calcium current activated by depolarisations from a holding potential (H.P.) of -50 mV to the potential shown. The two top traces show the duration of the depolarising pulses. The neurone was bathed in a 25 mM calcium containing solution. Temp. 21°C.

B. Plots of the peak inward current amplitudes at a number of potentials for one cell bathed first in 25 mM Ca (Q) and then in 25 mM Sr containing (Δ) solutions. H.P. -50 mV, T. 18°C.
containing solution (see methods). It shows a small increase in the current at the lower potentials and a shift in the current-voltage relationship of about -10 mV. For the purposes of the injection experiments described below an estimation of the strontium current at 0 mV was made using brief (20 msec) depolarisations to zero millivolts from a holding potential of -50 mV, giving inward currents close to the maximum size. Control experiments showed that during trains of these short depolarisations a 10 second delay between pulses was sufficient to allow for recovery from inactivation, giving constant current amplitudes over the whole series.

Calcium injection results

Iontophoretic injection of calcium ions was used to increase [Ca]$^{2+}$ and so study the resulting effects on calcium channel inactivation. In all the experiments where calcium ions were successfully iontophoretically injected into voltage clamped neurones a reduction followed by slow recovery of the peak inward strontium current was observed (Fig. 3.2). Control experiments using 25 mM calcium solution, instead of strontium, in the bath gave similar results producing a similar reduction of inward current in response to calcium injections. The average reduction in peak inward current, in strontium solution, from fourteen cells and eighteen injections for a 100 nA injection of calcium ions, of 10 seconds duration, was to $0.83 \pm 0.18$ ($n=18$) of the original current amplitude. Doubling this duration to 20 seconds produced an average reduction, for seven cells and eight injections, to $0.72 \pm 0.12$ ($n=8$) of the original. Using a value of 0.3 for the transport number (Gorman and Hermann, 1979) for the intracellular iontophoretic injection of calcium ions, it was
A. Records of membrane potential (above) and current (below) before (i), 5 sec. (ii) and 55 sec. (iii) after a 20 sec., 100 nA Ca injection. The currents were activated by 20 msec depolarisations to 0 mV from a holding potential of -50 mV. The second pulse in each record was a hyperpolarisation to -90 mV used to check for changes in the leakage current. 25 mM Sr containing solution, T. 17.5°C.

B. The time course of effect of Ca injection. The graph shows the peak inward current relative to its pre-injection value (I/I₀) as a function of time. The period of the 100 nA injection is shown at the top.

Figure 3.2
Effect of calcium injection.
possible to calculate the total quantity of calcium carried into the cell by injection currents of 100 nA for 10 sec and for 20 sec, the respective quantities being $1.55 \times 10^{-12}$ and $3.11 \times 10^{-12}$ mol. The actual increases in intracellular calcium produced by the injection are discussed later in the chapter. Fig. 3.2B shows the time course of the response to a typical calcium injection; the strontium current amplitude is expressed as a function of the mean current amplitude before the injection ($I/I_0$). Although the time course and general nature of the effects of calcium injections were similar in all the cells tried there was considerable variation between the maximum reduction obtained in different cells for identical injections. It was felt that the most likely causes of this variation were differences in the pattern of diffusion following a calcium injection and differences in the cells' abilities to sequester the calcium load. A smaller and less metabolically active cell is likely to have less buffering capacity, resulting in larger increases in intracellular free calcium concentration ($[Ca]_i$) for similar injected loads. Although there were large variations between responses to injections in different cells, identical injections into the same cell produced consistent results provided that the period of time between injections was sufficient to allow for full recovery. This ability to produce constant reductions in inward currents allowed the effects of changing the intensity and duration of the injections to be examined.

Fig. 3.3 shows the reductions in inward current, in one cell, created by a series of injections all of 10 second duration but of increasing amplitude. To avoid the effect of summation of inactivation created by the injections, the cell was left for over 6 minutes between injections and on all occasions the
Figure 3.3

The effect of amplitude of Ca injection

A. A plot of the peak inward current relative to its pre-injection value ($I/I_0$) as a function of time for four injections from a series of nine injections of increasing amplitude with periods of at least 3 minutes between injections.

25 mM Sr containing solution, H.P. -40 mV, depolarisations to 0 mV.

B. A plot of the maximum degree of reduction of inward current ($1-I/I_0$) against injection amplitude for all of the nine injections in the series.
inward current recovered to at least 0.95 of the current value before the injection. The plot of maximum reduction against injection current gives a good fit to a linear relationship, up until the largest 300nA injection, supporting the idea that there is a relatively direct relationship between the increase in $[\text{Ca}]_i$ and the reduction of the inward current.

The injection of calcium ions has been known to activate a potassium channel conductance for a number of years (Meech, 1972). Fig. 3.4 A. gives an example of the outward current activated by a 20 sec., 150 nA injection of calcium ions in a neurone bathed in normal snail Ringer. Gorman and Thomas (1980a,b) have demonstrated a linear relationship between $[\text{Ca}]_i$ and the peak calcium activated potassium current in *Aplysia* neurones for concentrations of up to 1.4 μM. The similarity between the time course of the two responses to intracellular calcium injection, the activation of outward current and the reduction of inward current, supports the idea that the calcium permeability is also mediated by $[\text{Ca}]_i$. Gorman and Thomas (1980a) found the same delay between the end of the injection and the maximum response when calcium injections were used to activate the calcium activated potassium current. This was explained as being due to the time taken for the diffusion of calcium from the tip of the injection electrode to the site of action of the calcium ions. They demonstrated that the delay before the maximum response could be significantly increased by forcing the tip of the electrode further into the cell cytoplasm away from the cell membrane, arguing that the difference reflected changes in the rate at which calcium reached the cell membrane. This also supports the assumption made earlier that the large variation in the effect of the calcium injection in different cells partly reflected changes in the rate at which calcium reached the cell.
Figure 3.4
The calcium activated potassium current.

A. A recording of the increase in outward current caused by a 20 sec. 150 nA injection of Ca ions into a neurone bathed in the normal snail Ringer in the absence of any potassium channel blockers and held at -50 mV. The period of the injection has been drawn above the trace. T. 17.5°C.

B. A graph showing the change in the holding current at a holding potential of -50 mV (O), caused by a 20 sec. 150 nA injection of Ca ions into a neurone bathed in 25 mM Sr Ringer also containing the potassium channel blockers TEA (75 mM) and 4-AP (2 mM). A selection of points from the recording shown in A (□) have also been plotted in order to demonstrate the blocking of the outward current by the potassium channel blockers. T. 18°C.
membrane. However, the similarity between the time courses of the two effects highlights one of the major problems that could seriously affect the results obtained here for the reduction of inward current. It was possible that the effect attributed to a reduction of inward current could equally be caused by the activation of an outward current with the same time course. For this reason a large number of control experiments have been performed to rule out this possibility (see later). During the experiments where inward current was measured the outward potassium currents were blocked by TEA (75 mM) and 4-AP (2 mM). The effectiveness of the block at the holding potential is demonstrated in fig. 3.4 B. The change in the holding current, for a cell held at -50 mV in the 25 mM Sr solution containing the potassium channel blockers, in response to a 20 sec., 150 nA injection of calcium ions, is plotted against time. It can be seen by comparing the plot with the plot of selected points from the outward current activated in a different cell held at -50 mV in normal snail Ringer in the absence of the potassium ion blockers, that the block of the current is virtually complete at least at the holding potential (a number of other control experiments are also shown later).

There are a number of major factors that will affect the relationship between the size of the injected load and the degree of reduction of the inward current. These may be divided into two groups: the factors that affect the proportion of the injected load that reaches the receptor sites responsible for inactivation, assumed to be on the inner membrane surface, and secondly the factors affecting the dose-response relationship between Ca\(^{2+}\) concentration and calcium channel inactivation.

Injection of strontium and barium ions instead of calcium
appeared to give similar results producing the typical reduction and recovery of the inward current. Fig. 3.5 shows the effect of a series of Sr injections into a single snail neurone. The average reduction of Sr inward current for 10 sec., 100 nA injections of Sr and Ba ions were $0.73 \pm 0.07$ (n=7) and $0.77 \pm 0.02$ (n=6) respectively. Since both barium and strontium have been shown to cause less inactivation when they are the permeating ion, one would expect injection of strontium or barium to cause a smaller reduction in inward current amplitude as opposed to the slightly larger effects obtained here. Two possible explanations of this anomaly are that the ions either displace calcium ions within the cell, thus causing a very similar rise in $[Ca]^+$ as a calcium injection, or that the cell has a lower buffering capacity for strontium and barium than for calcium allowing a greater increase in $[Sr]^+$ and $[Ba]^+$ than $[Ca]^+$ and thus compensating for the reduced effect of the ions on inward current inactivation. Brown, Morimoto, Tsuda and Wilson (1980) have reported that raised $[Ba]^+$ in perfused Helix neurones caused little inactivation of the calcium current which would suggest that the injected barium actually has its effect via the release of intracellular calcium. Meech and Thomas (1980) have also shown that pressure injection of BaCl$_2$ into Helix neurones causes release of intracellular calcium.
Figure 3.5
The effect of Sr injections.

A. The three traces at the top show the membrane potential (top) and currents (below) recorded before (i), 5 sec (ii), and 55 sec (iii) after a 10 sec, 150 nA injection of Sr ions. 25 mM Sr containing solution, H.P. -50 mV, depolarisations to 0 mV, T. 19°C.

B. The effect of the amplitude of Sr injection. The peak inward current is plotted relative to its pre-injection value (I/I₀) against time for a series of 10 sec Sr ion injections using different injection current amplitudes. 25 mM Sr containing solution, H.P. -50 mV, test depolarisations to 0 mV.
Control experiments

There are a number of possible processes by which the recorded reduction in inward current, demonstrated above, could be brought about other than as a result of the inactivation of the calcium channel by raised [Ca]_i. The most likely is the activation of an outward current by the injection which results in an apparent reduction of the inward current. The recording system only gives a measure of the total membrane current and, therefore, would combine an outward current with the inward current, producing a net reduction of the measured inward current. Control experiments were designed to isolate this possibility and a number of other mechanisms that could be responsible for the reduction of inward current other than as a direct result of raised [Ca]_i, for example artifacts of the injection technique or pH effects within the cell.

The activation of an outward current

If an outward current was activated by the calcium injection it could produce an apparent reduction in inward current, essentially identical to that observed in these experiments. It is well known that raised [Ca]_i can activate an outward current in many cell types. The calcium activated potassium current has been described specifically in molluscan neurones as a result of increases in [Ca]_i brought about by both influx of calcium as a result of membrane depolarisation (Meech and Standen, 1974, 1975) and direct injection into the cell (Gorman and Hermann, 1979, Gorman and Thomas, 1980a, Hermann and Hartung, 1982a). Fig. 3.4 shows how similar the time course of the effects of a
calcium injection on the outward currents is to the reduction of inward current. Therefore a number of control experiments were devised to check that the calcium activated current was satisfactorily blocked and that no other current was activated. Three main strategies were used: the complete removal of the inward current, the half block of the current and finally a change in the size of the test depolarisation. Initially, a change of the holding current was looked for and it was found that injections did not activate any outward current at the holding potential which had the same time course as the reduction in inward current (see above). This can be seen in the plot in fig 3.4 B. and by the steady level of the baseline in the current traces shown in figs. 3.2, 6, 7, 8. The possibility that there was activation of outward current during the test pulses was studied by removing the inward strontium current with the calcium channel blockers Cd$^{2+}$ or Co$^{2+}$ at concentrations of 1mM and 15mM respectively. Although these experiments revealed no activated outward current there was still the possibility that the calcium channel blocker also blocked any calcium activated outward current, even if this was activated by Ca injection.

For this reason the second set of control experiments were carried out. The effects of reducing the pre-injection inward current on the relative reduction in current caused by identical calcium injections were studied. Two methods of current reduction were used, either $6 \times 10^{-5}$M CdCl$_2$, a concentration close to the cited half block (7.2 $\times 10^{-5}$M, Kostyuk, 1980), was introduced into the bath between injections or the external strontium concentration was reduced from the standard 25mM to 2.5mM. These methods both lead to reductions of pre-injection current of over half and gave virtually identical results. Fig.
3.6 shows the reduction of inward current produced by a 10 sec., 200 nA Ca injection into a cell before and after 6 x 10^{-5} M Cd^{2+} was added to the bath solution and fig. 3.7 shows the effect of reducing the external Sr concentration between identical 20 sec. 100 nA injections of calcium. If the injections had activated any kind of outward current one would expect the relative reduction of inward current to be greater for the reduced current, assuming, of course, that the outward current was not reduced to the same extent, perhaps as a result of reduced strontium entry during the depolarisation.

The third type of control was designed to isolate any other permeability change brought about by the injection, other than the reduction in strontium permeability through inactivation of the calcium channels. Since the current-voltage relationship of the strontium current reaches a peak at a pulse potential to about 0 mV (see fig. 3.1), it was possible to select two potentials at which the current amplitudes were very similar, (see fig 3.1). It was shown that the reduction in inward current at both potentials was the same for similar calcium injections. If the injection was activating another permeability it would have to have a current voltage relation which produced current of the same size at the two different potentials for it not to affect these results. For example, any outward potassium current would increase in amplitude at the higher potential through an increase in driving force and would also be likely to have a potential dependent activation (Gorman and Thomas, 1980a) that would further increase the current amplitude.

An injection artifact

It was always possible that the effect of the injection was not
Figure 3.6
Effect of partial block of the inward current on the relative reduction of inward current caused by a Ca injection.

A. Records of membrane potential (above) and current (below) obtained from a cell bathed in 25 mM Sr containing solution before (upper series) and after (lower series) the addition of $6 \times 10^{-7}$M CdCl$_2$, which reduced the amplitude of the inward current to about 20% of its original value. In each case records are shown before (i), 5 sec. (ii) and 55 sec. after (iii) a 20 msec. 200 nA injection of calcium ions. The holding potential was -50 mV throughout, with test depolarisations to 0 mV. T. 17.5°C.

B. Plot of the peak inward current relative to its pre-injection value ($I/I_0$) against time for the injections shown in A before (□) and after (○) the addition of $6 \times 10^{-7}$M CdCl$_2$ to the 25 mM Sr containing solution.
Figure 3.7
Comparison of the effect of Ca injection on the inward current measured in 25 mM and 2.5 mM Sr Ringer solutions.

A. Records of membrane potential (above) and current (below) obtained from a cell bathed first in 25 mM Sr containing solution (upper series) and then in 2.5 mM Sr containing solution (lower series). This caused a reduction of the inward current to about 35% of its original value. In each case the records were taken before (i), 5 sec. (ii) and 55 sec. (iii) after a 20 sec., 100 nA injection of Ca ions. The holding potential was -50 mV throughout, with test depolarisations to 0 mV, T. 17.5°C.

B. A plot of the peak inward current relative to its pre-injection amplitude (I/I₀) against time for the injections shown in A. before (●) and after (○) the change of the bathing solution from 25 mM Sr to 2.5 mM Sr containing solutions.
related to the increase in $[\text{Ca}]_i$, but some other side-effect of the injection. The most obvious control was to inject calcium chloride by pressure injection as opposed to iontophoretic injection of calcium ions. On many occasions pressure injection produced effects very similar to the iontophoretic injections. However, it was impossible to say with certainty that on the occasions when the injection failed to produce a response, it did so because the electrode was blocked or not in the cell. The pressure injection system gave no indication as to whether the electrode was blocked when no resultant effect was recorded (see methods).

Another method of isolating artifacts of the iontophoretic injections was the injection of other monovalent and divalent ions.

The divalent ion used for the control injection was magnesium. Fig. 3.8 shows the time course of the response to a calcium injection and the responses to two similar magnesium injections into the same cell, one before the calcium injection and one after. The magnesium injections produced very different effects from the calcium injection, the reduction of the inward current was smaller than for the Ca injections and did not show any recovery over the few minutes after the injection during which the effect of the calcium injection was overcome.

Potassium ion injections of up to 200 nA for 10 sec. had virtually no effect on the size of the inward current compared to calcium injections in the same cells (see fig. 3.9 A). For example, a 10 sec., 100 nA injection of potassium ions produced a reduction in the current to 0.99 of the original value, 5 other injections of potassium gave similar results, whereas an identical injection of calcium ions, representing a considerably
Figure 3.8

Mg and Ca injection.

A. Records of membrane potential (above) and current (below) obtained before (i), 5 sec. (ii) and 55 sec. (iii) after a 10 sec., 100 nA injection of Mg ions (upper) and Ca ions (lower). The cell was bathed in 25 mM Sr containing solution with a holding potential of -50 mV throughout with test depolarisations to 0 mV. T. 21°C.

B. A graph of the peak inward currents relative to their pre-injection current against time, for the two injections shown above (Mg, Ca) and a third injection of Mg ions (Δ) of the same duration and amplitude in the same cell. The order of the injections was: Mg, Ca, Mg.
Figure 3.9
The effect of K and Na ion injection.

A. A plot of the peak inward current relative to its pre-injection amplitude (I/I₀) in response to 10 sec., 100 nA injection of K ions (□) and Ca ions (○) into the same cell. The cell was bathed in 25 mM Sr solution and held at -50 mV, the test depolarisations were to 0 mV. T. 18°C.

B. Plots of the peak inward currents relative to their pre-injection amplitudes (I/I₀) against time, in two different cells in response to 10 sec. injection of Na ions.
Cell 1, 50 nA (□), H.P. -50 mV, test depolarisations to 0 mV, T. 19°C.
Cell 2, 50 nA (△), 100 nA (○), H.P. -40 mV test depolarisations to 0 mV, T. 19°C.
smaller number of injected ions, reversibly reduced the current to 0.30 of the original. The high internal potassium concentration of the snail neurone results in the injected potassium having little effect on the total intracellular potassium concentration. A 10 sec., 100 nA injection into a cell 200μm in diameter would increase the total cell potassium by about 1mM, under 2 percent of the intracellular concentration, assuming a transport number of 0.4, (Gorman and Hermann, 1979). In contrast a control injection of sodium ions will cause a considerably greater relative change in the total cell sodium concentration as a result of the relatively low intracellular sodium concentration. The same calculation as for the potassium ions gives at least a 20 percent change in intracellular sodium, taking a value of 3.6 mM for [Na]₀ (Thomas, 1972). The results of three Na injections in two separate cells are shown in fig 3.9 B. The complete lack of any reversible reduction in inward current by sodium ion injections (n=16) in three separate cells, whilst acting as another good control was also slightly surprising since Rahamimoff, Lev-Tov and Meiri (1980) suggested that increases in intracellular sodium in nerve ending at the neuromuscular junction causes increased transmitter release as a result of release of intracellular calcium. It would appear these injections are either too small to provoke the same calcium release in the cell or that sodium ions do not affect the intracellular calcium store in the snail neurone in the same way. A release of calcium in the neurone would presumably cause a reversible reduction of inward current in the same manner as for a calcium injection.
Hydrogen ion injection

Meech and Thomas (1980) demonstrated that the injection of calcium chloride into snail neurones causes a release of H\(^+\) ions and a consequent lowering of intracellular pH. They estimated that in normal cells there is approximately a 1:1 ratio between H\(^+\) released and Ca\(^{2+}\) sequestered. Although the majority of the injections used by Meech and Thomas were much larger than those used here, it was felt necessary to establish that it was changes in [Ca\(_i\)] and not some concomitant pH change that caused the reduction of the inward current. Iontophoretic injections of H\(^+\) ions from electrodes containing 100mM HCl were used to establish the effects of intracellular pH changes on the inward current. As an example, see fig 3.10, a 100nA injection of H\(^+\) ions for 10 seconds produced a reduction of current to 0.98 of the original, which showed no signs of recovery over the 40 sec. after the injection, in a cell in which an identical injection of Ca\(^{2+}\) ions caused a reduction to 0.82 of the original amplitude from which the cell recovered to a current amplitude of 0.98 of the original. In all a series of 4 injections of 20, 40, 60 and 100 nA of H ions for 10 sec. produced reductions in inward current that showed no signs of recovery over the same time scale as the calcium injection and produced a maximum reduction to 0.92 of the pre-injection current. Since H\(^+\) ions have a transport number greater than 0.5 (Thomas, 1976) and a valency of only one the H\(^+\) injection represents a substantially greater number of H\(^+\) ions than Ca\(^{2+}\) ions.
Figure 3.10
Injection of H ions.

A. Recordings of membrane current obtained from cells bathed in 25 mM Sr solution before (i) and 5 sec. (ii) after 10 sec., 100 nA injections of H ions (upper traces) and Ca ions (lower traces). The cell was held at -50 mV and the test depolarisations were to 0 mV. T. 19.5°C.

B. A plot against time, of the peak inward currents relative to their pre-injection amplitude for the two injections shown in A. H ions (○), Ca ions (●).
EGTA injection and its effect on the response to Calcium injection

Another means of showing the direct nature of the action of raised [Ca]ᵢ after a calcium injection on the current reduction, was by the use of the calcium chelator ethyleneglycol-bis-(beta-amino ethylether)N,N',tetra acetic acid (EGTA). This binds with calcium at least 100 fold more strongly than to other ions (Martell and Smith, 1974) normally found within the cell. It has already been shown that EGTA injections can increase the inward calcium current in *Paramecium* (Brehm and Eckert, 1978; Brehm, Eckert and Tillotson, 1980) and in molluscan neurones (Eckert and Tillotson, 1981; Plant and Standen, 1982; Plant, Standen and Ward, 1983), the increase being attributed to a reduction of [Ca]ᵢ caused by the calcium buffering by the EGTA. Fig. 3.11 shows the increase in calcium inward current brought about by a 100nA injection of EGTA for 3 minutes. The current reaches a maximum at about 1.25 of its original amplitude. This is lower than the mean increase found by Plant, Standen and Ward (1983) because the EGTA injection was not continued until the current reached a maximum value. Fig. 3.12 shows the effects of 10 sec., 100 nA injections of calcium ions both before and after the EGTA injection. After the EGTA injection the normal response is much reduced and no longer shows recovery over the same time course. The simplest explanation is that the EGTA has buffered the large transient increase in [Ca]ᵢ normally caused by the calcium injection, only allowing a small rise in [Ca]ᵢ, but has maintained this increase for a longer period of time thus explaining the apparent lack of recovery from the injection.

Since the buffering of calcium by EGTA releases more H⁺ ions than the normal cellular buffer system (Gorman and Thomas, 1980)
Figure 3.11
The effect of an EGTA injection on the inward current amplitude.

A. Records of membrane potential (above) and current (below) obtained from a cell bathed in 25 mM Sr solution and held at -50 mV. The records were taken before (i), 45 sec.(ii), 105 sec.(iii) and 165 sec.(iv) after the start of a 3 min., 100 nA injection of EGTA. The last record (v) is the current just prior to the calcium injection shown in fig. 3.12 and was taken 135 sec. after the end of the EGTA injection. Test depolarisations were to 0 mV, T. 18°C.

B. The graph shows a plot of the peak inward current relative to its pre-injection amplitude \( I/I_0 \) against time during the EGTA injection shown in A. The last point (●) represents the current amplitude reached just before the following Ca injection (see fig. 3.12)
Figure 3.12
Effect of prior EGTA injection on the response to a Ca injection.

A. Records of the membrane potential (above) and current (below) obtained from a cell bathed in 25 mM Sr containing solution before (upper series) and after (lower series) the EGTA injection shown in fig. 3.11, which increased the inward current amplitude by about 25%. In each case the records were taken before (i), 5 sec. (ii) and 55 sec. (iii) after a 20 sec., 100 nA injection of Ca ions. H.P. -50 mV, test depolarisations to 0 mV, T. 18°C.

B. Graph of the peak inward current relative to its pre-injection value (I/I₀) against time, for the two injections shown in A. before (○) and after the EGTA injection (□) shown in fig 3.11.
this experiment shows by a different means that at least the major part of the reduction of current normally seen with a calcium injection is not due to a reduction of intracellular pH and supports the idea that the normal response requires a raised level of \([\text{Ca}]_i\).
Absolute intracellular calcium concentration and channel inactivation

In all the above experiments it has been assumed that calcium injections cause an increase in \([\text{Ca}^+]_i\). However, the experiments yield neither direct confirmation of this nor any information about the absolute value of \([\text{Ca}^+]_i\) and the sizes of the increases caused by the injections. In an attempt to gain some idea of the \([\text{Ca}^+]_i\), intracellular measurements were made using calcium ion sensitive electrodes. The electrodes were based on an ion sensitive gel and were made using the technique described by Tsien and Rink (1980). They were calibrated both before and after insertion into a cell and the results from any that showed excessive loss of sensitivity or, more commonly, excessive voltage drift during the experiment were ignored. However, the electrodes were never perfectly stable and therefore changes of less than about 10 mV/hour were assumed to have occurred steadily between the calibrations before and after each experiment, a linear change with time being used as a correction for the recorded electrode potential before conversion to intracellular calcium ion concentration.

For the six experiments in which the electrodes were most stable between the two calibrations the average resting intracellular calcium ion concentration was \(2.66 \pm 0.65 \times 10^{-7}\) Molar. Alvarez-Leefmans, Rink and Tsien (1981) using similar electrodes obtained a mean value \(1.70 \pm 0.25 \times 10^{-7}\) Molar for \([\text{Ca}^+]_i\) of snail neurones bathed in 2 mM calcium containing Ringer. They also showed that snails that had been hibernating for several months had significantly raised intracellular calcium levels. This may account for the small difference between their estimate and the one obtained here, since the snails used in
these experiments were kept in the hibernating state for a short period before use (see methods). It should be noted that the saline used for all the experiments in which the ion-sensitive electrodes were used contained 25 mM calcium as opposed to the strontium saline used earlier. This was to avoid any complications brought about by the electrodes' differing sensitivities to calcium and strontium. The change in external saline to a high calcium, sodium free solution could possibly have caused an increase in the resting \([\text{Ca}]_i\) through an increased influx or a disruption of any calcium removal mechanisms such as a sodium-calcium exchange system. Therefore, on a few occasions the calcium-sensitive electrode was inserted into the cell before the external medium was changed from the normal Ringer solution. In these cases no change in the measurement of \([\text{Ca}]_i\) over a 10 minute period was seen as a result of the change to the sodium free high calcium saline.

The electrodes were also used to follow the changes in intracellular calcium brought about by iontophoretic injection of calcium and by calcium loading with long depolarisations in the high calcium saline. Fig. 3.13 shows the potential recorded in the calcium sensitive electrode during a 100 nA calcium injection for 20 seconds. The increase recorded represents an increase of only 1.73\(\mu\text{M}\) compared to the calculated increase of 938\(\mu\text{M}\) for this cell (185 \(\mu\text{m}\) diameter). This represents an increase of only 0.2\% of the total injected quantity 20 seconds after the start of the injection. For injections of similar amplitude in \textit{Aplysia} neurones Gorman and Thomas (1980b) found that after a 30 second injection the peak rise in \([\text{Ca}]_i\), measured using arsenazo III, was 1.6\(\mu\text{M}\) per \(\mu\text{M}\) (0.16\%) of injected load, the figure being much higher at 2.0\%, about 400 msec after the start of the injection. The value of 0.2\% after 20 seconds obtained for these experiments
Figure 3.13
Recording of intracellular Ca$^{2+}$ during a Ca injection.

The recording from a Ca ion-sensitive electrode during the course of a 20 sec., 100nA Ca injection. The cell was bathed in 25mM Ca saline. H.P. -50 mV, T. 19°C.
lies between the above values, but it should be noted that due to the slow response time of the calcium electrode the peak increase in intracellular calcium and the recovery rate may be heavily influenced by the slow response time of the electrode. The electrodes generally required between 1 and 5 minutes to give a level response after a change of [Ca]_i from 1.0 to 0.1 µM during calibration.

Recovery from inactivation

If the inactivation of the calcium current is assumed to be totally dependent on the intracellular free calcium concentration then the recovery of the inward current from an injection of calcium will be related to the rate of return of the calcium levels to the resting concentration. If the levels of calcium are not uniform within the cell then it is the calcium concentration in the areas where the calcium-sensitive sites, responsible for inactivation, are situated that is important. In double-pulse experiments where the entry of calcium during the first pulse causes a reduction of the current seen during a second depolarising pulse, the recovery is normally fitted to a double exponential (Adams and Gage, 1979; Plant and Standen, 1981). The onset of inactivation has been modelled as being a result of the accumulation of calcium at the inner mouth of the calcium channel (Standen and Stanfield, 1982; Eckert and Chad, 1984). The recovery from inactivation can be considered to be a result of the return of these localised high calcium concentrations to the normal resting [Ca]_i. Using this model it is then possible to explain the differences in the onset and recovery from inactivation seen during double pulse and calcium injection experiments. In the 'domain' hypothesis (Eckert and
entry of calcium during a depolarisation causes the accumulation of calcium at the inner face of the calcium channel as a result of the slow diffusion of calcium from this point. It can then be seen that the removal of the calcium from this area by diffusion and sequestration could be responsible for the two time constants $\tau_1$ and $\tau_2$ seen during the recovery period. It is then obvious that the onset and recovery from inactivation caused by a calcium injection will not have the same time course. The onset of inactivation will be related to the rate at which calcium builds up at the receptor sites, assumed to be close to the inner mouth of the calcium channel. The injection, however, will produce a rather complex time course of action as a result of the time taken for the calcium to diffuse from the high point at the electrode tip to the site of action at the membrane. As can be seen in fig. 3.2, for a short period after an injection the degree of inactivation continues to increase, presumably because of the continued diffusion of calcium from the accumulation at the electrode tip. This idea is supported by the experiments of Gorman and Hermann (1979) who showed that the depth of an injection electrode in the cell significantly affects the rate of activation of the calcium activated potassium current by calcium injection. An increase in the depth of the electrode tip was found to cause an increase in the delay before activation. In these experiments the exact position of the electrode tip was unknown. The electrodes were pushed up against the cell until they just penetrated the membrane. It is assumed that this tended to put the electrode tip a short distance into the cell and therefore there will have been a difference in the time taken for injected calcium to reach different areas of the cell membrane. The recovery from inactivation after the initial complex onset should have followed the rate at which free calcium
was removed from around the receptors. Since the whole cell had been loaded with calcium rather than the small areas adjacent to the membrane the rate of recovery is likely to have been strongly influenced by the rate of calcium sequestration from the bulk of the cell cytoplasm. Fig. 3.14 shows a plot on a logarithmic scale of 1-\(I/I_0\), a measure of the reduction of the current after the injection, with respect to its pre-injection amplitude, for a range of the injections shown in Fig. 3.2 The fitted line over the recovery from the 150 nA injection has a time constant of 17.5 sec., close to the mean of 18.0 ± 1.2 sec. obtained from 17 separate injections. No sign of a faster time constant was found in experiments where the injection time was altered in relation to the test pulses. For double pulse experiments using Helix neurones Plant and Standen (1981) gave mean values of 121 msec and 9.4 sec for the two time constants of recovery \(\tau_1\) and \(\tau_2\). The slower time constant is about half the mean value for recovery obtained here. If it is assumed that the rate of diffusion from the area adjacent to the inner face of the calcium channel to the rest of the cell is at least as fast as \(\tau_2\), then when the concentration gradient is reversed, the constant for diffusion from the bulk of the cell to the inner mouth of the channel will be about the same. Therefore, since \(\tau_2\) is faster than the recovery from calcium injection, it appears reasonable to assume that the free calcium within the cell during recovery from an injection will be relatively evenly distributed and consequently the recovery of the calcium current after an injection will follow closely the rate of removal of calcium from the cytoplasm.

The two main areas of calcium sequestration in the bulk of the cell are the mitochondria, where calcium is actively taken up, and the buffering in the cytoplasm. The mitochondrial uptake
Figure 3.14
Recovery from Ca injection.

A graph showing the recovery of the peak inward current relative to its pre-injection current (1-I/Io) plotted on a logarithmic scale against time starting 5 sec. after the four 10 sec. injections shown in fig. 3.3 A. plus the results from a 60 nA injection from the same series of nine injections. The cell was bathed in 25 mM Sr solution, was held at -40 mV with depolarising test pulses to 0 mV.
of calcium has been shown to be an important process in the recovery of $[\text{Ca}]_i$ after a calcium load has been introduced into the cell. Meech and Thomas (1980) showed that the rate of decline of the calcium activated potassium current in Helix neurones was severely slowed in the presence of carbonyl cyanide m-chlorophenyl-hydrazone (CCmP), which has been shown to block calcium uptake by mitochondria in rat kidney (Vasington and Murphy, 1962). Figs. 3.15 and 3.16 show clearly the effect of a calcium injection after 2µM CCmP had been added to the bath, the degree of current reduction caused by the calcium injection is increased and the rate of recovery slowed. Comparison with fig. 3.14 shows how under normal conditions increasing the degree of inactivation, by increasing the size of the injection, causes an increase in the rate of recovery from inactivation, probably by further stimulating the sequestering mechanisms. However, the increased reduction after the addition of CCmP shows a slower rate of recovery, this effect of CCmP can be explained by a loss of the active uptake and possible reduction in the sequestering ability of the cell. Cells treated with CCmP tended not to recover as fully from inactivation created by calcium injection as cells bathed in the normal CCmP free 25mM strontium or calcium solutions.

Monitoring of Intracellular Calcium and Recovery

Under normal conditions the calcium-sensitive electrodes did not respond to changes of $[\text{Ca}]_i$ rapidly enough to follow the increase of $[\text{Ca}]_i$ caused by many long depolarisations, nor the subsequent return to the resting intracellular pCa levels. As a result, in attempts to correlate intracellular calcium changes and degree of inactivation, the calcium electrode records lagged
Figure 3.15
The effect of CCmP on the effect of injection of Ca ions.

A. Records of membrane potential (above) and current (below) obtained from a cell first bathed in 25 mM Sr containing solution before (upper series), and then 6 min. (middle series) and 12 min. (lower series) after the addition of 2μM CCmP to the bath solution. In each case the records show the current before (i), 5 sec. (ii) and 55 sec. (iii) after each Ca injection. H.P. -50 mV, test depolarisations to 0 mV, T. 19°C.

B. Plot of the peak inward current, relative to its pre-injection value (I/I₀) against time for the injections shown in A. Before (○), 6 min. (□) and 12 min. (△) after the addition of 2μM CCmP to the bath solution.
Figure 3.16

Effect of CCmP on the recovery on the inward current from Ca injection.

A graph showing the recovery of the peak inward current relative to its pre-injection current ($1-I/I_0$) plotted on a logarithmic scale against time after the three 20 sec. 80 nA Ca injections shown in fig 3.15, before ($o$), 6 minutes ($D$) and 12 minutes ($A$) after the addition of the 2μM CCmP to the bath. In each case zero time is taken as the end of the calcium injection. H.P. -50 mV, T. 19°C.
behind the measured changes in the inward current amplitude. However, by using the slowing effect on recovery of CCmP it was possible on one occasion to load a cell with calcium, using long depolarisations, and follow the steady recovery of both $[Ca]_i$ and the inward current. Fig 3.17 shows the record from the calcium electrode in a cell loaded by many 900 msec depolarisations to 0 mV. The pulses were repeated until the inward calcium current at the start of each pulse was close to zero. The bottom half of the same figure is a plot of $I/I_0$, the current amplitude obtained using periodic 20 msec, 50 mV depolarisations relative to its value before loading. Plots of the two recoveries on logarithmic scales, shown in fig 3.17 give very similar time constants of approximately 1.5 minutes implying that the electrode was able to keep up with the recovery of $[Ca]_i$. Since the loading was brought about by influx of calcium during depolarisations there may have been fast components in the recovery of the current. However, with fast time constants in the same range as those seen by Plant and Standen (1981) the fast recovery will have been over well before the first test pulse was applied to the cell, 40 seconds after the last loading pulse. The recovery seen after the very severe loading was assumed to represent the recovery of $[Ca]_i$ in the bulk of the cell rather than the localised effects seen during double pulse experiments. It was also assumed that with such a slow rate of recovery $[Ca]_i$ at the inner membrane surface would be very similar to that in the rest of the cytoplasm. By correlating the reduction of the inward current with the measured $[Ca]_i$ it was possible to construct a dose-response relationship between $[Ca]_i$ and the degree of calcium channel inactivation. In order to compare the relationship with the one obtained by Plant, Standen and Ward (1983) using injection of EGTA/calcium buffers a number of
Figure 3.17

$[\text{Ca}]_i$ during Ca loading of a cell.

A. A record of intracellular calcium, measured using an ion sensitive electrode, obtained from a cell loaded using 900msec depolarisations to 0mV. The capacity artifacts caused by the pulsing of the cell can be seen on the rising phase of the trace. The cell had been treated with 10μM CCmp before this calcium loading. 25 mM Ca$^{2+}$ containing solution. H.P. -50 mV, T. 19°C.

B. The recovery of $[\text{Ca}]_i$ and of inward current after Ca loading. A plot on a logarithmic scale of the difference between the measured $[\text{Ca}]_i$ and the resting level (taken as $4.5 \times 10^{-7}$M) after the loading shown in A. ($\square$), and of the recovery of the peak inward current relative to its pre-loading amplitude $(1-I/I_0)(0)$ measured using periodic brief depolarisations to 0mV. The time constant of the two fitted lines are 1.4 and 1.6 minutes for $[\text{Ca}]_i$ and current amplitude respectively.
assumptions were made. The normal current amplitude for this cell was assumed to be that measured at the beginning of the experiment before treatment with CCmP. The maximum current amplitude for the cell \(I_{\text{max}}\), the value when no inactivation is present, was then calculated using the mean increase from the normal current amplitude caused by EGTA injections of 31.1% (Plant, Standen and Ward 1983). This same principle was then applied to the points for buffer injections with 500mM Hepes taken from fig. 10 of Plant et al (1983) and a new value for \(I/I_{\text{max}}\) calculated for both sets of results. The values obtained by the two different methods complement each other relatively well giving a not unreasonable approximation to the theoretical curve drawn for a 1:1 ratio of calcium ions to receptor with a \(K_d\) of \(4.8 \times 10^{-7}\) Molar. Plant (1982) calculated a \(K_d\) for the calcium receptor using just the mean increase seen with EGTA injections and the resting calcium concentration and got a value of \(6.7 \times 10^{-7}\) Molar, not dissimilar to the value obtained here. Two other points added to the plot in fig. 3.18 were obtained from experiments in which the calcium electrode responded too slowly to follow the recovery of the intracellular calcium concentration. Therefore the only points that could be taken were at the turning point of the calcium measurement. The measured peak \([\text{Ca}^+]_i\) was matched with the degree of inactivation of the inward current, and the results were converted in the same manner as those already plotted in fig. 3.18. The two points both fit quite convincingly with the original data plot.

The estimation of the \([\text{Ca}^+]_i\) level within the cell and its effect on calcium channel inactivation offers a simple method of evaluating the effect of raised \([\text{Ca}^+]_i\) on the inward current caused by a reduced driving force for \(\text{Ca}^{2+}\) ions across the membrane. Drawn in on fig. 3.18 is the expected reduction of
A plot to show the relationship between the degree of inactivation of the calcium current and the intracellular calcium concentration. Data from two types of experiments are included in the graph. $I/I_{\text{max}}$ was calculated assuming that $I_{\text{max}}$ was 1.31 times greater than the inward current amplitude before the cell had been loaded with Ca or put in CCMp containing solution (see text) and $I$ was the current amplitude measured at each $[\text{Ca}]_{i}$. One set of values ($\bullet$) was obtained from the recovery experiment shown in figure 3.18. Two other points ($\Delta$) were obtained from two other similar recovery experiments (see text). Finally, the results obtained by Plant, Standen and Ward (1983) ($\square$) from experiments in which $[\text{Ca}]_{i}$ was changed by injection of EGTA/Ca buffer solutions were added to the graph.

The solid line drawn over the data is the theoretical dose response curve assuming 1:1 binding with a dissociation constant of $4.8 \times 10^{-7}$M.

The broken line shows the relative changes in current amplitude that are predicted by the constant field equation (see below) to occur simply as a consequence of the rise in $[\text{Ca}]_{i}$ reducing the driving force on Ca ions, assuming a resting $[\text{Ca}]_{i}$ of $2.7 \times 10^{-7}$M and a $[\text{Ca}]_{o}$ of 25 mM.

$$I_{\text{Ca}} = \frac{V F_{\text{Ca}}}{RT} \left[ \frac{[\text{Ca}]_{i} e^{(2 VF/RT)} - [\text{Ca}]_{o}}{e^{(2 VF/RT)} - 1} \right]$$
inward current at OmV assuming a constant membrane permeability, using the constant field equation (see fig. legend). No correction was made for the effect of this reduction of current on the original results because it was so small compared to the accuracy of the intracellular calcium measurement and the assumptions made about the maximum current with no inactivation present.
Introduction

In recent years metabolic control of the calcium permeability has been suggested by several workers to explain certain of the calcium current characteristics in neuronal membranes. It is now generally accepted that the calcium permeability of cardiac tissue is strongly influenced by changes in intracellular cyclic adenosine monophosphate (cAMP) levels brought about by changes in adenyl-cyclase activity primarily in response to adrenaline and noradrenaline concentrations in the extracellular solution. Reuter (1974) suggested that the activity of the calcium channels could be controlled indirectly by cAMP via protein kinase phosphorylation reactions causing an increase in calcium uptake by the sarcoplasmic reticulum, thus affecting the intracellular free calcium concentration. More recently certain workers (Doroshenko, Kostyuk and Martynyuk, 1982; Cachelin, de Peyer, Kokubun and Reuter, 1983; Sperelakis, 1984) have suggested that the calcium channel activity may be controlled directly by enzyme phosphorylation of the channel protein.

During perfusion experiments using *Helix pomatia* neurones (Doroshenko, Kostyuk and Martynyuk, 1982) it was found that the calcium currents suffered from some form of wash out. The currents were shown to decline steadily during intracellular perfusion. Byerly and Hagiwara (1982) found that the decline of the current in *Lymnaea stagnalis* was accelerated by increasing the perfusion rate, as measured by an increased rate of intracellular ion exchange. They also attempted to retain the calcium channel activity by introducing cAMP, ATP, phosphate and
Ms²⁺ to the perfusate and found that certain ATP-containing solutions could arrest the calcium current decline for a short period but that eventually the decay resumed. (All their intracellular solutions contained 5 mM EGTA which was assumed to maintain the [Ca]ᵢ below 10⁻⁸ M.) Doroshenko et al. (1982) used a larger variety of intracellular perfusates and discovered that cAMP, ATP and Mg²⁺ ions all played a significant role in arresting the decline of the current but again found no solution that could permanently maintain the calcium current in the perfused neurones, suggesting that this could be due to the wash out of essential enzymes or co-factors. Both Byerly and Hagiwara (1982) and Doroshenko et al. (1982) suggested that the decline was due to the loss of some metabolic activity required for the maintenance of active calcium channels, naturally drawing parallels with the cardiac system, in which the neurotransmitter control of the calcium permeability is mediated by intracellular cAMP (Reuter, 1974, 1983).

Further evidence that suggests that the channel requires some specific factor within the cell for its normal functioning comes from patch clamp results. Fenwick, Marty and Neher (1982) recorded single channel calcium currents in bovine chromaffin cells using the patch clamp technique and discovered that the channel activity was completely lost from the patch within a few seconds of excising the membrane from the cell. They assumed that this was the result of the loss of some specific factor that is present within the cell.

There are now many descriptions of experiments using intact molluscan neurones under voltage or current clamp which suggest that there is considerable metabolic control of the membrane
permeability by cAMP levels via a chain of enzyme phosphorylation reactions. However, it must be said that there is enormous variation in the types of responses found by different workers using different means of affecting intracellular cAMP systems. The most commonly reported effect of raising the cAMP levels is the activation of an inward calcium current that increases the rate of firing in a bursting molluscan neurone (Treistman and Levitan, 1976; Castellucci, Kandel, Schwartz, Wilson, Nairn and Greengard, 1980; Pellmar and Carpenter, 1981; Green and Gillette, 1983; Kononenko, Kostyuk and Shcherbatko, 1983). The calcium activated potassium current of snail neurones has also been shown to be altered by changed cAMP levels or as a result of injection of cAMP-dependent protein kinase. Ewald and Eckert (1983) have described an increase in the current in Aplysia as a result of exposure to cAMP analogues whereas Gillette, Gillette and Davis (1982) have reported a cAMP-induced effect that appears to reduce this current. Injection of the catalytic subunit of the cAMP-dependent protein kinase has been shown to increase the calcium activated potassium current in perfused Helix neurones (de Peyer, Cachelin, Levitan and Reuter, 1982) and reduce an undefined potassium current in Aplysia (Kaczmarek, Jennings, Strumwasser, Nairn, Walter, Wilson and Greengard, 1980). The experiments described here were designed to investigate the effects of changing intracellular cAMP concentration on the voltage activated calcium current. Kononenko, Kostyuk and Shcherbatko (1983) have already shown that cAMP injection can reduce this calcium current in intact Helix neurones in contrast to the results obtained in perfused Helix neurones (Doroshenko et al., 1982).
Results

The first experiments performed were designed to look at the effects of simple pressure injection of cAMP on the voltage-activated calcium permeability of the intact snail neurone. Injection electrodes containing 10mM Na cAMP solution were inserted into voltage clamped *Helix* neurones bathed in the standard 25mM Ca\(^{2+}\) Ringer solution (see methods). This removed both the sodium current and potassium currents which would normally be present during the depolarisations to 0 mV used in these experiments (Plant and Standen, 1981; see also chapter 3). The calcium current was then measured by using depolarising pulses to 0 mV every 30 seconds. Using a variety of injection pressures and durations from 140 kPa for 10 msec to 700 kPa for 900 msec to inject the Na cAMP into the cell, no reproducible change in current amplitude could be found except after the largest injections when the current was temporarily reduced. Since estimates of intracellular cAMP levels normally set the concentration at about 10\(^{-6}\) M (Robison, Butcher and Sutherland, 1971) it was thought that these effects were not due to a specific action of cAMP. To increase the cAMP concentration 100 fold from 10\(^{-6}\) M would require an injection of only one hundredth of the cell volume. However, since the cells visibly swelled after the largest injections giving up to a 10% increase in diameter, it would appear that the larger injections represented increases in cAMP concentrations well outside the physiological range.

A second approach to the problem was the application of the phosphodiesterase inhibitors Iso-butyl-methyl-xanthine (IBMX) and theophylline to prevent the breakdown of cAMP. However, neither
of the two drugs was found to significantly change the calcium current amplitude over long periods of exposure to 0.5 and 2.0 mM IBMX (n=2) and theophylline (n=3) respectively.

A third method was the use of the cAMP analogue di-butyryl cAMP which will readily cross the cell membrane and is converted to cAMP within the cell via enzyme action. 1mM di-butyryl cAMP added to the bath produced no obvious effect on two occasions and again produced no effect when used in conjunction with 2 mM theophylline in the bath to inhibit any compensating breakdown of cAMP within the cell. Fig. 4.1 shows the steady current amplitude maintained over the 40 minutes after the application of di-butyryl cAMP and then 15 minutes with theophylline as well.

A fourth technique was to stimulate the production of cAMP within the cell by means of injected fluoride ions. F$^{-}$ has been shown to be a potent stimulant, increasing the production of cAMP by adenyl cyclase up to 10 fold in in vitro experiments with cell fractions from many different tissues (Robison, Butcher and Sutherland, 1971). In these experiments the F$^{-}$ ions were injected iontophoretically from an electrode containing 100 mM NaF. Fig. 4.2 gives two typical responses to F$^{-}$ injections showing a slightly delayed increase in inward current followed by a sharp decline (see also fig. 4.3). The subsequent block of the calcium channels by intracellular F$^{-}$ has been found in tunicate egg (Takahashi and Yoshii, 1978) and in *Helix* neurones. Doroshenko et al. (1982) noted that in *Helix* neurones intracellular perfusion with F$^{-}$ containing solution caused an increase in the calcium current before producing the block. They explained this as being a result of the stimulation of the adenyl cyclase system.

One final assault on the cAMP system of the cell was the
Figure 4.1
The effect of di-butyryl cAMP and theophylline on inward current.

The amplitude of the inward calcium current, relative to its original current value ($I/I_0$), from a cell pulsed to $0 \text{mV}$ every 30 sec is plotted against time. The first arrow represents the time at which di-butyryl cAMP was added to the bath solution, to give a final concentration of 1 mM. The second arrow marks the time at which theophylline was added giving a bath concentration of 2 mM. 25 mM Ca containing solution, H.P -50 mV, T 18°C.
Figure 4.2
The effect of fluoride injection on inward current.

Two examples of the changes in current amplitude relative to its initial value \( (I/I_0) \) brought about by iontophoretic injection of fluoride ions. In the first case a continuous 25 nA injection was used. In the second example three separate 25 nA injections 5.5, 3.5 and 5.0 min. long, were used. 25 mM Ca containing solution, H.P -50 mV, test depolarisations to 0 mV, T 18°C, in both cases.
application of the drug forskolin. This has been shown to be a potent stimulator of cAMP systems in a number of tissues (Seamon and Daly, 1981; Rugg and Simmons, 1984). Forskolin was added to the bath solution during a series of depolarisations used to measure the inward current amplitude. Three attempts at adding 100μM forskolin proved unsuccessful at stimulating any significant changes in current amplitude.

Preliminary experiments with the calmodulin binding drugs chlorpromazine, trifluoperazine and pimozide were unsuccessful partly because of the membrane destabilising affects of the drugs. They appeared to have no effect at concentrations up to 200, 100 and 0.1μM respectively but caused large increases in the leakage current at concentrations significantly above these values.

Discussion

As a whole the experiments presented here do not appear to support the hypothesis that this particular calcium permeability is under the influence of a cAMP mediated system within the cell, except perhaps for the fluoride injection experiments. I feel that there are two possible explanations for the fluoride effect.

The first is that suggested by Doroshenko et al. (1982); fluoride ions stimulate adenyl-cyclase activity and increase the intracellular cAMP concentration, this then leads to an increase in the number of calcium channels available to be activated by a depolarisation of the membrane. The second mechanism is by a direct reduction of [Ca]_i caused by precipitation of CaF_2. However, I find the first mechanism difficult to accept for two main reasons. The most persuasive is the failure of all the other experiments, designed to alter cAMP levels, to cause any
reproducible change in inward current amplitude, even though the techniques used have all been shown to have effects on cAMP mediated systems. There is also the problem of the reduction of $[\text{Mg}]_i$ by precipitation as $\text{MgF}_2$ (see below) when it has been shown that phosphorylation reactions using ATP require $\text{Mg}^{2+}$ ions as a co-factor. It is therefore, difficult to envisage a mechanism mediated by cAMP that could cause an increase in channel activity at these very low Mg concentrations, (see below).

The second mechanism is a direct effect of the reduction of $[\text{Ca}]_i$ by intracellular $\text{F}^{-}$, in a similar fashion to the reduction caused by EGTA injections. Since calcium fluoride and magnesium fluoride are both virtually insoluble the injection of $\text{F}^{-}$ ions will significantly reduce the total free $[\text{Ca}]_i$ and $[\text{Mg}]_i$, the solubility products for the two compounds being $8.61 \times 10^{-12}$ and $1.82 \times 10^{-9} \text{ mol. dm}^{-3}$. A fluoride injection would, therefore, reduce the $[\text{Mg}]_i$ to about 57 $\mu\text{M}$ before beginning to lower $[\text{Ca}]_i$ to values below the estimated mean concentration of 0.27 $\mu\text{M}$ (see chapter 3). If the concentration of Mg within the cell is assumed to lie between the estimated free Mg concentration of 0.66 mM (Alvarez-Leefmans, Gamino and Rink, 1984) and about 6 mM, the total Mg content of squid axon (Baker and Crawford, 1972) then the equivalent of between 1.2 and 12 mM $\text{F}^{-}$ ions must be injected to cause a reduction in $[\text{Ca}]_i$. Taking a cell with a 200 $\mu\text{m}$ diameter, this would require between 24 and 240 seconds using a continuous 25 nA injection and assuming a transport number for $\text{F}^{-}$ ions of 0.4. This could explain the delay seen after the start of an injection before the rise in inward current.

Comparison between the change in current time course during a single depolarisation especially at the higher potentials, after EGTA injections, and the change brought about by fluoride
injection also supports the idea of a similar mode of action of the two anions within the cell. Fig. 4.3 consists of three sets of traces from a fluoride injection experiment, each set is an example of the varying current amplitude and shape with different size depolarisations. Before the injection of fluoride the calcium currents appear to inactivate relatively normally and become partially contaminated by outward potassium current at the higher depolarisations (Plant and Standen, 1981; Heyer and Lux, 1976), (see fig. 3.1), most probably the calcium activated potassium current. The injection of fluoride ions can be seen to reduce both the inactivation of the current and the contaminating potassium current, and increases the peak amplitude of the inward current. This is virtually identical to the change in the current seen as a result of EGTA injections which are assumed to act by reducing [Ca]_{i}, and by buffering changes in [Ca]_{i} thus reducing inactivation (Plant, Standen and Ward, 1983. Eckert and Chad, 1984).

In conclusion it appears that the alteration of cAMP has no effect on the calcium current, at least studied under these conditions, and that the increase in current brought about by F\(^{-}\) injection is most likely to be a direct consequence of the precipitation of CaF\(_{2}\).
Figure 4.3
The effect of fluoride injection on the inward current.

Three sets of current traces taken from a single cell using 100 msec depolarisations to -20, 0, 20 and 40 mV from a holding potential of -50 mV. The sets were taken before a fluoride injection (i), at the peak of the current increase 5 min. after a 4 min., 50 nA injection (ii) and 1.5 min. after a second 4 min., 50 nA F⁻ injection (iii). 25 mM Ca containing solution, T 18°C.
5. SNAIL NEURONE SINGLE CHANNEL RECORDING

The primary aim of these experiments was to identify and characterise a number of the different types of channel activity found in the snail neurones. From the recordings of macroscopic currents in this preparation there appear to be five major currents that are activated, either directly or indirectly, by depolarisation of the membrane. The two inward currents present are the sodium and calcium currents which are both voltage activated (Chamberlain and Kerkut, 1969; Standen, 1975b; Kostyuk and Krishtal, 1977). The three major outward currents are all potassium currents but show different activation kinetics. Two are purely voltage activated; the early outward (A) and delayed rectifier currents, but can be separated by their very different activation and inactivation kinetics (Connor and Stevens, 1971; Neher, 1971; Thompson, 1977). Finally, there is the calcium activated potassium current described by Meech (1974), and Meech and Standen (1975) that is primarily activated by raised intracellular calcium concentrations but also shows a certain degree of voltage dependence (Hermann and Hartung, 1983).

A number of different channel types were observed in these experiments when recordings were made with normal snail Ringer in the patch pipette. However, only one of the channel types was studied to a sufficient degree to give an idea of its identity in terms of the macroscopic currents present in these cells.

Results

Fig. 5.1 shows a number of sections of current record exhibiting this type of unitary current. In this case the patch, which was cell attached, was depolarised by 30 mV from the
Figure 5.1
Single channel potassium currents.

Examples of the unitary potassium currents obtained from a patch on a snail neurone. The membrane was held at a depolarised potential 30 mV from the cell resting potential. The pipette contained 5 mM potassium Ringer solution. Filter 1 kHz, sample frequency 4 kHz, T.18°C.
resting potential. The channels were generally found to be more active when the patch membrane was depolarised and showed no apparent inactivation over a long period of depolarisation of the membrane. The figure also shows that the channel openings occurred in bursts.

Channel Conductance

The unitary current amplitude was measured with normal (5 mM), and high, (40 mM and 80 mM), potassium Ringer solution in the pipette, potassium being exchanged for sodium on a mole for mole basis to produce the high potassium solutions. The unitary current amplitudes were estimated from histograms of current amplitude, a typical example of which is shown in fig. 5.3. On a number of occasions the reversal potential of the current was obtained using ramped changes of the patch membrane potential. This was found to be most useful when the channels showed little activity at potentials below the reversal potential. By applying a hyperpolarising ramped potential to a depolarised patch it was possible to catch the channel in the open state even at potentials at which the activity was otherwise rarely seen. A number of the current voltage relations for separate patch recordings are shown in fig. 5.2. It should be noted that the voltage axis of the plot represents the negative of the pipette potential, and not the patch potential, which would be the negative of the pipette potential plus the resting potential of the cell. As a result one sees shifts in the plots of current amplitude along the voltage axis depending on the potential and intracellular potassium concentration of the cell. With 5mM potassium solution in the patch pipette the average estimated single channel slope conductance was $14.3 \pm 0.4\text{pS (n=4)}$. This
Figure 5.2

Single channel current voltage relationship.

A The current voltage relationships for the unitary potassium current seen in three patches formed with 5 mM (○), 40 mM (△) and 80 mM (○) potassium containing solution in the pipette. The voltage axis gives the potential by which the patch was depolarised from the cell resting potential. T.18°C.

B. The theoretical current given by the constant field equation (see below) taking the internal potassium concentration to be 90 mM and the external concentrations as quoted above and assuming a permeability to potassium of 5.5 x 10^-18 cm^3 sec^-1.

\[ I_K = P_K \times \frac{E P^2 [K]_i - [K]_o \exp(-F E/RT)}{RT \left( 1 - \exp(F E/RT) \right)} \]
increased substantially for the patches with 40 and 80 mM potassium to values of about 21 and 23 pS for the outward potassium current.

At the higher external potassium concentrations conductances for the inward current, observed at potentials negative to the reversal potential, showed a larger difference for the change from 40 to 80 mM potassium than did conductances for the outward current as is predicted by the constant field equation (See figure legend). A number of theoretical plots for this relationship are given in fig. 5.2 and were formed assuming a constant intracellular potassium concentration of 90 mM, taken from estimates made by Alvarez-Leefmanns, Gamino and Rink (1984) and assuming a constant channel permeability of $5 \times 10^{-14}$ cm$^3$ sec$^{-1}$ for all three external concentrations. The channel was also assumed to be completely selective for potassium over the other ions present. The limited data obtained here does not appear to show any significant deviation from the theoretical plot, unlike the delayed rectifier current of the frog sarcolemma (see chapter 6).

Independence of channel activity

It has been reported (Miller, 1983) that single channel chloride currents recorded from reconstituted Torpedo electroplax are dimers which behave in a linked fashion; the probability of obtaining two openings compared to one being much higher than expected. Miller (1983) described this as a shotgun effect for the chloride channel.

The method used to check for any behaviour of this type in these channels was to compare the relative times during which specific numbers of channels were open in a patch containing a
few channels. For this purpose current amplitude histograms were formed from long continuous sections of data in order to obtain unbiased samples. The area for each peak in the histogram, representing the time spent with a specific number of channels open, was then compared with the expected binomial distribution. The probability of being open for any one channel was calculated from

$$\sum_{j=1}^{n} \frac{t_{jj}}{Tn}$$

where \(n\) = the number of channels in the patch, \(T\) = the total time of the sample and \(t_{jj}\) = the total time spent with \(j\) channels open.

Fig. 5.3 is a distribution formed from about 60 seconds of current record. From the areas under the open current levels a value of 0.18 was obtained for the probability of being open for any one channel. With a total number of 3 channels in the patch the predicted distribution of areas under the closed level was 0.56 and under the open levels were 0.36, 0.08, 0.006 and compared well with the actual values of 0.56 for the closed level and 0.37, 0.068, 0.009 for the open levels.

Open times

Open times were obtained from recordings in which the number of channels and the channel activity were low enough to give large sections of data in which only one channel was opening. The open times were then estimated using two cursors as the criteria to identify channel opening and closing. The cursor levels were set by eye to give the best results for the first few records and were then left at these levels for the whole of the analysis. Sections of records in which multiple openings occurred
Figure 5.3

Histogram of current amplitude.

A histogram of the current amplitudes from a continuous recording sampled at 4 kHz. The patch was depolarised from the cell resting potential by 100 mV and the pipette contained 5 mM potassium Ringer solution. 0 marks the peak created by the baseline current and 1, 2 and 3 the peaks when 1, 2 or 3 channels were open. T.18°C.
were ignored. A total of 10 separate open time distributions were obtained from three separate patches. All the distributions could be fitted closely with a single exponential function giving time constants that varied from 10.0 to 16.5 msec (see fig. 5.4). The presence of only one exponential in the open time distribution shows that the channel regularly entered only one type of open state under these conditions.

With the very small set of mean open times estimated from all the data it was not possible to find any significant correlation between the mean open time and the different pipette potentials or potassium concentrations, the values appearing to vary randomly. In view of the possibly complicated effects of membrane potential, [Ca]i and other controlling factors within the cell (e.g. cAMP) on both [Ca]i and the kinetics of the channel it was felt better to simply calculate the average of the mean open times giving a value of 12.1 ± 0.8 msec, thus predicting a value of 0.083 msec^-1 for the sum of the rate constants leaving this open state.

Possible channel identity

This potassium channel was found to have a conductance, measured for outward currents of approximately 14 pS with 5 mM K in the external solution and about 21 and 23 pS when the external K was increased to 40 and 80 mM respectively. The channel appeared to show a weak voltage dependence of its activation kinetics, becoming more active at depolarised potentials. It, however, did not show signs of inactivation over long periods of depolarisation of the patch membrane. The weak voltage dependence seems to rule out the possibility that these channels are either delayed rectifier or early outward channels. Both of
Figure 5.4
Histogram of open times.

The histogram shows the distribution of open times obtained from a patch recorded with 5 mM potassium containing Ringer in the pipette. The patch was depolarised by 100 mV from the cell resting potential. The graph contains a logarithmic plot of the number of open times in each bin of the histogram. The straight line on the graph, which was fitted by eye, gives a mean open time of 16.5 msec.
these channels would inactivate with prolonged depolarisation and would show strongly voltage dependent activation. Other main types of potassium channels present in the membrane are the calcium activated potassium channel and transmitter mediated channels, for example the serotonin mediated channel (Siegelbaum, Camardo and Kandel, 1982).

One further characteristic of the channel behaviour noted during the experiments was the great increase in channel activity caused by excision of the membrane patch from the cell membrane. One possibility is that this was a result of the increase of the calcium concentration at the inner face of the membrane as it became exposed to the extracellular Ringer solution. However, this behaviour has also been demonstrated for serotonin mediated channels in Aplysia neurones (Siegelbaum, et al. 1982). Comparison of the reported conductance of these two types of channel favours the idea that the channel described here is the calcium activated potassium channel. Lux, Neher and Marty (1981) obtained an average value of 18.5 pS from single channel recording in Helix pomatia neurones and Hermann and Hartung (1982a) estimated a value between 15 and 18 pS from noise analysis of the calcium activated potassium permeability similar to the 14 pS found here. In both of these cases the external potassium concentration was 4 mM as opposed to the 5 mM used in the present experiments. For a serotonin sensitive channel in Aplysia Siegelbaum et al. (1982) obtained the much higher value of 55±6 pS for the conductance at 0 mV.

In an attempt to support the idea that the channels were calcium activated potassium channels a preliminary experiment was performed on two patches. After excision the patches were transferred to a calcium free EGTA (5 mM) containing Ringer solution. It was found that in this solution no channel activity
was observed with a pipette holding potential of 0 mV (see fig 5.5), suggesting that activity of the channel was inhibited by very low calcium concentrations on the cytoplasmic face of the patch. This lends some support to the tentative identification of the channels as the calcium activated potassium channel.
Figure 5.5
The effect of calcium free solution on channel activity.

A. The typical channel activity recorded in a non-excised patch from a snail neurone. The pipette contained 5 mM potassium Ringer solution and the patch was depolarised by 50 mV from the cell resting potential.
B. The same patch as in A. after excision and transfer to a calcium-free external solution (80 mM K, 5 mM EGTA, 5 mM Mg, 5 mM Tris). The membrane patch was held at 0 mV. Filter 1 kHz, T.18°C.
6. MEMBRANE VESICLE PATCH CLAMP RESULTS

Introduction

During patch clamp recording from frog muscle vesicles at least four different types of single channel currents have been observed, these include what were considered to be unitary sodium currents, two or three potassium currents and a chloride current. The only means of identification at this stage was the voltage dependence of the unitary current amplitude and any obvious voltage dependence of the channel activity. Only two of the channel types have been studied in depth and are presented here, the sodium and delayed rectifier channels. They were probably the easiest of the channels to isolate due to their strong voltage dependent activity. With a holding potential of -100mV on the patch membrane it was found that a depolarisation caused activation of the channels, as would be expected in view of the macroscopic channel behaviour. The potentials quoted for the experiments on the vesicles represent the potential of the inner face of the vesicle membrane, i.e. the face of the patched membrane that is in contact with either the internal vesicle solution or the bath solution when the patch is excised relative to the outer face. The vesicles were assumed to have internal potassium and sodium concentrations identical to those of the bath solution, 0 and 120mM, and no significant resting potential. This was supported by the fact no change in unitary current amplitude was observed during the excision of the patch membrane from the vesicles. It has since been shown by direct measurement using intracellular recording electrodes that the vesicles have a very small resting potential the mean value being 2.4 ± 0.2 mV (n=59); (Standen, Stanfield, Ward and Wilson, 1984).
Sodium channels

Fig. 6.1 shows a series of inward single channel currents activated using a 60mV depolarisation from the holding potential of -100mV. The patch was pulsed at a frequency of 0.5 Hz which was found to remove the majority of the delayed rectifier currents by inactivation but allow sufficient time for the sodium channels to return to an activateable state. The unitary currents can be seen as fast downward steps of approximately 1pA in the traces. The arrow marks the mean unitary current amplitude for estimates made by eye from a large number of traces.

Three main factors were used to establish that these currents were of the same type as those responsible for the macroscopic sodium current found in frog skeletal muscle fibres.

i/ The current carrying ion.

ii/ The characteristics of the summed currents.

iii/ Pharmacological identification.

Single channel recordings were also used to measure the current voltage relationship of the unitary currents and to estimate the mean open time.

The current carrying ion

With normal Ringer solution in the patch pipette single sodium channel activity was often seen in the first few msec after the start of a depolarising pulse. However, this channel activity was never seen in patches formed with sodium free, 120mM potassium solution in the pipette showing that the current is at
Figure 6.1
Unitary sodium currents.

A series of current records showing unitary sodium currents, the arrow at the side of the second record shows the estimated average single channel current at this potential. The larger currents seen in the lower two traces are the result of two channels opening at the same time. The channels were activated by 60 mV depolarisations from a resting potential of -100 mV, the start of the pulse is shown in the voltage trace at the top. The records were sampled at a rate of 4 kHz and filtered with an eight pole Bessel filter 3 dB down at 2 kHz. The seal resistance was in the region of 30 Gohms.
least dependent on the presence of sodium ions at the outer face of the membrane and implying that sodium is the current carrying ion.

Characteristics of the summed current

The single channel current behaviour was compared directly with the normal macroscopic current by means of summed currents. These were formed by combining many cleaned traces to produce an estimate of the macroscopic current that would be produced by many separate channels of this type. The two examples in fig. 6.2 clearly exhibit the rapid activation and slightly slower inactivation so typical of the macroscopic sodium current (Ildefonse and Roy, 1972). The theoretical curves fitted to the summed currents were created using the \( m^3_h \) kinetics of the Hodgkin and Huxley (1952d) model for the squid axon. The value of the time constants \( \tau_m \) and \( \tau_h \) were obtained by fitting the summed currents using a routine for least squares estimation of nonlinear parameters (Marquardt, 1963). There was considerable variation in the values obtained from the fits but generally they gave values slightly larger than those quoted by Ildefonse and Roy (1972) for macroscopic currents measured at the same temperature as here. The differences could possibly be a result of the different means of recording or of the vesicle forming technique. With respect to the recording system, the effect on the activation kinetics could be a result of the delay in the rise time of the headstage input, in response to a voltage step in the command potential and/or the filtering of the data. Although the analogue capacity subtraction system removes the majority of the capacity current seen at the headstage input there is still likely to be a certain delay in the changing of
Figure 6.2
Summed unitary sodium currents.

The two summed currents were formed from a series of traces. The top example (A) was created from records obtained from a patch pulsed to -40 mV from a holding potential of -100 mV and the other (B) from a patch pulsed to -30 mV. All the original records were sampled at 4kHz and filtered at 2kHz (8 pole Bessel). The continuous lines are drawn to the expression

\[ I_{Na} = A[(1 - \exp(-t/\tau_{m}))^3 \exp(-t/\tau_{h})] \]

derived assuming the channel obeys Hodgkin-Huxley m^3h kinetics (Hodgkin and Huxley, 1952d). The best fit values of the parameters \( \tau_{m}, \tau_{h} \) were: (A) 0.70, 1.86, (B) 0.75, 0.94 (msec).
the pipette tip and patch potential as a result of the resistance between the electrode tip and the input. Such a delay would obviously delay the activation of the sodium channels. The rounding of the channel openings and the reduction of current amplitude of brief openings, caused by the filtering, may also contribute to the slower kinetics seen in the summed currents. In the light of the reported loss of channel activity as a result of cytoskeletal disruption in tissue cultured cells (Fukuda, Kameyama and Yamaguchi, 1981), it is possible that intracellular disruption caused by the vesicle forming technique has altered the channel behaviour. It does, however, appear unlikely that disruption at the inner membrane surface should cause such similar slowing of both the activation and inactivation kinetics.

However, even with the differences between the time constant obtained and those quoted for macroscopic currents, it is apparent that the summed current still gives a very good approximation to the sodium current.

Pharmacological identification

Finally, perhaps the strongest evidence supporting the proposed identity of the channels is the block of the currents by tetrodotoxin (TTX). The presence of TTX at a concentration of $3 \times 10^{-6} \text{M}$ was found to completely inhibit this type of channel activity during the experiments on the potassium current with normal Ringer solution, when the toxin was used to help isolate the potassium current. Spalding (1980) has shown that TTX blocks more than 99% of the sodium current in frog muscle at a concentration of only $0.1 \times 10^{-6} \text{M}$. 
The very brief nature of the channel openings made estimation of the current amplitude by histogram formation impractical, even with the selection of only the relevant portions of the recordings, because of the filtering of the data which caused brief openings to be reduced in amplitude, producing a large scatter of data points in the histogram, and obscured the open level peak. Therefore, the unitary current levels were evaluated by averaging a large number of separate estimations made by eye. Fig. 6.3 is a histogram formed from the estimates of channel open levels made by eye. Fig. 6.4 is a plot of the current voltage relationships obtained from two separate patches with normal Ringer solution in the pipette and sodium free high potassium solution within the vesicles. They give values of 12 and 15 pS for the conductance of the channel. These are smaller than the value of 18 pS obtained by Sigworth and Neher (1980) from cultured rat muscle but agree well with the values of 12 and 16 pS quoted by Sigworth and Neher (1980) for the expected amplitude calculated from the fluctuation analysis results obtained at lower temperatures in frog node of Ranvier by Conti, Hille, Neumcke, Nonner and Stampfli (1976) and Sigworth (1980).

Open times

Although the channel openings were rather brief and the membrane patches contained more than one channel it was possible to produce reasonable histograms of open time distribution from a number of separate sets of recordings. The mean open time, $\tau_{\text{op}}$, for each set of records was estimated from semi-logarithmic of plots of the histogram. Fig. 6.5 gives an example of an open time distribution with a single exponential
Figure 6.3  
Current amplitude histogram.

A histogram formed from the estimated unitary sodium current amplitudes, made by eye, using a 115 mM sodium containing solution in the pipette. The patch was depolarised to -40 mV from a holding potential of -100 mV. The mean current amplitude was 0.967 ± 0.018 pA.
Figure 6.4
Sodium channel current voltage relationship.

The plot of the estimated unitary sodium current amplitude against voltage for two separate patches. 115 mM NaCl containing Ringers solution was used in the pipette for both patches. They gave slope conductances of 12 (○) and 15 (□) pS. Seal resistance 25 Gohms (○), 30 Gohms(□).
Figure 6.5

Two histograms of sodium channel open times taken from patches pulsed to -40mV (A) and -30mV (B) from a holding potential of -100mV, and formed using bins 0.5 msec wide. The single exponential drawn over the distribution in A has a time constant of 0.6msec and was fitted by eye to a logarithmic plot of the number of openings in each bin. 115 mM NaCl containing solution in the pipette.
function fitted to it by eye. All the distributions could be fitted quite closely by a single exponential function implying that the recorded times are the result of the sodium channel passing into only one major open state. For this situation, where the distribution of times represents the times spent in one state, the reciprocal of the mean open time should give a good estimate of the sum of the rate constants leaving the open state.

For the Hodgkin and Huxley model (1952d) used to fit the summed currents there are two other states which can be entered directly, the inactivated state and the closed state C3 (see below). For the distribution in fig. 6.5 A the calculated sum of the rate constants B3 and a is 1.67 msec⁻¹.

Discussion

The combination of the information from the summed currents and the open time distribution offers an opportunity to make an estimate of the values of the rate constants assuming a Hodgkin Huxley state model for the channels.

\[
\begin{align*}
\text{A1} & \quad \text{A2} & \quad \text{A3} \\
\text{C1} & \quad \text{C2} & \quad \text{C3} & \quad \text{OP} \\
\text{B1} & \quad \text{B2} & \quad \text{B3} \\
b & a & b & a & b & a \\
\text{Cl}^* & \quad \text{C2}^* & \quad \text{C3}^* & \quad \text{C4}^* & \quad (* \text{INACTIVATED})
\end{align*}
\]

The time constant of the open times \( \tau_{op} \) will equal \( 1/(B3+a) \). From Hodgkin and Huxley \( 1/(b+a) \) will be given by the time constant \( \tau_{in} \) fitted to the summed current. Taking the inactivation of the summed current in fig. 6.4 as an example it
can also be seen that inactivation is complete causing the current to return to the base line. This suggests that the ratio of $b$ to $a$ is very small. Therefore, $\tau_\text{off}$ should be approximately equal to $a^{-1}$ and then $B_3$ can be evaluated. Since $B_3$ equals $3B_1$ and $B_2$ equals $2B_1$ all the back rate constants can be calculated. $\tau_\text{on}$ for the fitted curve gives $1/(A_3+B_1)$ and $A_1$ equals $A_2/2$ and $A_3/3$ so that it is possible to obtain values for all the rate constants.

The forward rate constants can thus be evaluated from the relationships. For the recording used to produce the summed current in fig. 6.2 A the evaluated rates are (msec$^{-1}$) 0.374, 0.749, 1.12 for $B_1$, $B_2$ and $B_3$, and 3.14, 2.09, 1.05 for $A_1$, $A_2$ and $A_3$, and finally 0.537 for $a$. At this potential (-40 mv) the calculated values using the data from Ildefonse and Roy (1981) are 0.76 for $B_1$ and 0.57 for $A_3$. 
Delayed rectifier channels

The second type of channels in the frog muscle membrane vesicles which have been convincingly identified are those of the delayed rectifier potassium current which plays a major part in the repolarisation of the muscle membrane during an action potential. In these experiments four main characteristics of the channel were matched with the macroscopic current in order to identify the channel.

1. The dependence of the current on potassium.
2. The kinetics of the summed current.
3. The block of the channel by TEA.

Having identified the channel two further characteristics of the unitary current behaviour were then studied, the dependence of the current on membrane voltage and the potassium concentration within the pipette, and the distribution of the times spent in the open (conducting) and closed (nonconducting) states.

Dependence on potassium ion concentration

Fig. 6.6 shows two sets of single channel recordings obtained using 2.5 and 120 mM potassium containing solutions in the patch pipettes (see methods). The bath and internal vesicle solutions both contained 120 mM potassium. Comparison of the two sets of traces clearly shows the shift in reversal potential after changing the potassium concentration in the pipette. With the 120 mM potassium solution the reversal potential, estimated using a ramped voltage pulse, is almost exactly 0 mv which is the predicted value. With normal Ringer solution in the pipette the reversal potential could not be directly measured although
Figure 6.6

Unitary potassium currents recorded with 2.5 and 120 mM potassium in the pipette solution.

A. Records made using a 2.5 mM potassium containing pipette solution with a holding potential of -100 mV. The duration of the depolarising pulse, to the voltage indicated on the right of each trace, is shown at the top. Seal resistance 20 Gohms.

B. Records made using 120 mM potassium containing pipette solution with a holding potential of -100 mV. The duration of the depolarising pulses is shown at the top. The single channel currents reverse at a depolarisation to 0 mV. Seal resistance 30 Gohms.

C. The lower trace shows the current record obtained using a voltage ramp. From a pipette with 120 mM potassium containing solution. The voltage pulse applied to the patch (upper trace) first depolarised the membrane to +100mV and then repolarised the patch at a rate of 2 mV/msec for 100 msec taking the patch potential to -100 mV at the end of the pulse. It can be seen that the current reverses close to 0 mV. Seal resistance 40 Gohms.

Filter 1 kHz, sample frequency 2 kHz, T 18-23°C, in each case.
extrapolation of the current voltage relationship, (see fig. 6.9), puts the value very close to the potential of 97 mV predicted by the Nernst equation assuming perfect potassium selectivity.

Kinetics of the summed current

The summed currents shown in fig. 6.7 were formed using traces recorded from a patch pipette containing 120 mM potassium. The channel was activated by depolarising the membrane by 160 mV (a) or 60 mV (b) from a holding potential of -100 mV for 100 msec. The activation kinetics of the currents have been fitted with the $n^4$ kinetics of the Hodgkin Huxley (1952d) model for the squid axon giving values of 1.06 and 1.73 for $\tau_n$. These agree well with the values obtained for frog skeletal muscle by Stanfield (1970) and Adrian, Chandler and Hodgkin (1970).

TEA block

It has been shown that tetraethylammonium chloride (TEA) applied externally (Stanfield, 1970) blocks the delayed rectifier current in frog muscle. Stanfield (1970) obtained a value of 8 mM for the dissociation constant of TEA for the channel receptor.

In the present experiments, the block of the single channel currents was studied by the addition of TEA to the pipette solution. The effects on the unitary potassium current of the presence of 10 mM TEA can be seen in Fig. 6.8. The reduction of the unitary current is quite clear and can be seen in the histograms of current amplitude. The Gaussian fits to the peaks in the histograms give values of 1.46 and 0.75 pA for the unitary current amplitudes, a reduction to 51%. The average reduction of the current amplitude at four different potentials, with respect to the average unitary current for normal Ringer was to 41.7
Figure 6.7
Summed currents.

Two summed currents formed by adding 359 (top) and 349 (bottom) traces from the same patch formed using a pipette with 120 mM potassium containing solution. The currents were activated by depolarising pulses to +60 (top) and -30 mV (bottom). The solid lines are drawn to the expression

\[ I_K = A \left[ 1 - \exp \left( -t/\tau_n \right) \right]^4 \]

derived assuming Hodgkin-Huxley \( n^4 \) kinetics (Hodgkin and Huxley, 1952d), using a least squares algorithm (see methods) to find the best fit values of \( A \) and \( \tau_n \). No attempt was made to fit the slow decline in the summed current caused by inactivation. The values (\( A, \tau_n \)) used were: A. 320 pA, 1.06 msec, B. -150 pA, 1.73 msec.
Figure 6.8
The effect of TEA on single channel amplitude.

A. A current amplitude histogram for records obtained using 2.5 mM potassium containing pipette solution. The single channel currents were activated by a depolarising pulse from -100 mV to +20 mV. The estimated unitary current amplitude was 1.46 pA. Seal resistance 20 Gohms.

B. A current amplitude histogram from records obtained using 2.5 mM potassium containing pipette solution also containing the 10 mM TEA. Holding potential and depolarisations the same as in A, above. The estimated unitary current amplitude was 0.75 pA. Seal resistance 20 Gohms.
Filter 1 kHz, sample frequency 2 kHz, in each case.
+5.6%, which is very close to the predicted value (Stanfield, 1970). This apparent reduction of the unitary current amplitude by the partial TEA block suggests that the rate of the blocking and unblocking reaction is extremely fast. If the rate of reaction was within the frequency band of the recording set up the block would reduce the probability of the channel being open but would not affect the unitary current amplitude (Neher, 1983, Methfessel and Boheim, 1982). In this case the block appears to be much too fast for the maximum recording frequency and thus the brief closings caused by the block are filtered so that an apparent reduction of current amplitude is observed. Raising the filter frequency to 5kHz produced no significant change in the amplitude of unitary current which means that the mean rate of the block is well above the 5kHz range. Stanfield (1973) reported a time constant for the rate of onset of block, with external application of TEA of 3-4 seconds, for frog muscle, the block being measured as an increase in the duration of the action potential and twitch. Vierhaus and Ulbricht (1971) also reported a rate of onset in the region of 2 seconds in the frog node of Ranvier, again measured as an increase in action potential duration. These figures are greater by a factor of at least 1000 than the rate of onset predicted by the single channel information. This puts the rate of binding well above 0.2 msec, the cutoff frequency of the recording setup. However, Vierhaus and Ulbricht (1971) did obtain values for the offset of the block that was limited by the diffusion of TEA away from the node of Ranvier. They suggested that the difference was possibly due to a two stage block of the current.

Single channel conductance

The current voltage relationship for the unitary current was
estimated with both normal Ringer solution and a number of high potassium solutions in the patch pipette. Fig. 6.9 is a plot of the mean current voltage relations for the channel with 120, 60 and 2.5 mM potassium in the pipette and with 120 mM potassium in the bath and vesicle. For the two higher concentration potassium solutions the current amplitude could be measured at potentials on both sides of the reversal potential. The current voltage relations appear to rectify slightly giving larger conductances for the inward current than for the outward currents. In 2.5 mM external potassium it was impossible to obtain inward current amplitudes at potentials more negative than the reversal potential because the channels closed too rapidly at these hyperpolarised voltages, when stepped from depolarised potentials. The second graph in fig. 6.9 is a plot of the predicted current voltage relationships for a channel which conforms to the constant field equation assuming complete selectivity for potassium and a constant permeability at all the pipette potassium concentrations. It is obvious from the comparison of the two plots that the constant field equation does not fit the experimental data. The most striking differences are the rectification of the current at the higher potassium concentrations and the saturation of the unitary current at the higher potentials with 2.5 mM potassium in the pipette. The assumptions behind the constant field equation are that there is independence of ion movements, there is a linear potential field across the membrane and that ions partition instantaneously between the solution and the membrane. An alternative theoretical approach to predict the channel currents is a barrier model in which the channel is considered as a pore containing a set of energy barriers over which the permeating ions must pass (Hille, 1975). The inward rectification of the potassium channel seen at the higher potassium concentrations can then be explained.
Figure 6.9

Current voltage relations for the potassium channel.

A. Plots of the mean current voltage relationship for the unitary potassium current recorded with 2.5 (Δ; n=3 to 10), 60 (□; n=2 to 4, ■ n=1) and 120 mM (○; n=3 to 5, ● n=2) potassium containing solutions in the pipette. The vesicle contained 120 mM potassium.

B. The theoretical current given by the constant field equation (see below) using the potassium concentrations quoted above and assuming a permeability to potassium of $5.5 \times 10^{-14}$ cm$^3$sec$^{-1}$.

$$I_K = P_K \times \frac{E_F^2 [K]_1 - [K]_0 \exp(-FE/RT)}{RT \ 1 - \exp(FE/RT)}$$
by an asymmetrical distribution of the position or heights of the barriers in the channel (Corey, 1979), where the height of a barrier is the energy required by an ion to pass over the barrier. The positional asymmetry causes a difference in the energy supplied to the ions as they approach each barrier thus changing the voltage dependence of the current in the two directions.

The saturation of the potassium current at the higher depolarisations, seen here in the results using 2.5 mM potassium in the pipette, can also be explained by a barrier model. If the saturation is considered to be caused by a lack of the voltage dependence of the unitary outward current then a high energy barrier close to the inner face of the membrane could produce this independence of voltage. The reason for this is that ions passing outwards over this barrier would not obtain energy from the membrane potential gradient if the barrier was located at the inner face of the membrane so that the number of ions able to pass over it would be related solely to the proportion of the ions in the solution with enough energy to surmount the barrier (Corey, 1979).

Open and closed time analysis

The slower kinetics of the potassium channels, compared to the sodium channels shown earlier, gave much more scope for the accurate estimation of the open and closed time distributions. The open times were measured in records in which only a single open level was observed, using a single cursor as the basic criterion to recognise open/close transitions, but with corrections being made by eye for incorrect or missed openings and closures. Open time distributions could be taken from many patches since they always represent the distribution of times for
a single channel as long as records containing openings to only
the single level were used. The argument behind this is that if
only a single open level is observed then only one channel is
open and therefore the period of that opening represents the
period of time a single channel remains in the open state. There
are three major exceptions to this; the first is if one channel
closes and another opens at the same instant so that no closure
or double opening was observed. This was considered to be a rare
enough event to be ignored during the analysis. The second
possible source of error came from the open times recorded that
were terminated by the end of the pulse rather than by the closing
of the channel. In the results shown here these openings were
excluded from the final distributions and from the calculation of
the mean open time. This will have caused a bias in the final
data. The extent to which a distribution is distorted depends on
the length of the pulse in relation to the mean open time and
probability of opening at the end of the pulse, because a larger
proportion of the longer openings will have been terminated by
the pulse and lost. However, the evaluated mean open times were
normally considerably less than 10 msec and, therefore, are
unlikely to have been seriously effected by the truncation of
some openings. The third source of error and the most difficult
to rule out, is related to the selection of events exhibiting
openings to only one level. In a patch containing more than one
channel the selection procedure will tend to bias the results
towards a greater number of short openings because there is a
higher probability of a second channel opening during a long
opening. However, Standen (unpublished results) has suggested
that a slow inactivation of these channels results in channels
often being inactivated during the whole of a pulse thus
temporarily removing them from the pool of activateable channels
in the patch for whole events. Therefore, when only single open
levels are observed they are likely to be the result of openings of only one channel.

In patches in which more than one channel was present the number of separate traces exhibiting openings to only the first level was increased by pulsing the patch regularly in order to keep a certain proportion of the channels in an inactivated state. This procedure was assumed not to affect the open time distribution for the reason given above. Fig. 6.10 shows an example of an open time distribution taken from a patch, in this case containing only a single channel. The distributions gave good fits to single exponential functions, the time constants of which were taken from the calculated mean open time (Total time open/Number of openings). The distribution shown was obtained from a patch depolarised to +60 mV from a holding potential of -100 mV and gave a value of 6.08 msec for the mean open time. The presence of only one exponential in the distributions suggests that there was only one commonly visited open state in the records. If a state diagram based on the model of Hodgkin and Huxley (1952d) is assumed for the closed and open states with a sequential inactivating state or with independent inactivation the only possible means for the channel to leave the open state is by the path to either the last closed state or the inactivated state.

A1 A2 A3 A4 Ah
C1 → C2 → C3 → C4 → OP → IN
B1 B2 B3 B4 Bh

The mean open time, the time constant of the exponential distribution, represents \(1/(B4 + Ah)\) where \(B4\) and \(Ah\) are the rate constants for the paths to the last closed state and the inactivated state from the open state. The rate of inactivation
Figure 6.10
Open time distribution.

A typical open time distribution obtained from a patch formed with a 120 mM potassium containing solution in the pipette. The currents were activated by depolarising pulses to +60 mV. The exponential fitted to the distribution (see text) has a time constant of 6.08 msec. Bin size 1 msec, H.P. -100 mV. Filter 1 kHz, sample frequency 2 kHz, seal 30 Gohms.
of the summed currents suggests that B4 will dominate in this relationship on the assumption that the channel does not return from the inactivated state at a rate approaching the return from the first closed state. This is supported by the observation that the channels generally take many seconds between pulses to recover from inactivation and that the channels are often closed for long periods of time towards the end of the pulse. If the return from the inactivated state is slow then the rate constant for passing to this state must also be small otherwise inactivation of the current would be rapid.

Closed times

The majority of the closed time distributions could not be used to give reliable results for the rate constants leaving the closed states since there was generally more than one channel present in the patch. Since it is impossible to know which channel is opening if more than one channel is present, the closed times measured represent the time spent when all the channels are closed and not just when one particular channel is closed. This is further complicated because the closed time distributions contain more than one exponential and as a result the change in the distribution caused by the presence of more than one channel is not simply related to the number of channels in the patch. However, a number of closed time distributions were taken from a patch which contained only one potassium channel. Fig. 6.11 shows two of these distributions. The solid lines superimposed onto the histograms were produced by a fit of a triple exponential to the data. Initially the two slower exponentials were fitted to the distribution, using 2.0 msec wide bins to form the histogram and ignoring the first few bins, thus excluding the fastest exponential component. The fastest
Figure 6.11
Histograms of the closed time distribution.

The closed times were obtained from records from a patch containing a single delayed rectifier channel with 120 mM potassium containing solution in the pipette. The channels were activated by depolarising pulses to +60 mV from a holding potential of -100 mV.

A. A histogram of the closed times formed using 0.5 msec wide bins. In this case the fastest of the three exponentials fitted to the data dominates the distribution.

B. A histogram of the closed times using 2.0 msec wide bins. This clearly shows the two slower exponentials that were fitted to the data.

The time constants for the fitted triple exponential curve in both A and B were 0.52, 2.5 and 20.7 msec with relative areas of 0.726, 0.208 and 0.062 respectively. Filter 1 kHz, sample frequency 2 kHz, seal 30 Gohms.
exponential was then fitted to the data by reforming the histogram with 0.5msec bin sizes and taking the values obtained from the first fit as constants. Both the fitting procedures used the nonlinear least squares method of Marquardt (1963) as a basis for the computer routines. The triple exponentials were found to give a consistently better fit to the data than the simple double exponentials which tended to leave a large proportion of the longer closings unaccounted for. Colquhoun and Hawkes (1982) have shown that a distribution of closed times for a channel with a number of distinct closed states will consist of a sum of exponentials, one for each closed state. From the histograms in fig. 6.11 it is obvious that these channels have at least three closed states. As with the open time distributions the loss of all the closed times terminated by the end of the pulse will cause a relative reduction of the longer closed times in the distribution. Using the recovery period required between pulses as a means of estimating the rate constant for the return from the inactivated state suggests a value less than 0.01 msec⁻¹. An important consequence of this is that the closed times during which the channel is in the inactivated state will tend to be excluded from the final distribution, in fact with such a slow recovery from inactivation one would expect them to be almost totally excluded. If this assumption is correct then the three exponentials are a consequence of three closed states accessible, either directly or indirectly, from the open state. It should be emphasised that with a sequential state diagram similar to the Hodgkin and Huxley system the exponentials produced for each closed states are not simply related to the rate constants leaving each particular state.

Therefore, in order to gain a better understanding of the closed time distribution I used a simple model with two sequential closed states before the open state and considered the
total closed time distribution to be composed of a series of
distributions each related to the particular ordered set of
closed states entered during a closed time. Fig. 6.12 is based
on a histogram constructed from a computer model, using 0.1 msec
bins, in which a random system was used to mimic the path of a
channel through the two closed states, one closed time being
measured each time the channel returned to the open state. In
the diagram above there are four closed states before the open
state as for the proposed model of this channel. Of these four
states only states C3 and C4 were assumed to be entered in the
model system. It can be seen that the pair of exponentials that
can be fitted to the complete distribution are composed of one
exponential plus a series of more complex distributions. The
first exponential, resulting from entry into only the first
closed state before returning, represents a subset of the dwell
times in the first closed state. It is $A_4/(A_4+B_3)$ of the total
number of dwell times and has a time constant equal to $1/(A_4+B_3)$.
The second largest distribution is a result of the channel
passing from the first closed state to the second before
returning via the first closed state to the open state. It is
not a simple exponential and is the distribution of the sum of
the three waiting times spent in each state encountered on route.
When the total distribution is compared with these subsets it
becomes apparent that if there is a reasonable separation between
the time constants fitted to the total distribution then the
reciprocal of the first time constant should give an
approximation to the value of $A_4+B_3$. 
Figure 6.12
Modelled close time distribution.

The distribution of closed times obtained from a computer model of a channel with just two closed states and a single open state. The solid line is the curve drawn through a histogram of all closed times formed using 0.1 msec bins. The other two lines were drawn over the histograms of closed times resulting from the closed times created when the two most common pathways were used. O to C1 to O and O to C1 to C2 to C1 to O.
Discussion

The kinetics of the potassium channel in the frog vesicle preparation were considered using two different methods of analysis. The summed currents were analysed in terms of the traditional Hodgkin Huxley (1952d) model. However, single channel recording also offered a second form of kinetic analysis based on the distribution of the channel open and closed times. The most reliable information that can be obtained from the open and closed times gives information on the rate constants closest to the open state, whereas the analysis of the summed current gives information on the overall rate of activation and inactivation of the potassium channel. As a means of comparing the two sets of results the values obtained from one particularly good set of records were used to model the channel behaviour in a computer simulation program using a linear four state model with a final inactivating state (see above). The results used came from the summed current, open time and closed time distributions for a patch depolarised by 160 mV from -100 mV and they are shown in the figures earlier in this section. For the first attempt at modelling, $\tau_{op}$ and $\tau_{cl1}$, the time constants from the open state and the first exponential of the closed state, were used to calculate the rate constants. The evaluation of $B_4$ from $\tau_{op}$ required some knowledge of the rate constant for inactivation which was obtained from the inactivation of the summed current. It was assumed that the return from inactivation was negligible since long or frequent depolarisations caused a complete loss of the channel activity and therefore, the decline of the current was directly related to the rate of passage to the inactivated state giving a value of $0.01 \text{ msec}^{-1}$. Also, since $B_4$ dominates out of the two rate constants leaving the open state the accurate
evaluation of \( A_h \) was not considered to be important. The two rate constants, \( A_4 \) and \( B_3 \), leaving the first closed state were then calculated from \( \tau_{c11} \) by assuming that \( B_3 = 0.75B_4 \) and that \( \tau_{c11} = 1/(B_3 + A_4) \). Assuming the same relationship between the rate constants as predicted by the Hodgkin and Huxley (1952d) model all the constants can be calculated. The values were then used in the computer model of the channel to create some simulated data. The resultant open times distribution was, as expected, a single exponential with a time constant approaching \( 1/(B_4 + A_h) \). However, neither the closed time distribution or summed current gave an approximation to the experimental data. The summed current activated too quickly, reaching a peak after only 3 to 4 msec. The closed time distribution produced a set of exponentials which did not show the large separation of time constants or any of the longer closed times seen in the experimental results. The total closed time distribution declined to virtually zero after only 5 msec suggesting that either the estimated forward rate constants were too fast or the back constants too slow. The forward rate constants were then changed to give the correct activation rate for the summed current using the relationship \( \tau_a = (\text{fitted rate of activation of the summed current}) = 1/(A_4 + B_1) \). This gave values of 3.64, 2.73, 1.82 and 0.911 for \( A_1 \), \( A_2 \), \( A_3 \) and \( A_4 \). The back rate constants were set as before. Although this obviously gave the correct activation and open time distribution the closed time distribution still contained no closed times approaching the values obtained from the experimental results. Attempts to produce a closed time distribution similar to that produced from the experimental data were made by either increasing the back rate constants so that the channel, on average, visited more closed states per closure or by decreasing the forward rate.
constants so that the waits in each closed state increased. During these attempts $B_4$ was kept at 0.154 and $A_h$ at 0.01 so that the open time distribution and degree of inactivation stayed constant. It was found that it was impossible to produce closed time distributions approaching the experimental and at the same time maintain the speed of the summed current activation. Any attempt that produced close times as long as 20 or 30 msec produced summed currents that did not reach a peak until well after 10 msec.

This failure of the model obviously points to a difference in the layout of the states through which the channel can pass and suggests that there is a closed state in which the channel can reside but one that it does not normally enter during activation. Proportionally, the area of the distribution accounted for by the longer closed times is small, implying that the proposed closed state is not entered regularly, but that there is a very slow rate of return from the state. If the data is assumed not to fit the linear model discussed above then there are endless possible models that could be created to account for the data obtained in these experiments. I have restricted this discussion to two small changes in the model that could be used to create results similar to the experimental data. The first change is the addition of a second inactivated state sequential with the open and first inactivated states. This state can be used to create the long periods of inactivation seen between pulses, found to be in the region of seconds. It is then possible to give the rate constant for the return from the first inactivated state a larger value so that entry into the first inactivated state would produce closed times similar to the longest ones found in the experimental data. This idea of a second inactivated state is also supported by work on the
macroscopic currents in frog skeletal muscle by Stanfield (1970) and Adrian, Chandler and Hodgkin (1970) where a slowly recovering component of inactivation was found.

The second change does not require a change in the state diagram of the model but instead simply removes the assumption that the channel returns to the state C1 before activation. If for example, the channel were predominantly in state C2 when the membrane potential was at the resting level it would still be possible to create a similar rate of activation as a result of the movement from C2 to the open state and also create long closed times by infrequent prolonged visits to the state C1.

To obtain a better picture of the different states that underlie the potassium channel activity it will be necessary to obtain data from single channels over very long periods of time. Although the data used in the discussion was obtained from over 400 separate pulses of the membrane patch it can be seen that there is still a large amount of random variation in the both the summed current and the closed time distribution. It will also be necessary to lengthen the pulse duration from the 150 msec usually used to catch more of the longer closed times which are important for the separation of the different closed states.
7. DISCUSSION

Calcium current inactivation

When the inactivation of the calcium current in many different tissues was first studied it tended to be considered in terms of a voltage dependent system similar to that of the sodium current in squid axon. The kinetics of the inactivation was even fitted to a similar model to that described by Hodgkin and Huxley (1952d; Standen, 1975b; Shakhovalov, 1977; Adams and Gage, 1979). However, after some initial experiments that demonstrated the dependence of calcium current inactivation on calcium entry in Paramecium (Brehm and Eckert, 1978), Aplysia (Tillotson, 1979) and in insect skeletal muscle (Ashcroft and Stanfield, 1980, 1982) the inactivation of many different calcium permeabilities has been found to be better described by a calcium rather than voltage dependent mechanism (see review by Eckert and Chad, 1984).

With respect to molluscan neurones a range of different experiments have been performed which support the idea of calcium dependent inactivation of the voltage activated calcium currents. The degree of inactivation has been correlated to the total calcium entry during depolarisation by changing the calcium entry. For example, by using depolarisations of different amplitude and duration, by voltage independent means using partial block by Cd$^{2+}$ and changing the external calcium concentrations. The calcium current has also been demonstrated to be dependent on [Ca]$^\text{i}$ using calcium chelators to alter [Ca]$^\text{i}$. Addition of EGTA to the internal solution during perfusion of Lymnaea neurones (Kostyuk and Krishtal, 1977) and injection of
EGTA into intact *Aplysia* (Eckert and Tillotson, 1979) and *Helix* neuronules (Plant, Standen and Ward, 1983) have all been shown to increase the calcium current and reduce inactivation. Kostyuk and Krishtal (1977) and Plant, Standen and Ward (1983) using EGTA/calcium buffers showed that the calcium currents could be both increased by lowering \([Ca]_i\) and decreased by increasing \([Ca]_i\) and thus were also able to estimate the resting \([Ca]_i\).

In experiments described in chapter 3, increases in \([Ca]_i\) were brought about by injection of calcium ions rather than as a consequence of calcium entry during membrane depolarisation. It was possible to clearly demonstrate calcium dependent inactivation and isolate it from potential dependent mechanisms. The results of some of the largest injections from which the cells subsequently recovered produced a reduction of the current of over 70%, suggesting that more than this proportion of the total calcium permeability could be inactivated by raised \([Ca]_i\). On the assumption that the calcium permeability can be completely inactivated by raised \([Ca]_i\), the plot of inactivation against \([Ca]_i\) was fitted to a 1:1 binding model and gave a value of 5.2 x 10^-7 M for the inactivation of half of the calcium permeability. This agrees quite well with the values obtained by Plant (1982) and appears to be in a sensible region with respect to the measured resting calcium concentrations (Alvarez-Leefmans, Rink and Tsien, 1981). As can be seen in fig.10 of chapter 3 with half the current inactivated at 5.2 x 10^-7 M only a small proportion of the current will be inactivated at the resting \([Ca]_i\) and any increases, up to 10 fold, in \([Ca]_i\) affect the channels over their most sensitive region thus maximising the response. The results of Kostyuk and Krishtal (1977) gave values for the \([Ca]_i\) when the current was half blocked in the region of 2.7 x 10^-8 M. However, Byerly and Moody (1984) have recently
shown that the method of perfusion with EGTA/calcium buffers does not lead to adequate control of $[Ca]_i$ and they suggested that this could explain the much lower value obtained by Kostyuk and Krishtal (1977).

Using models of the calcium permeability it has been found that the accumulation of calcium at the inner face of the membrane, accompanied by purely calcium dependent inactivation of the channel, can explain the majority of the characteristics of calcium channel inactivation. Plant et al. (1983) using a simple model of calcium accumulation, found it possible to reproduce the apparent double exponential decline of the calcium current during a depolarising voltage pulse. In a more complex model Standen and Stanfield (1982) could recreate the results of double pulse experiments also showing that when the prepulse was taken to a potential at which no inward current was observed, a certain degree of inactivation of the second pulse was caused by entry of calcium during the tail current at the end of the prepulse. Collectively these results all suggest that the inactivation of the current in molluscan neurones can be completely accounted for by a calcium dependent mechanism. However, Brown, Morimoto, Tsuda and Wilson (1981) and Yatani, Wilson and Brown (1982) argue that there is a certain degree of voltage dependent inactivation of the current. They accept that calcium plays a major role in inactivation but give a number of reasons as to why there must also be some voltage dependent inactivation. Their two strongest arguments for the presence of voltage dependent inactivation relate to the remaining inactivation seen when Ba$^{2+}$ is the permeating ion or when high intracellular concentrations of EGTA are used to buffer changes in $[Ca]_i$.

There are however, equally convincing arguments that explain the remaining inactivation in both these cases, other than by a
voltage dependent mechanism. For the Ba$^{2+}$ currents the remaining inactivation could be due to accumulation of Ba$^{2+}$ at the inner face of the membrane or the release of Ca$^{2+}$ by Ba$^{2+}$. To counter this argument, they showed that when 1mM Ba$^{2+}$ was introduced to the intracellular perfusate of cells bathed in a 10mM Ba$^{2+}$ containing solution, only a minimal reduction of inward current was observed. The current was measured using depolarisations to +24mV. However, a strong argument that points to a lack of control of the internal cellular environment is that with the 10 and 1mM Ba$^{2+}$ on either side of the membrane the reversal potential of the inward current would be reduced to about +29mV. This would undoubtedly considerably reduce the inward current implying that the intracellular barium concentration never reached values as high as 1mM.

The failure of EGTA to completely block inactivation can also be related to a similar phenomenon. Byerly and Moody (1984) discovered that the perfusion of neurones with calcium buffered solution with [Ca] below the resting level of the cell results in the reduction of [Ca]$^i$ to a value somewhere between the resting level and the concentration of free calcium in the buffer solution. It is therefore unlikely that the EGTA buffer could completely prevent increases in [Ca]$^i$, especially near the membrane, during calcium entry, thus allowing some inactivation of the inward current.

These arguments, then favour a calcium dependent system of inactivation. However, recently Lux and Brown (1984) have proposed an alternative view as a result of experiments on Helix pomatia using patch clamp and whole cell voltage clamp to look at the calcium channel activity. They showed that the inactivation of the channels in the patch was not affected by calcium entry elsewhere in the cell thus ruling out inactivation by general
accumulation of calcium. They then showed that the increase in mean closed time towards the end of the pulse, which they considered to be the cause of inactivation, was not correlated with increased opening at the beginning of the pulse, as would be expected if local calcium accumulation was the cause of inactivation. But, given the wealth of evidence for calcium-dependence of inactivation reviewed above (see also Eckert and Chad, 1984), these results must remain controversial. Clearly more single channel recording is required to help to resolve this argument.

The cAMP dependence of the calcium permeability

Doroshenko et al. (1982) suggested that the effect of raised $[\text{Ca}]_i$ on the calcium permeability may be a result of changes in the cAMP level of the cell caused by the effects of $[\text{Ca}]_i$ on phosphodiesterase or other enzymes. This was based on the results of perfusion experiments in which the addition of cAMP, ATP and Mg$^{2+}$ to the perfusate was shown to either temporarily increase or at least maintain the voltage dependent calcium permeability of the perfused neurones. However, other experiments by Kononenko, Kostyuk and Shcherbatko (1982) on this current in intact molluscan neurones showed a negative effect of cAMP, injected cAMP reducing the inward current. They explained these contrasting results as being caused by some peculiarities of the internal media in the two preparations. As an example they considered the different calcium buffering in the two systems. Using an assumption that increases of cAMP cause a release of calcium within the cell, they explained the decrease of current in the intact neurone as being a direct result of raised $[\text{Ca}]_i$. 
The increase seen in the perfused cells was then assumed to be a different result of raised cAMP that is normally masked by the effects of raised [Ca]$_i$, which in this case is held relatively constant by the EGTA buffer of the perfusate. In the results shown in chapter 3, changes in [Ca]$_i$ have been demonstrated to affect the voltage activated calcium current. However, attempts to cause changes in current amplitude by cAMP injection were not successful. The series of experiments shown in chapter 4 were considered not to support any link between cAMP and the voltage activated calcium current in the intact neurone. The argument used by Kononenko et al. (1982) to explain the differences between their results and those for the perfused cells do not hold here since no change in current was seen implying that there was no change in [Ca]$_i$ caused by cAMP injection in these neurones. However I feel that there is a further possible mechanism to explain the effects of raised intracellular cAMP in the perfused cells. If it is assumed that, under normal conditions, the cAMP level within intact neurones is high enough to keep saturated any metabolic systems controlling the voltage activated calcium permeability, so that a maximum number of channels are in an activateable state, then increasing the cAMP concentration should have no effect. On the other hand, during the perfusion of a neurone, cAMP along with other metabolites will tend to be washed out of the cell. It is then possible to envisage how reintroducing cAMP along with ATP and Mg$^{2+}$ could temporarily increase or at least maintain the calcium permeability until other necessary, but unreplaced compounds, are reduced to concentrations too low to maintain the channel activity. This last statement also implies a rather nonspecific role of cAMP in the maintenance of the calcium permeability, the cAMP, ATP and Mg$^{2+}$ ions just tending to create a slightly more normal
environment within the cell. Such a system would obviously not play a role in the inactivation of the current by \([Ca]_i\) already demonstrated above. There appears to be no reason to suggest any mechanism other than a direct effect of calcium on the calcium channel to account for the effects of changing \([Ca]_i\) on both the inactivation of the current during depolarisation of the membrane and the longer term inactivation after prolonged calcium loading of the cell.

Membrane vesicle preparation

The large vesicles formed from the adult muscle fibre sarcolemma do not represent a totally original preparation since enzyme treatment of cells has been used before for the production of similar vesicles (Scott, 1978). However, the preparation has not until now been used in patch clamp studies and its use has been found to offer a number of advantages over the intact muscle fibre preparation. The vesicles were originally used when it was discovered that the pipettes sealed more readily to the vesicle membrane than to the intact sarcolemma of the muscle, but it was soon realised that they offered a novel method for the analysis of a number of the membrane channel activities. The vesicles have been shown to have only a minimal resting potential and an internal ionic concentration very similar to that of the solution in which the final enzyme treatment was performed (Standen, Stanfield, Ward and Wilson, 1984). These two facts immediately give an advantage over the use of the intact fibres since both the patch membrane potential and solution composition on both sides of the patch are known. Also, the fact that the bath solution was identical to the internal vesicle solution was found to be a great asset during the experiments since it then became
unnecessary to determine whether the patch was excised. When using the intact fibres the resting potential of the fibres and the composition of the internal solution were not known, even though the muscle had been put through contracture, thus making the measurement of the voltage dependence of the channel current and activity rather unreliable. Control of the internal solution composition was also useful for the control of the calcium activated potassium permeability by reduction of $[Ca]_i$ using 5mM EGTA in the solution. Finally, it has since been found (Spruce, Standen, Stanfield and Wilson, 1984) that by changing the composition of the solution during the final enzyme treatment it is possible to produce vesicles with a variety of different internal ion compositions. Spruce et al. (1984) used this fact to produce vesicles containing a rubidium as opposed to potassium based solution in order to study its effects on the delayed rectifier currents.

During the experiments described here the two membrane permeabilities of the vesicles studied were those responsible for the delayed rectifier and sodium currents. As far as could be determined the delayed rectifier potassium currents behaved with normal kinetics with respect to macroscopic currents previously recorded from intact muscle fibres (Stanfield, 1970, Adrian, Chandler and Hodgkin, 1970). However, the sodium currents did appear to show some slowing of the activation and inactivation kinetics when compared with the macroscopic currents recorded under similar conditions (Ildefonse and Roy, 1972). As discussed before this difference in the measured kinetics could be due to differences in either the recording system or changes in the channels brought about by the vesicle formation. The possibility that the vesicle formation could affect the sodium channel behaviour is supported by the experiment of Fukuda, Kameyama and
Yamaguchi (1981) and Tank, Wu and Webb (1982). Tank et al. (1982) showed that enzyme treatment that promoted the formation of vesicles increased the mobility of certain large molecules in the membrane. Using the technique of fluorescence photobleaching recovery, they obtained values for the diffusion coefficient for some molecules over 100 times greater in the vesicle membrane than in the intact cells. This increase was attributed to the breaking of cytoskeletal connections during the vesicle formation that normally anchored some of the membrane proteins. Fukuda et al (1981) showed that cytoskeletal disruption caused a loss of sodium channel activity in tissue cultured mammalian neurones, when specific enzymes were used to break the cytoskeletal connections in the cell. This result is in disagreement with those obtained here since if the vesicle formation involves breaking of the cytoskeletal connections one would expect to see a loss of sodium current. A likely explanation of this anomaly is that although the vesicles apparently contain little of the original fibre contents there may well be enough of the cytoskeletal attachments remaining to maintain sodium channel behaviour, perhaps marginally slowed, but still allow for the increased mobility seen by Tank et al (1982). The loss of activity seen by Fukuda et al (1981) can then be explained as being a result of a different form of cytoskeletal disruption, perhaps much closer to the inner membrane surface. It would be interesting to look at the vesicle membrane using an electron microscope to discover how much of the cytoskeleton remains attached to the inner membrane surface after the vesicle forming procedure.

The recordings from the snail neurone preparation highlight some of the problems that are avoided by the use of the vesicles. If the channel type shown in chapter 5 is assumed to be the
calcium activated potassium channel then the unitary current amplitude and the kinetics of the potassium current are affected by the concentration of $K^+$ and $Ca^{2+}$ ions within the cell. In this preparation neither of the two concentrations were known during the experiments and in fact the kinetics of the channel opening over long periods of time appeared to alter, the most noticeable change being the frequency of bursting of the channel. This combined with the lack of knowledge of the cell resting potential, and the probability of this changing during the experiment, meant that no correlation of these factors with the channel behaviour could be made. The differing cell resting potentials and intracellular potassium concentration also made evaluation of even the current-voltage relationship a problem.

These experiments on the calcium activated potassium current only touch on the mechanisms behind the channel activity. The most obvious step forward would be to perform experiments on excised patches under conditions in which the solution at the original intracellular face of the membrane patch could be controlled. This would offer an opportunity to examine the channels dependence on both $[Ca]$ and potential separately.
Appendix 1.

A. The circuit for the patch clamp amplifier head stage used for the snail neurone recordings was very similar to that described by Hamill, Marty, Neher, Sakmann and Sigworth (1981). It consisted of an operational amplifier (a; Burr Brown 3523J) with a matched pair of FET transistors (b; UA401) across the inputs to increase the impedance and reduce the noise of the input. The transistor (c; 2N3904) was used to supply a constant current to the FET to maintain the device at a constant raised temperature. The feedback resistor was found to be a very critical component, a number of resistor types were tried and were found to produce quite different levels of noise on the output. A 10 Gohm (Welwyn) resistor was finally used.

The layout of the components was also found to have a considerable effect on the output noise. In the final version the connections to the FET inputs and the resistor were kept clear of the circuit board, reducing any stray connections to the very high impedance points. The feedback resistor was shielded from the rest of the circuitry and also kept as far away as space permitted from the shielding and case to keep capacitative links to a minimum. The operational amplifier was mounted upside down to avoid crossing of the FETs outputs and to keep the connections between the FET's and the amplifier as short as possible.
Appendix 2.

The circuit of the patch clamp amplifier used to obtain single channel recordings from the snail neurones. It was adapted from a circuit originally designed by D. Corey.

Amplifiers A1, A2 and A3 deal with the processing of the current signal returned from the head stage.

A7, A8 and A9 produce the capacity subtraction signal which is fed to the head stage.

A12 sums the outputs of the auto zero, holding potential and the pulse input producing the final command pulse for the control of the pipette potential.


A11, AD515.

A12, NE5534.


Takahashi, K. and Yoshii, M. (1978). Effects of internal free calcium upon the sodium and calcium channels in the tunicate egg analysed by the internal perfusion technique. J. Physiol. 279:519-549.


VOLTAGE AND PATCH CLAMP STUDIES OF THE IONIC CURRENTS IN SNAIL NEURONES AND FROG SKELETAL MUSCLE

The inactivation of the calcium permeability in Helix aspersa neurones was studied in relation to [Ca]. Injection of calcium ions was shown to reduce the voltage activated calcium current by increasing \([Ca]_i\). This was proved to be by a process of inactivation of the calcium current, rather than a direct effect of increased \([Ca]_i\) on the driving force for calcium across the membrane. \([Ca]_i\) was measured using ion sensitive electrodes, and a mean resting level of \(2.66 \pm 0.65 \times 10^{-7}\)M was obtained. The inactivation of the calcium current was found to fit 1 to 1 binding between calcium and the receptor responsible for inactivation giving a \(K_d\) of \(4.6 \times 10^{-6}\)M.

A number of techniques were used to influence the intracellular cAMP concentrations of the snail neurones in an attempt to isolate some form of metabolic control of the calcium permeability. However, no consistent responses could be obtained.

The patch clamp technique was used to look at the unitary current in frog skeletal muscle. Membrane vesicles formed from the sarcolemma by enzyme treatment, were found to reduce the number of problems associated with the use of the technique on intact muscle fibres. Sodium and delayed rectifier potassium channels were identified in the vesicles, having conductances in the range 12-15pS and 16-24pS, for the two types of current. The kinetics of the sodium channels in the vesicles were found to be slightly slower than those reported for the intact frog skeletal muscle. However, they could still be fitted to the m-n model of Hodgkin and Huxley. Studies of the kinetics of the delayed rectifier potassium channels suggested a model for the channel different from the four-closed state system proposed by Hodgkin and Huxley.

Finally, experiments were performed on snail neurones in order to observe the unitary calcium activated potassium currents.