

Ion Channels of the
Tonoplast Membrane
of *Nitella hookeri*

Thesis submitted in accordance
with the requirements of the
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for the degree of
Master of Science in Biochemistry

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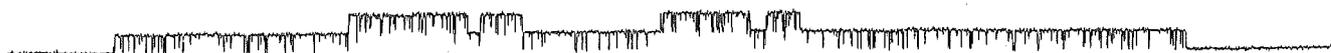
Abstract

Ion channels in cytoplasmic droplets of the characean algae *Nitella hookeri* have been studied using the patch clamp technique. This technique enables the measurement of the ionic current through single membrane channels. Several channels have previously been identified and characterised in characean tonoplast membranes. These include at least one type of potassium channel (in *Chara australis*; Lühring, 1986, *C. gymnophylla*; Andjus *et al.*, 1999 and Djurišić & Andjus, 2000, and *C. corallina*; Tyerman & Findlay, 1989) and one chloride channel (in *C. corallina*; Tyerman & Findlay, 1989). The tonoplast membrane of *N. hookeri* was surveyed for its similarity to those in previous studies. A potassium channel was observed that correlated with reports of either a 60pS channel (Tyerman & Findlay, 1989), or a 170pS channel that showed a predominant subconductance state that had a conductance of 60pS (Lühring, 1999). Other potassium channels observed conducted at ~40pS, ~20pS, and ~80pS. A chloride channel was recorded that resembled that reported in the tonoplast membrane of *C. corallina* (21pS). Other Chloride channels observed conducted at ~78pS, and ~100pS.

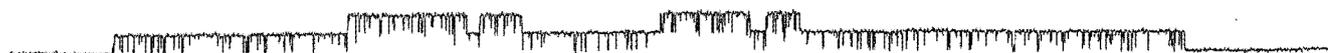
A channel protein's reaction to glycation, via the Maillard reaction, was studied using the improved patch clamp technique. Preliminary results show that methylglyoxal (a glycating agent) addition decreases channel conductance while a control of time (no addition) showed no similar reduction. It is hypothesised that cross-linking and other covalent modification, known to result from the Maillard reaction, has altered the channel's structure to induce this change. A similar experiment using glutaraldehyde (another glycating agent known to react at a greater speed) gave similar preliminary results.

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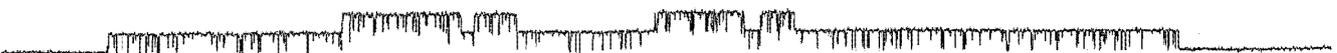


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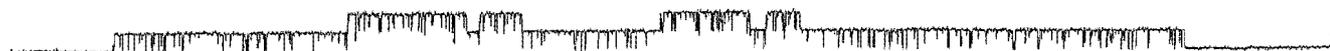
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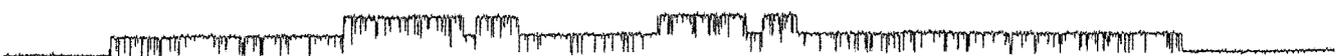


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List of Abbreviations and Definitions

ATP	adenosine triphosphate
Ca ²⁺	calcium ion
CholCl	Choline ([2-Hydroxyethyl]trimethylammonium) Chloride
Cl ⁻	chloride ion
E _{Ca}	reversal potential of calcium
E _{Cl}	reversal potential of chloride
EGTA	ethylene Glycol-bis(b-Aminoethylether)-N,N,N',N'-Tetraacetic Acid
E _K	reversal potential of potassium
E _{Na}	reversal potential of sodium
Glutaraldehyde	Pentane-1,5-dial
IV curve	Current (I) versus voltage (V) plot which allows identification of a channel
K ⁺	potassium ion
M	molar (moles per litre)
MES	2-[N-Morpholino]ethanesulfonic Acid
Methylglyoxal (MG)	2-oxopropanal
mM	millimolar
mM	millimolar
ms	millisecond
mV	millivolt
Na ⁺	sodium ion
pA	picoAmpere
Reversal potential	The potential (clamped voltage) at which ion flux equals 0 pA
s	second
[S]	concentration of ion S
t	time
TLA	three letter acronym
V	volt
μL	microlitre
μm	micrometre

"I am here on this earth to explore."

-Jamie Gaskill (1979—)

To whom this thesis is dedicated.

Chapter 1

Introduction

*“The most beautiful and the most profound emotion we can
experience is the sensation of the mystical.*

It is the source of all true science.”

-Albert Einstein

Found in Spurgens Rock Shelter—Kahurangi National Park, New Zealand.

1.1 Importance of Ion Channels in Biological Membranes

This thesis is a study of ion channels in the membrane that surrounds cytoplasmic droplets isolated from the characean alga, *Nitella hookeri*. Membranes surround all living cells and, as endomembranes, enable compartmentalisation of cellular apparatus. Regardless of their cellular location, they play a key role in the fundamental processes of life. The function of a membrane was originally thought of as simply a separator, to delineate the cell and its environment. While this is true, they serve a number of other important purposes (Garrett & Grisham, 1999).

The fluid mosaic model for membrane structure suggests that a membrane is a dynamic structure made up of proteins and other molecules embedded to varying degrees in a phospholipid bilayer. This model was proposed in 1972 and still provides an insight into the complexity and importance of membranes (Singer & Nicolson, 1972). As well as performing the function of defining 'self' and 'non-self', membranes function to:

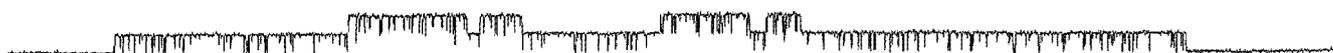
- avoid various toxic molecule accumulation by excluding and secreting them from the cytoplasm by acting as a "skin" to the cell (Purves *et al.*, 1998)
- accumulate nutrients by allowing their entry through specialised transport molecules (Alberts *et al.*, 1994)
- assist in reproduction of the organism or the cell; e.g. it is responsible for the block of polyspermy (>1 sperm binding to an egg; Alberts *et al.*, 1994)
- assist with interactions between cells (Garrett & Grisham, 1999)
- assist with locomotion in motile cells (Purves *et al.*, 1998)
- control and pass signals to the cytoplasm and is the usual site for signal cascade initiation (Brandon & Tooze, 1999a)
- provide a site for, and link together for efficiency, an enormous range of biochemical reactions (Alberts *et al.*, 1994)



Perhaps the most important function of membranes is their role in the compartmentalisation of a cell. Even in prokaryotic cells, which have only a plasma membrane, the membrane is used to localise reaction pathways, signal cascades, and other linked processes. In eukaryotic cells, numerous intracellular organelles that perform specialised tasks at a faster speed than in the prokaryote example have allowed eukaryotic cells to evolve into significantly larger structures (Garrett & Grisham, 1999).

Lipid bilayers, the basis of all membranes, are effectively impermeable to ions (Van Winkle, 1995). The permeability coefficient is a measure of the relative permeability of a molecule, in this case, through a lipid bilayer (or a protein-free membrane). Small uncharged polar, and hydrophobic, molecules can permeate a membrane while larger polar molecules and charged ions cannot. The relatively membrane impermeable potassium ion, for instance, has a permeability coefficient of $\sim 10^{-12}$ centimetres per second (cm/s). This can be compared to water molecules, which are small enough to pass through a membrane, and have a permeability coefficient of $\sim 10^{-2}$ cm/s (Alberts *et al.*, 1994); In some cells movement of water is, however, still too slow to allow cellular survival; these cells therefore allow water to move through specialised water pores (Schutz & Tyerman, 1997). It has also been shown that potassium and chloride selective channels conduct water (Andjus *et al.*, 1999). As well as being too slow to support a living cell, this permeability of molecules through a membrane lacks the selectivity that is required for cellular survival.

Cells, to liaise between the inner and outer environments of membrane-bound 'compartments', produce a large numbers of some types of membrane-bound proteins. Some, in the form of channels and transporters, control the internal composition of a cell, or organelle. Transporters, such as the H^+ ATPase pump (an example of a primary active transporter) or the proton driven antiporter (a secondary active transporter), use metabolic energy (in this case adenosine triphosphate (ATP), or a proton concentration gradient) to drive the ions across the membrane (figure 1.1; Taiz & Zeiger, 1998; Alberts *et al.*, 1994; Mathews & van Holde, 1990). A range of energy sources are used



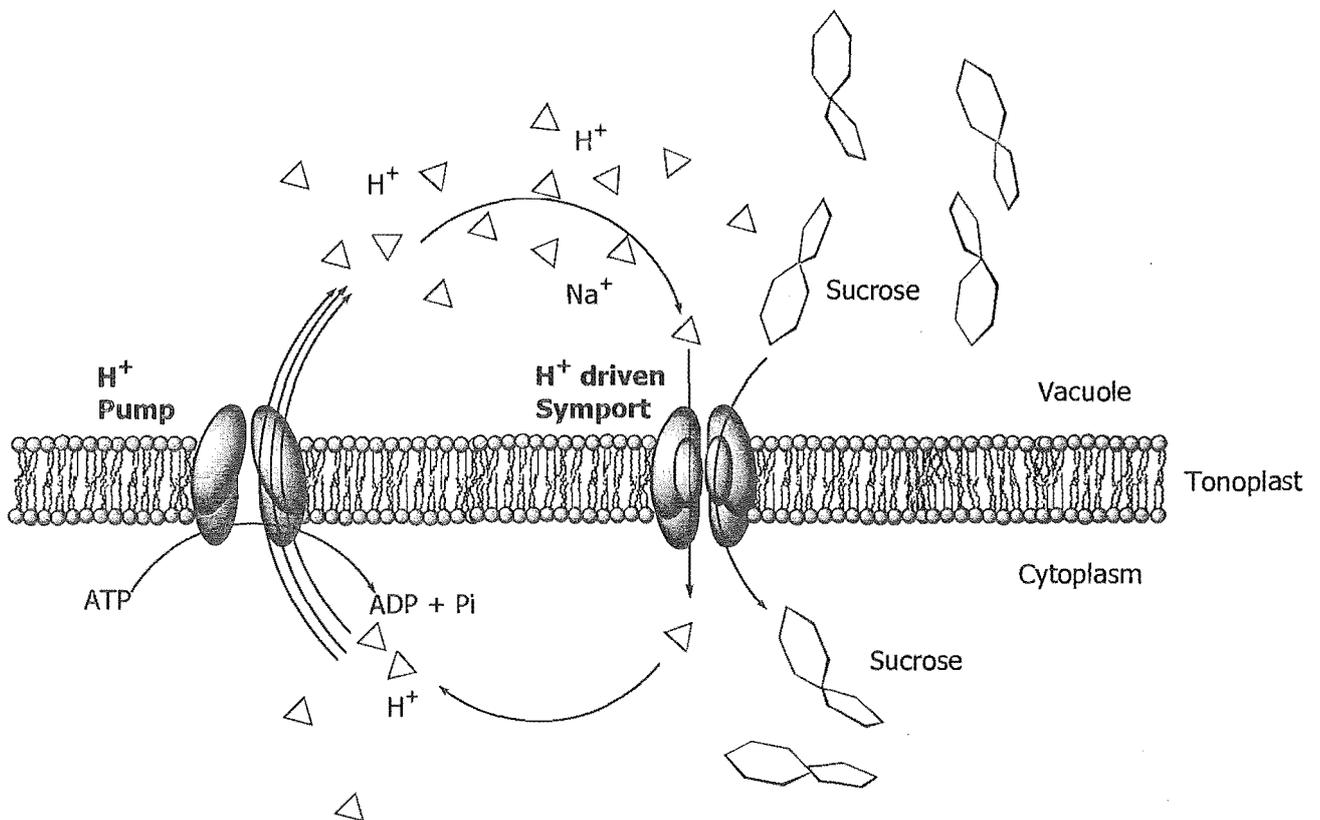
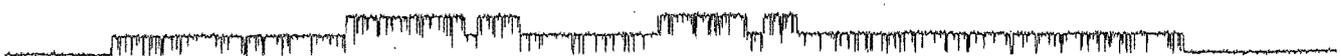


Figure 1.1 Schematic view of an example active transport. To the left is proton-pump, or H⁺ ATPase that uses adenosine triphosphate to 'pump' protons (hydrogen ions) into the vacuole. This is an example of primary active transport. The proton gradient created by the ATPase is used to drive sucrose into the vacuole (right) in an example of secondary active transport. Note that the globular structures depicted as transport proteins, above, bear no resemblance to the actual proteins.

by active transport systems including, ATP in the sodium-potassium ATPase, redox chemistry in the respiratory chain, and light in bacteriorhodopsin.

Channels, however, are passive; they rely on the physical laws of entropy (i.e. the tendency of molecules to move to a state of lesser energy) to move ions down their concentration gradients through the channel pores (Garrett & Grisham, 1999). Regulation of flux through a channel, or gating, occurs in the same way as in enzyme regulation, i.e. with the use of inhibitors, coenzymes, signalling molecules etc that convert the protein between catalytic and partial- or non-catalytic forms (Purves *et al.*, 1998).

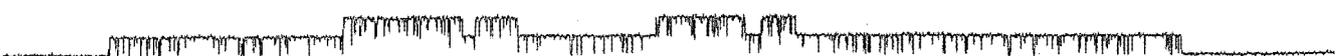
The molecular constituents of the cytoplasm and vacuolar sap are controlled by a range of ATPases, symports, antiports and channels (see figure 6.12 in Taiz & Zeiger, 1998).



1.2 Why Study Membrane Channels?

Membrane channels, both individually and collectively, have a profound impact on a cell's ability to survive and to adapt within its environmental niche. The appropriate quantity, type and regulation of ion channels in a membrane within or surrounding a cell is critical for maintaining the desired electrochemical gradients and osmotic potentials across that membrane.

Modern biology has evolved through an attempt to understand how structure confers function on any scale since at least the time of Aristotle, when he described the variety of structures within animals (Aristotle, 1961). As any structure is usually made up of smaller parts, the structure and function of the smaller parts need to be studied for understanding of the larger structure (Eisenberg, 1999). Membrane channels are a significant structure of molecular scale that contributes to the physiology of the cell; so to understand the cell the channel must be understood.



1.3 What is known About Membrane Channels?

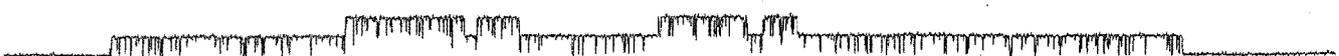
1.3.1 Membrane Channels as Enzymes

Membrane channels are analogous to enzymes in that, where an enzyme converts a substrate, A, to product, B, a channel transports a substrate from position X to position Y. Thus a channel catalyses the ionic movement. Assuming conductance is thought of as a chemical reaction, channels can be viewed as 'enzymes' (Klieber & Gradmann, 1993). As 'enzymes', membrane channels:

- catalyse ion flux (down the ion's concentration gradient)
- are 'gated' or controlled by molecular 'switches', e.g. inhibitors, cofactors, coenzymes, product inhibition, etc, or membrane potential (figure 1.2)

1.3.2 Relationship with the Membrane

Channels are membrane spanning, or integral, proteins and are therefore in contact with two different environments (not including the interior of the lipid bilayer). A channel typically consists of a number of transmembrane structures (usually α -helices or β -sheets) on one or more polypeptide subunit. The transmembrane structures create an aqueous pore through which ions can flow. It is thought that channels are often anchored to other membrane, or cytoplasmic proteins such as the cytoskeleton. Most channels are glycoproteins; i.e. they have oligosaccharides (that sometimes number in the hundreds) attached to extracellular amino acid residues. These sugar residues are generally considered to be part of the manufacturing and transportation process of the channel (Hille, 1992) although some recent studies suggest that the sugar residues may also assist the action of the channel (see section 1.7 below and Rho *et al.*, 2000 or Shi & Trimmer, 1999). Gated channels (that open and close allosterically) have some mechanism(s) that react to the gating factor (allosteric effector) by changing the ion pores conductive properties. For example, a voltage-gated channel has a transmembrane device which "senses" the voltage across the membrane and causes a conformational change that opens, or closes the channel (Hille, 1992). Ligand-gated or mechanosensitive



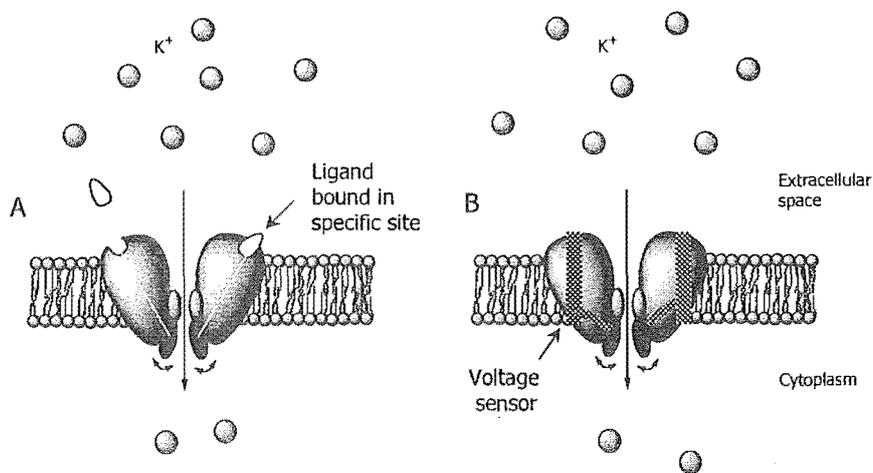


Figure 1.2 Schematic representation of the structural model hypothesised currently. This channel allows conductance of potassium ions as an example only. (A) Example of a ligand-gated channel, 'gate' is linked to a ligand-binding site on the extracellular side of the channel. (B) A voltage-gated channel. A transmembrane voltage sensor signals the 'gate' when the voltage passes a specific value.

channels operate in much the same way, with molecular apparatus in place to sense the changes that control the conductance of those channels.

1.3.3 Subconductance States

Subconductance states (substates) have been observed in channels from most membranes that have been studied thus far (Fox, 1987; Laver & Gage, 1997). They can be described as states of partial conductance, where the channel is conducting at neither fully open nor at the fully closed configuration (Laver & Gage, 1997). There are theories both that substates are cell-induced states to control conductance, or that they are products of a modified or damaged channel. It is probable that both causes of substates occur. Channels activated by second messengers can show prominent substates; suggesting a control mechanism, i.e. an increase in the binding of specific molecules may alter the conductance of the channel (Laver & Gage, 1997). The fact that a range of experimental manipulations (below) can induce substates suggests that they may be induced by protein damage. The following methods have been employed to increase the probability of substate observation:

- UV irradiation (at 280 nm) of membranes. (Busath & Waldbillig, 1983) Since this effect peaks at the absorbance maximum of tryptophan residues suggesting that some tryptophan residues are particularly important in modulating currents in the channel looked at in this study (Gramicidin A)
- Low temperature (Gustin *et al.*, 1986; Hamill & Sakmann, 1981)
- The drug curare (Takeda & Trautmann, 1984; Trautmann, 1982)
- Membrane composition (Pope *et al.*, 1982; Rosenberg & Finkelstein, 1978)
- Membrane charge and the ionic composition of the bathing medium (Schindler *et al.*, 1984)

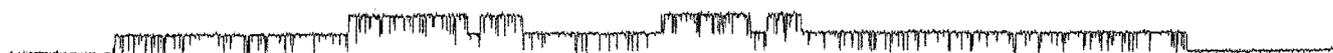
1.3.4 X-ray Crystallography

An X-ray crystal structure of a potassium channel (known as KcsA, found in *Streptomyces lividans*) determined by Doyle *et al.* (1998) was the first visualisation of a channel pore's dimensions (figure 1.3). This pore was formed by a large number of polar amino acid residues, from the transmembrane domains of the channel protein.



The majority of the pore length is far wider than the ion for which it is specific. At one point, near the extracellular side of the membrane, there is a constriction. This is the point at which, based on the size, charge and hydration energy of the ion, the protein selects those ions for which to allow passage. Since this selectivity filter is also the narrowest point along the pore, it defines the rate at which the channel allows ions to pass through it. The rate of flow, or conductance, usually is inversely correlated to how selective the channel is, although this is not the case in KcsA (Doyle *et al.*, 1998). Figure 1.4 shows the selectivity filter of KcsA. This filter is lined with residues that specifically select ions based on charge (negative to attract positive potassium ions in this case), while the size of the constriction controls diameter (therefore species) of ions allowed to pass.

The size and environment in which these proteins exist makes it hard, in most cases, to apply three dimensional visualisation techniques. For example, X-ray crystallography (see section 1.4.1) which has to date solved the structures of approximately 20 membrane-bound proteins to a relatively high resolution (Fyfe *et al.*, 2001). This means that biophysical work is still the main source for structural models of channel proteins.



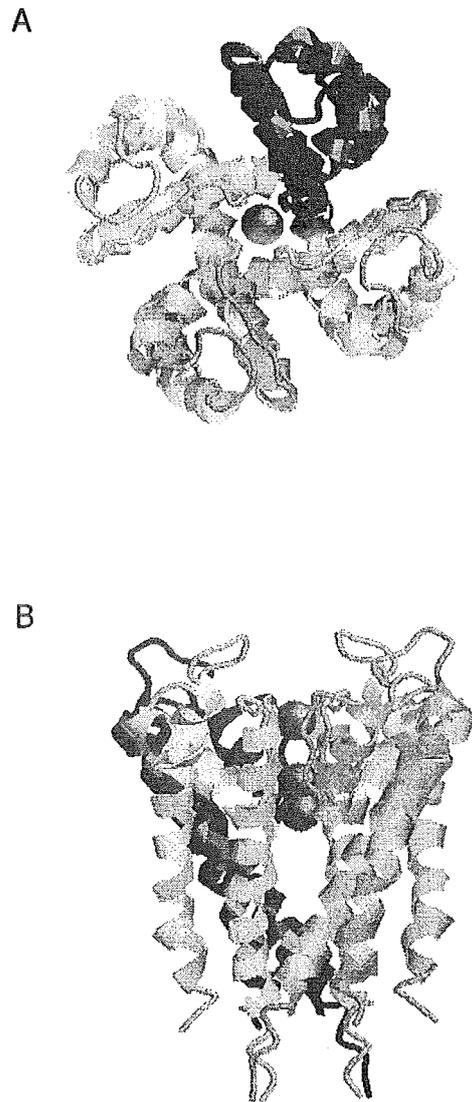


Figure 1.3 Plan (A) and elevation (B) views of the structure of the potassium channel KcsA from *Streptomyces lividans*. Solved to 3.2 angstroms by Doyle *et al.* (Doyle *et al.*, 1998). NB the spheres in the centre of the channel pore represent three dehydrated potassium ions. The four subunits of this channel protein are represented by the different shades. (A) is viewed from the extracellular side and in (B) the extracellular side is up.

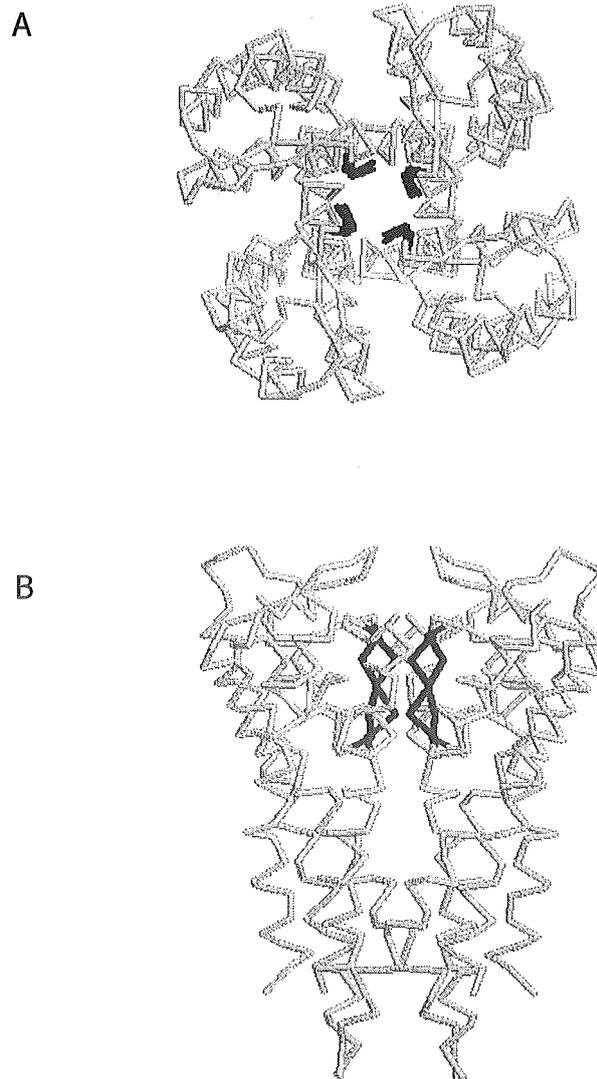
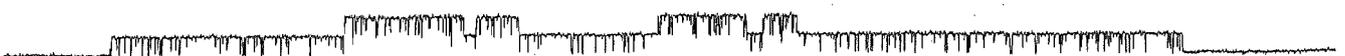


Figure 1.4 Plan (A) and elevation (B) views of the backbone conformation of the potassium channel (KcsA) from *Streptomyces lividans* with the selectivity filter shown in black. Note that the selectivity filter forms the smallest diameter section of the pore. This constriction is responsible for determining which ions can pass to the other side of the membrane (Doyle *et al.*, 1998). (A) is viewed from the extracellular side and in (B) the extracellular side is up.



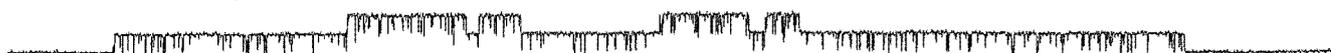
1.4 How are Channel Proteins Studied?

The study of proteins may be divided into two approaches: structural and functional. Structural studies look at the protein's shape and suggest a model of how it might work. Working the other way, functional studies look at how a protein reacts to various stimuli then can suggest how the protein might be structured. This thesis makes use of a functional observation technique known as patch clamping. The structural studies of others have provided defined parameters around which models based on functional data can be fitted therefore these will be reviewed first.

1.4.1 Structural Research

The refinement of X-ray crystallographic technique for gaining accurate molecular structures means that it is often the first method employed in the study of a protein. Unfortunately, X-ray crystallography has, up until recently, been of limited use to the study of channel. Due to the polarity of various regions within the channels (i.e. some parts prefer an aqueous environment while other parts prefer lipid) it is very difficult to form crystals from these proteins. Relatively few channel proteins have been well defined by this method although the use of novel crystallisation techniques is now providing good results (Rosenbusch *et al.*, 2001). A striking example of the power of these techniques is the study of Doyle *et al.* of the first X-ray structure of an ion channel (see section 1.3.4 and figure 1.3; Doyle *et al.*, 1998) and the recent extension of this work (Zhou *et al.*, 2001). Hence, a number of other techniques have been explored to gather data that infer structural information from functional data.

Analysis of a protein's amino acid sequence can be used to develop models of how the protein might fold. Due to the complexity of proteins, however, the reliability of this data diminishes as the protein being studied gets larger. Secondary structures (α -helices and β -sheets) and some motifs are usually the extent of what can be reliably determined. Using the degree of hydrophobicity of each amino acid, or group of amino acids, a hydropathy plot can be constructed to suggest which portions of the protein



would reside in the cytoplasm, lipid bilayer, and extracellular space. These plots are of less-use with ion channels since the channels require polar side groups to face the pore lumen making it hard to define which parts will be confined to the membrane.

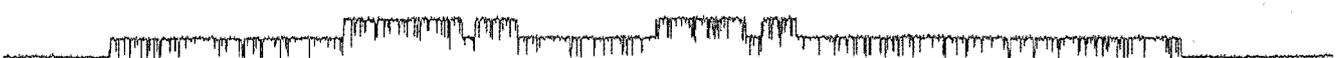
Motif comparison between peptides of channels with their structure defined and new channel sequences is of use when looking for specific structures (Mathews & van Holde, 1990). Many sections of the protein that lie outside the membrane may be cloned, as an individual polypeptide, and crystallised for X-ray structure elucidation. This is usually not useful, except to show the structure of a gating domain or binding site that may exist free of the membrane (Brandon & Tooze, 1999b).

1.4.2 Functional Research

Biophysics provides a number of techniques aimed at functionally evaluating a protein. This field studies the kinetics and electrophysiology of the channel protein. Because of their fluidity and relative strength, membrane patches have been manipulated (with respect to the rest of the membrane) in order to produce a number of techniques aimed at observing the flux of ions from one side to the other (Tester, 1997). These range from the use of radioisotopes and cell impalements, for the study of whole cells, to physical isolation of single channel proteins (patch clamping) and channel incorporation into artificial bilayers, for the study of single channels.

Data from each of these techniques can be combined through models to give a structural picture of a particular ion channel when structural studies have been unable to provide one (see above). A major problem when using data that implies structure, or function, rather than shows it directly, is the potential loss of relevance *in vivo*. Thus the question arises: Are the data an accurate representation of what happens in a living cell or are they a result of artefacts? Often an increase in data resolution means a decrease in the physiological relevance of the data. Examples relevant to channels are the various methods of observing ion flux through a channel (Tester, 1997) such as:

1. Unidirectional radioactive tracer fluxes
2. Intracellular impalements with fine tipped microelectrodes



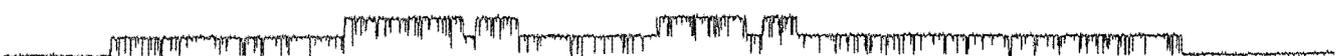
3. Patch clamp microelectrodes in 'whole cell' mode
4. Patch clamp microelectrodes with a membrane patch
5. Incorporation of channels into artificial planar lipid bilayers

When moving through the above examples from 1 to 5 each technique (figure 1.5):

- Increases definition of biochemical resolution (the conditions to which the protein is subjected)
- Decreases the physiological reality (the protein may not behave in the same way in these conditions compared with its 'natural' environment)
- Increases the resolution of the data collected, to the point that the last two techniques can resolve the movement of a few hundred ions

The technique selected depends on the question being addressed. For example, methods 3, 4 or 5 would be better for examining how the channel works, whereas the role of the channel in the physiology of the cell tissue would be better tested by method 1 or 2 (Tester, 1997).

One special note is the recent development of the microelectrode ion flux estimation technique that uses ion selective microelectrodes placed a short distance from the intact tissue. These measure multiple ion flux through the cell membranes with relatively good resolution and relatively little reduction in physiological reality (Lucas & Kochian, 1989; Shabala *et al.*, 1997).



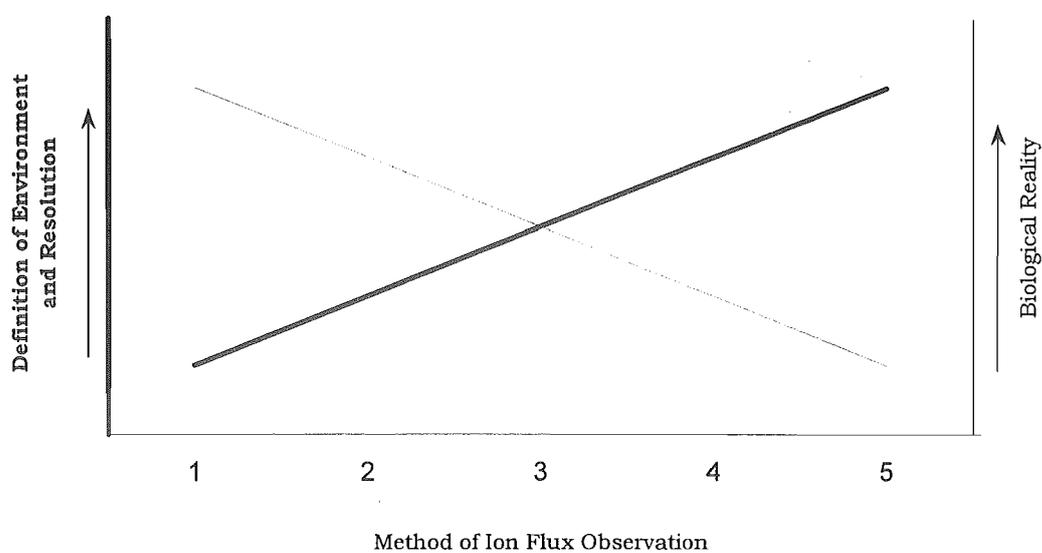
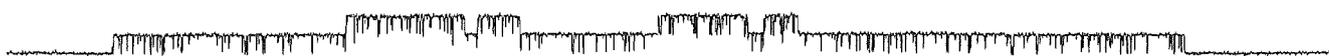
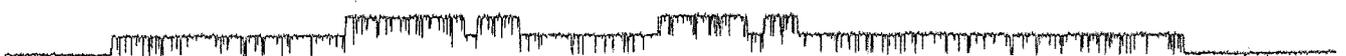


Figure 1.5 Pictorial representation of the concept outlined in the text (Tester, 1997). The methods of observation are: 1 unidirectional radioactive tracer fluxes, 2 intracellular impalements with fine tipped microelectrodes, 3 patch clamp microelectrodes in 'whole cell' mode, 4 patch clamp microelectrodes with a membrane patch, 5 incorporation of channels into artificial planar lipid bilayers.



1.4.3 Observing Single Channel Current

Given the relative ease of measuring current over a whole membrane, why try to isolate a single channel? The noise created by a large number of channels opening and closing at different times makes the contribution of most single channel events beyond the limit of resolution in whole cell experiments (Findlay *et al.*, 1994). The effects of antagonists or agonists are much easier to interpret in single channel recordings, therefore, single channel recordings are desirable even though they increase the possibility of artefact inclusion. The first recordings of a single channel were achieved by Neher and Sakmann (Neher & Sakmann, 1976) when a small fire polished electrode was pressed against a frog muscle membrane. This technique was later improved when negative pressure was applied to the microelectrode while it was pressed against a membrane. A high quality seal formed between the membrane being studied, and the glass around the circumference of the electrode tip. This allowed the measurement of ion flux through a single channel, and resulted in a 10-fold reduction in the background noise associated with the original technique (Hamill *et al.*, 1981). The background noise is caused by ionic leak between the pipette and the bath solution and this seal simply held the membrane more securely against the electrode tip. The seal is known as a gigaseal since the seal quality, which is measurable as electrical resistance, is in the order of gigaOhms ($G\Omega$) (Hamill *et al.*, 1981). This technique refinement produced the most sensitive protein assay known with the measurement of 60 monovalent ions moving through a channel possible (Tester, 1997). It was also found that the seal was mechanically strong enough to tear the patch of membrane away from the intact cell or organelle, allowing access to the solutions on either side of the membrane. These solutions were thus definable and could be altered during an experiment (Horn & Patlak, 1980). This refined technique can be used to observe and record, in real time, the ion flux through a single channel, making this technique optimal for studying the response of a channel to a range of stimuli.



1.5 What Membrane Channels?

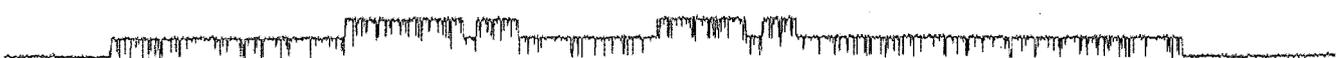
This thesis has used, as a model membrane system, the membrane surrounding the cytoplasmic droplet of the characean algae, *Nitella bookeri*. This membrane has been shown to originate largely from the tonoplast (vacuole membrane)(Lühring, 1986; Sakano & Tazawa, 1986).

The characean tonoplast membrane has been used extensively in the biophysical research of algal membranes. Characean algae have played an important role in biological research, including their use as the material where Corti (1776) first described cytoplasmic streaming. With respect to membrane transport this membrane's usefulness is akin to that of the squid giant axon used in the classical biophysical experiments that led to the electrophysiological knowledge that has revolutionised biology (Hope & Walker, 1975). Both characean algae and the squid axons are unusually large and durable cells (Baker *et al.*, 1962; Cole & Hodgkin, 1939; Hodgkin & Horowicz, 1959; Hodgkin & Huxley, 1952; Hodgkin *et al.*, 1949; for review see Katz, 1966). The plasma membrane, however, has proven problematic to biophysicists due to the cell wall being resistant to enzymatic degradation. This has been overcome in several cases by performing microsurgery on the wall with micromanipulated knives or a laser (Coleman, 1986; Laver, 1991; McCulloch *et al.*, 1997). The tonoplast has an advantage of being unencumbered by a cell wall, thus allowing approach to the membrane unobstructed.

Cytoplasmic droplets from *Nitella bookeri* (a characean algae found almost solely in New Zealand; Wood & Mason, 1977) were produced easily using the large internodal cells that commonly reach 5 cm in length and have been recorded up to 15 cm.

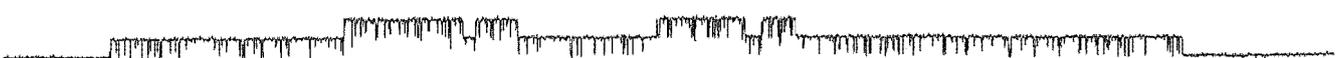
1.5.1 Previous Characean Tonoplast-Channel Research

The first research specifically aimed at distinguishing the tonoplast were conducted as an improvement to the voltage clamping technique recently adopted from animal cell biophysics (Findlay, 1961; Findlay, 1964). These studies developed the



voltage clamp technique (see section 2.1.3) for use on algal cells, thus paving the way for patch clamping. Patch clamp studies of the characean tonoplast membrane were initiated in the mid-eighties (Coleman, 1986; Lühring, 1986). These have shown a number of channel types and suggest the possibility that a large number are still undescribed (Tyerman & Findlay, 1989). The first to be characterised was a ~165 pS potassium channel which became saturated at ~20 pA inward and ~10 pA outward current. This channel was highly selective for potassium ions (permeability ratios P_{Na}/P_K and P_{Cl}/P_K estimated to be both ~0.01) and exhibited at least one additional conducting state that was less than maximal (a subconductance state) (Lühring, 1986). This channel has become known as the multistate-K, prime-K, and the maxi-K channel. It has similarities to the ubiquitous animal maxi-K or BK channel: it is sensitive to calcium and to the calcium-binding protein, calmodulin, suggesting an allosteric interaction (Laver *et al.*, 1997; Laver & Walker, 1991). Potassium ion current is affected by sodium ions at either side of the membrane (Bertl, 1989). Effect of pH was studied by Lühring and it was found that while an increase in acidity did not affect the current through the channel it did reduce the open time (Lühring, 1999). This channel has also been analysed kinetically to provide a model of the action of various inhibitors and competitors (Bertl, 1989; Djurišić & Andjus, 2000; Klieber & Gradmann, 1993). This large conductance channel has not yet been observed in plant cells other than in the characean algae (Laver & Walker, 1991) and in *Acetabularia* (Bertl & Gradmann, 1987).

In the study by Tyerman and Findlay, two potassium and one chloride channels were described (Tyerman & Findlay, 1989). The potassium selective channels conducted 100 and 60 pS with the latter being an outward rectifier (having a greater conductance at positive membrane potentials than negative). The chloride channel observed had a conductance of 30 pS and was also outwardly rectifying. It has been shown that this channel responds to calcium ions as a gating factor (Thiel & Dityatev, 1998) and the difficulty involved in obtaining an excised patch containing an active chloride channel (Tyerman & Findlay, 1989) suggests that this channel may require cytoskeletal elements to allow its gating mechanism to function. Tyerman and Findlay suggest that the chloride channel is of importance in the regulation of the vacuole to cytoplasm volume



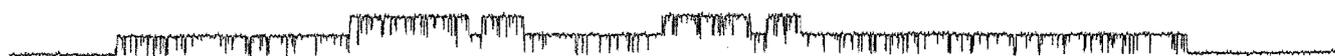
ratio, since an opening of the chloride channels creates a gradient down which potassium ions flow (to maintain electroneutrality) followed by water (to maintain osmolarity). Blockage of both the major channels described above causes a reduction in the water permeability of the membrane (Djurišić & Andjus, 2000).

In the Lühring study described above (Lühring, 1999), it was noted that, while other research had suggested that the frequently observed potassium channel was calcium-activated (Laver & Walker, 1991) there were in fact both calcium-activated and non-calcium-activated forms. There have also been reports of a variety of large-conductance potassium channels including the channel discussed above that had a conductance of 60pS (Tyerman & Findlay, 1989), a 60-80pS channel (Pottosin, 1990), and a 100-130pS channel (Pottosin *et al.*, 1993). Lühring suggested that these were conditions-dependent substates of the 170pS channel that had been reported in 1986 (Lühring, 1999). Because of the availability of a characean algae species and the previous success with the characean tonoplast membrane, *Nitella hookeri* was chosen as a model membrane for the glycation experiments described below.

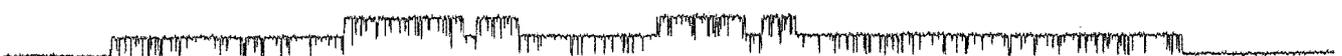
A recent study by Kikuyama and Tazawa (2001) shows that the calcium release that had been observed earlier (Reeves *et al.*, 1985; Tazawa, 1998) was due to the presence of stretch-activated channels that were permeable to calcium ions. This release of calcium due to pressure was observed most in the Characeae *Nitella flexilis* although it was observed to a lesser degree in other species.

1.5.2 *Nitella hookeri*

There are no endemic characean algae species in New Zealand although four genera are represented: *Chara*, *Lamprothamnium*, *Nitella*, and *Tolypella*. The most common species (*N. hookeri*), however is commonly found only in New Zealand, with rare populations in Australia and Kerguelen Island. The similarities between the characean algal species, and the characteristics of those species, suggest that all New Zealand species may have been derived from Australia (Wood & Mason, 1977).



Nitella hookeri is found throughout New Zealand in stagnant or slowly moving fresh water such as drains or ponds. The material used in this research was classified based on Wood and Mason's survey and classification of New Zealand Characeae (i.e. "*Nitella hookeri* can be recognised as the only New Zealand *Nitella* which has pluricellular dactyls (2 or more celled)" – continues, see Wood & Mason, 1977). It often forms a dark green 'undergrowth' around other aquatic plants such as watercress (*Nasturtium sp.*). Characeae have a dibiontic life cycle (also referred to as an alternation of generation life cycle) and undergo parenchymatous growth (Mauseth, 1991; Raven *et al.*, 1999). This combined with the fact that characean reproductive structures are multicellular (Hope & Walker, 1975), and it can be debated that this family should be classified as plants. These features have led to the conclusion that characeae are either a possible ancestor of all plants or an example of convergent evolution (Mauseth, 1991; Raven *et al.*, 1999).

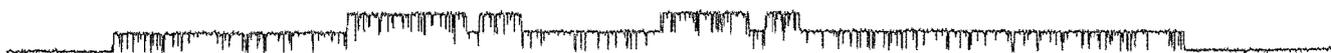


1.6 Maillard Reaction

'Maillard reaction' is the title given to the series of reactions that are initiated by nonenzymatic glycosylation of an amino group (Nagaraj *et al.*, 1996) and result in a complex and heterogeneous group of compounds (Singh *et al.*, 2001; see figure 1.6). A number of molecules are known to initiate the Maillard reaction. Examples of these are glucose (and its autodegradation products, such as methylglyoxal (MG)) and glutaraldehyde (a rapidly reacting glycating agent used as a model for reactions likely to occur in living systems).

Until relatively recently, the Maillard reaction, first described by Louis-Camille Maillard in 1912 (Maillard, 1912), has been studied with an emphasis towards food science since it occurs readily during food storage, commercial and domestic processing. The reaction alters food colour, appearance, taste, nutritive value, and toxicity; research has been aimed at understanding and controlling the reaction. The last 15 years have seen research starting to focus more attention on the Maillard reaction in living systems (Friedman, 1996). The majority of this research focuses on medical aspects rather than plant systems. However, relevant medical research has been reviewed below since it is anticipated that the biochemistry will be similar at the molecular level in all cells.

Maillard chemistry becomes relevant to this project when glycating agents come into contact with membrane channel proteins. It has been shown that the physiological concentrations of Maillard initiators (glycating agents such as MG) are sufficient to allow the reaction to occur (Nagaraj *et al.*, 1996). The Maillard reaction has been implicated in diabetes (diabetic cataracts, kidney disease), Alzheimer's disease (formation of the neurofibrillary plaques characteristic of Alzheimer's patients), cataracts, and the ageing process (similar to diabetics but at a slower rate) (Thornalley *et al.*, 1999). A symptom of diabetes mellitus is elevated blood sugar levels, therefore, the formation and accumulation of Maillard end products (known as advanced glycation endproducts, AGEs) is accelerated (Baynes *et al.*, 1986; Tessier *et al.*, 1999; Yamagishi *et al.*, 1997).



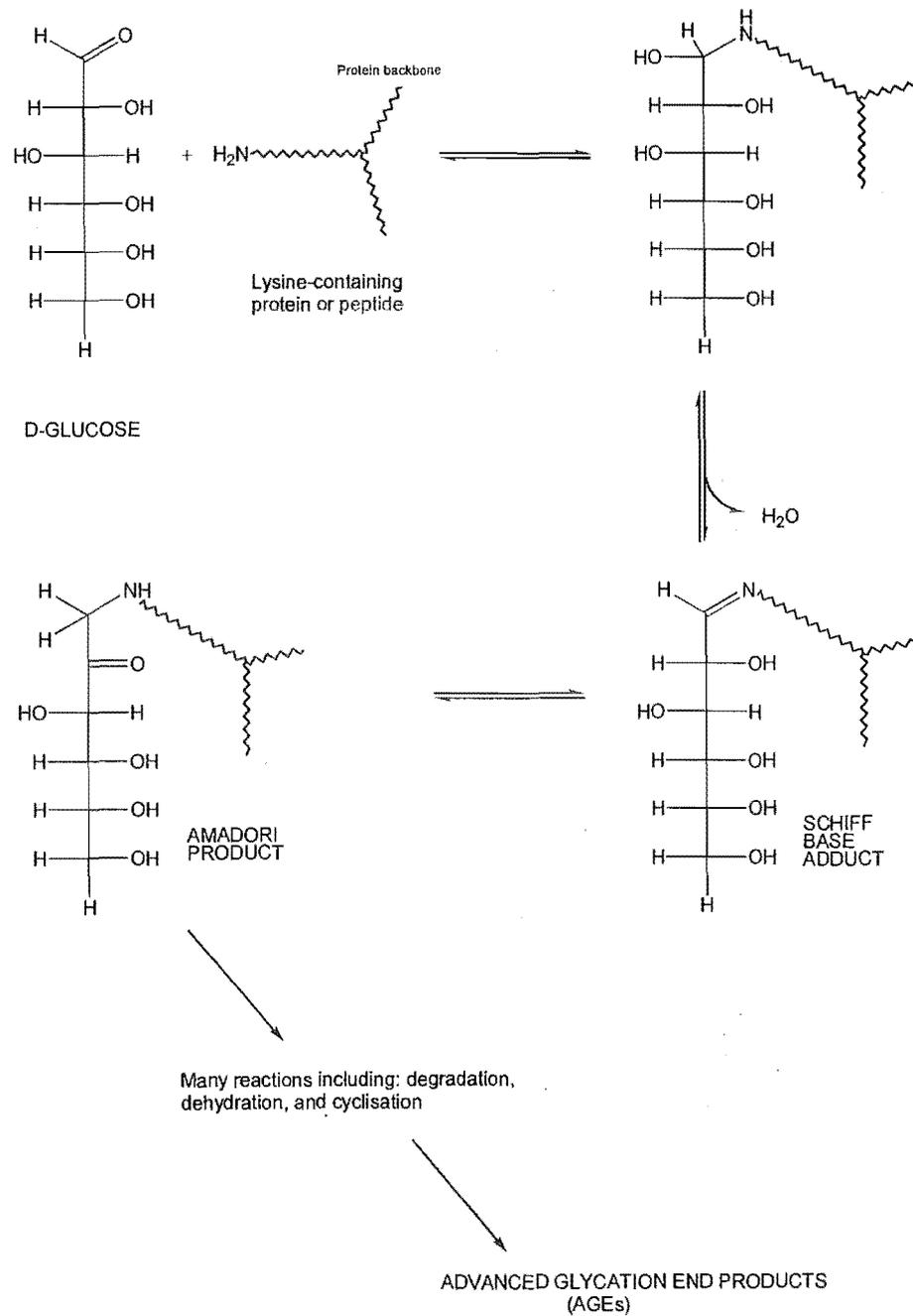


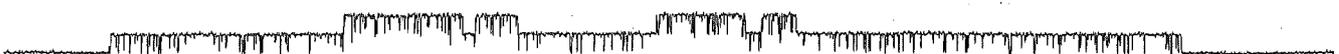
Figure 1.6 The initial steps of the Maillard reaction with a generalised protein.

It has been shown that type 1 diabetic patients can have a blood MG concentration 5-6 times higher than normal. Blood MG concentrations in type 2 diabetic patients are 2-3 times the normal levels (Nagaraj *et al.*, 1996). A study on plasma levels of an AGE breakdown product in Alzheimer's disease patients showed an increase over age-matched control subjects. Furosine (an acid hydrolysis product of the AGE ϵ -deoxyfructosyl-lysine) was found to be twice as concentrated as normal in Alzheimer's patients, but 50% lower than in diabetic patients (Riviere *et al.*, 1998).

When first described, the Maillard reaction was defined as the reaction between the free amino group of proteins and the carbonyl group of a reducing sugar. It has now been expanded to include aldehydes, ketones, and oxidised fatty acids as the carbonyl source, and free amines, free amino acids, nucleic acid bases, and some vitamins as an amino group source (Cox, 1991). All Maillard reaction pathways begin with the covalent attachment of a carbonyl group to a free amino group such as an amino acid, peptide, or protein. In proteins, the amino groups that react most often are the side chain amino groups of lysine and arginine residues, and those of the amino-terminal of a polypeptide. These are often most accessible because of their positioning away from the rest of the main protein chain (Thornalley, 1993; Thornalley *et al.*, 1999).

Because of the complexity of the latter stages of the Maillard reaction, the pathways followed are, as yet, largely unknown. The initial steps however have been well described (figure 1.6). In this example a glucose molecule binds to a protein-bound lysine residue to eventually form a Schiff's base adduct. This undergoes rearrangement to a more stable Amadori compound (1-amino-1-deoxy-2-ketose) that may then react via a number of pathways involving a complex series of dehydration, elimination, cyclization, cross-linking, and fragmentation reactions, resulting in a profusion of compounds (Friedman, 1996).

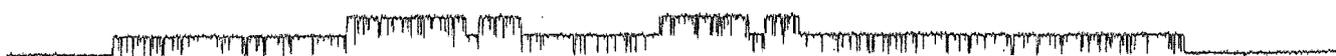
As previously mentioned, glycating agents are relatively common in living systems, for example MG and glyceraldehyde are in such abundance that they were



thought to be intermediates in alcoholic fermentation, a theory proposed by Carl Neuberg (1913), which held for 15 years (figure 1.7) (see Fruton, 1999). Since the Maillard reaction has a direct effect on proteins it is logical to predict that glycating agents may affect channels in living systems at some time.

Methylglyoxal is a small dicarbonyl molecule (figure 1.8) in most cases MG is produced by 'accidental' reactions (i.e. not part of normal metabolism and not enzymatically catalysed). The glyoxalase system is employed to reverse the formation of this toxic molecule (figure 1.9; Thornalley, 1993). Free MG (i.e. not scavenged by the glyoxalase system *in vivo*) forms reversible, then irreversible bonds to, and between, proteins that may inhibit or disrupt their activity (Oya *et al.*, 1999). Although *in vitro* versus *in vivo* studies show that the protection afforded by the glyoxalase system is great (Best *et al.*, 1999), a moderate increase of MG, or destruction of the glyoxalase system, can have a significant effect on a cell's protein structure and function (figure 1.10; Thornalley, 1993).

As well as looking at the results that this thesis produces from the perspective of normal channel blocker research, they will be of interest from the perspective of the pathophysiology of aging and the complications of diabetes (Thornalley *et al.*, 1999).



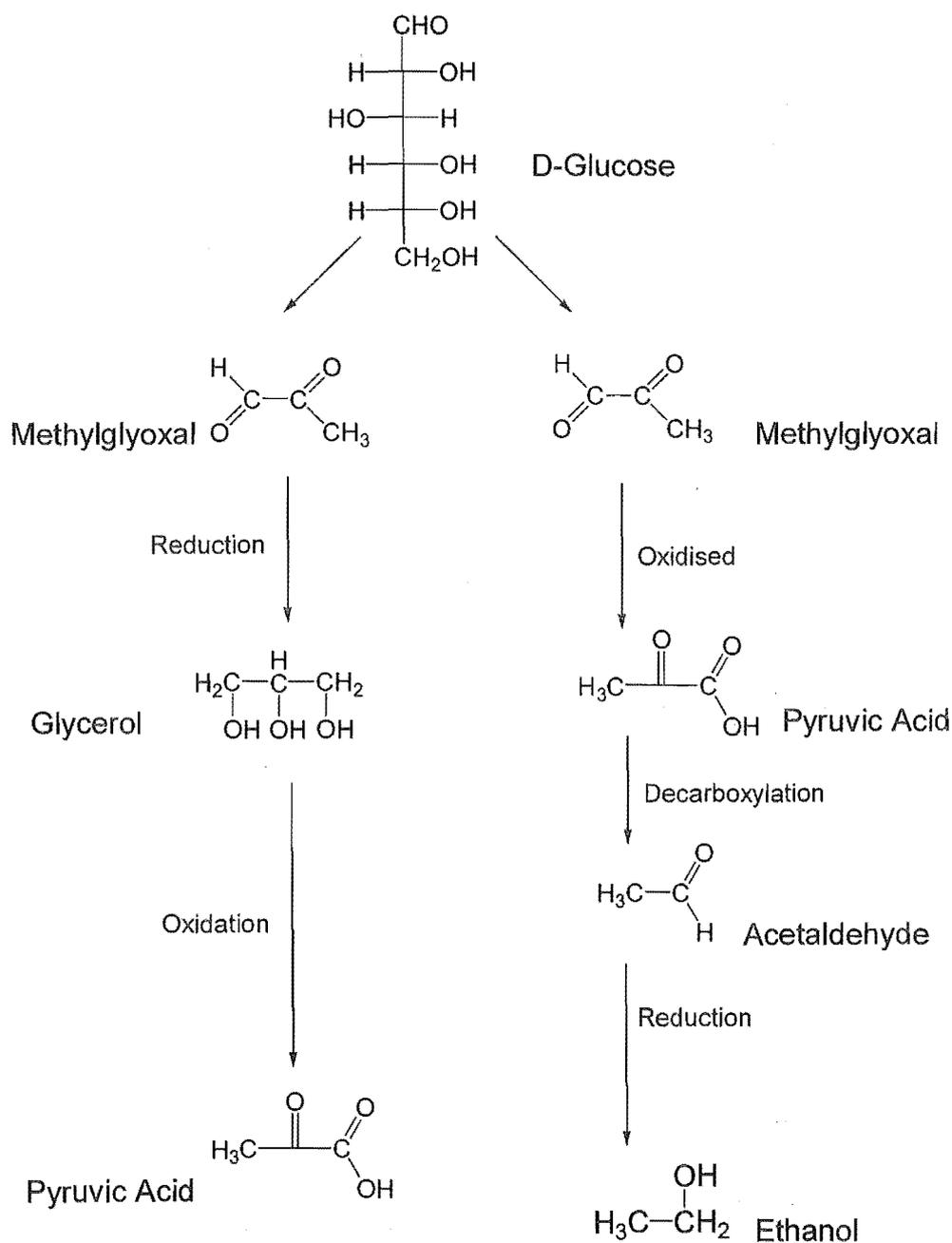


Figure 1.7 A schematic of Carl Neuberg's theory (1913) of alcoholic fermentation involving the cleavage of a glucose molecule into two molecules of methylglyoxal.

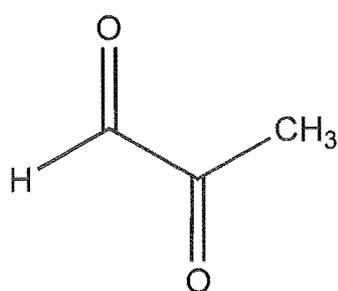


Figure 1.8 The chemical structure of Methylglyoxal.

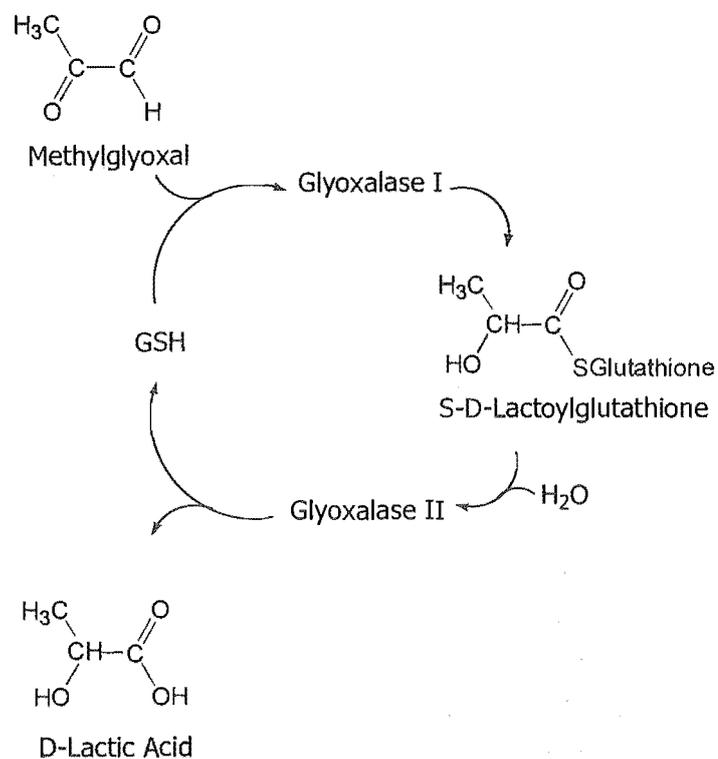


Figure 1.9 A schematic representation of the Glyoxalase system. The toxic methylglyoxal is converted to D-lactic acid with the reduction of glutathione (Thornalley, 1993).

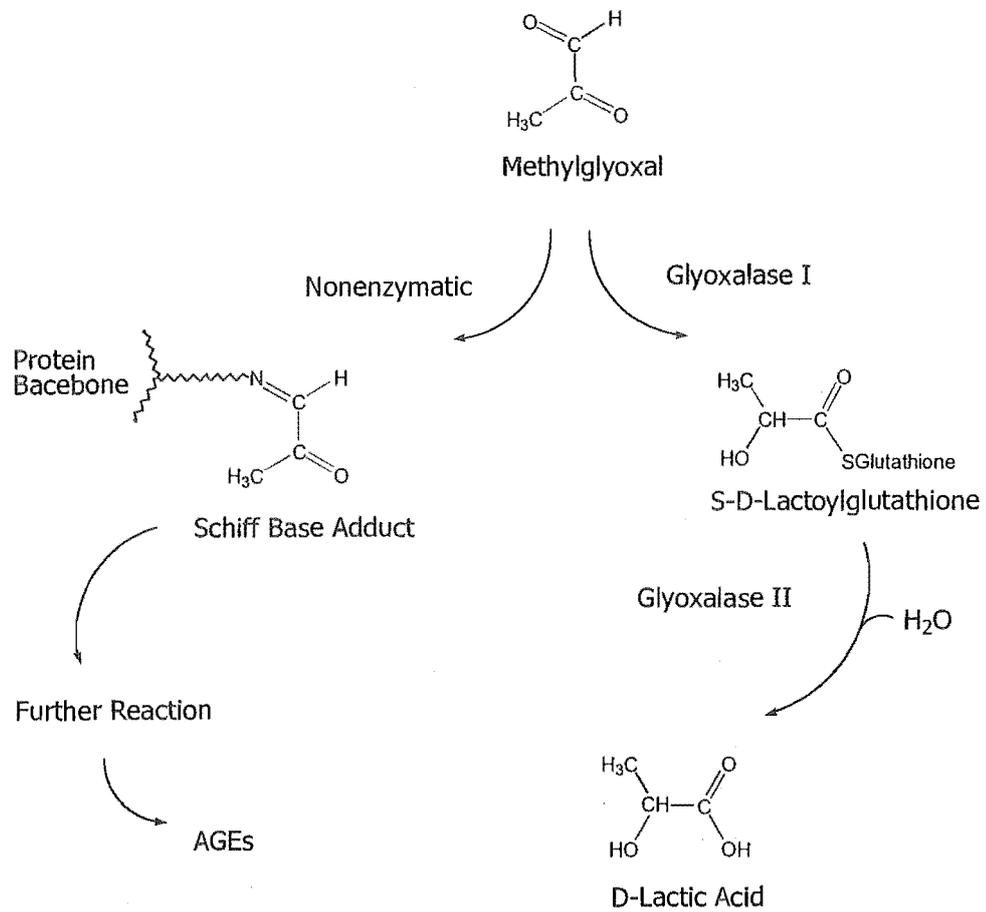
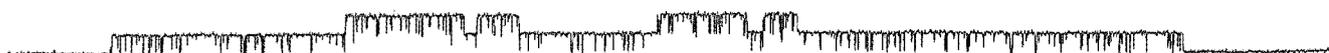


Figure 1.10 Scheme showing the competitive impact of the glyoxalase system on the Maillard reaction *in vivo*. The enzyme, glyoxalase I, scavenges for methylglyoxal stopping covalent modification of protein.

1.7 Glycation of Channel Proteins

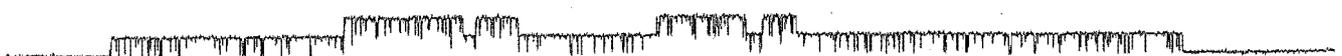
Proteins often undergo enzyme initiated, and regulated, glycosylation (addition of sugar molecules) during post-translational modification to allow proper functioning. This targeted glycosylation is thought to play a role in protein folding, sorting, and membrane targeting (Rho *et al.*, 2000) and some channels can be heavily glycosylated. An example is rat brain and skeletal muscle sodium channel that are 20-30%, by weight, carbohydrate (Zhang *et al.*, 1999). Little is known about the purpose glycosylation plays in channel production or function *in situ*, but a dramatic effect is often seen on the action of the channel when glycosylation is removed by mutational studies. It is suggested that since glycosylation often does not affect expression and functionality of the channel (yet is an evolutionary conserved feature of most channel families) the glycosylation may have a functional purpose (Freeman *et al.*, 2000; Rho *et al.*, 2000; Shi & Trimmer, 1999; Zhang *et al.*, 1999). That is, glycosylation may be of structural importance for a channel's action.

Random, non-enzymatic glycosylation, referred to in this thesis as glycation, can also occur on proteins in cellular systems. This is also known as the Maillard reaction (see section 1.6). Its effect in membrane transport systems have been observed in a calcium pump (González Fletcha *et al.*, 1990; González Fletcha *et al.*, 1999). Due to the toxicity of this protein modification, there are enzymatic systems in place to remove specific glycating agents from the living system. An example of this is the glyoxalase system (mentioned above) which catalyses the conversion of methylglyoxal (MG) to D-lactate (Figure 1.9; Thornalley, 1993). However, the glycation of certain proteins persists and the effects often accumulate, leading to metabolic complications over time. Thus glycation of proteins, and perhaps a genetic susceptibility to glycation, has been implicated in a number of human diseases and some complications of the aging process (Baynes *et al.*, 1986; González Fletcha *et al.*, 1999; Nagaraj *et al.*, 1996; Riviere *et al.*, 1998; Singh *et al.*, 2001; Tessier *et al.*, 1999; Yamagishi *et al.*, 1997). Since the frequency of the substrate occurrence in certain channels increases with time (Garrill, 2001) there is the



possibility that substates may be a result of glycation. Therefore it is of interest to study the effect of glycation agents on any ion channel.

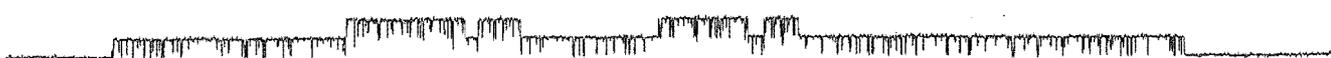
The research conducted for this thesis characterised the cytoplasmic droplet membrane of the characean algae, *Nitella hookeri* as a model system in which the effects of glycation could be studied. Specifically, it aims to assess the ability of glycation agents to alter substate frequency and/or current amplitude of the channels observed.



1.8 Aims of this Thesis

To summarise, ion channels are important, membrane-bound proteins which allow the cell to maintain and adapt its ionic composition according to its environment. Channel proteins are often glycoproteins; that is, they often have sugar molecules added to them after they are translated from their RNA. After this post-translational modification, proteins are still vulnerable to glycation through the non-cell directed Maillard reaction. In certain conditions, such as aging or disease, the probability of a protein becoming glycated via the Maillard reaction increases. Thus the aims of this thesis are:

1. To develop optimal conditions for patch clamping *Nitella hookeri* on which, to the best of this laboratory's knowledge, no channel characterisation work has yet been conducted.
2. To identify the channels present in *Nitella hookeri*.
3. To study the affects of protein glycation on membrane proteins.



Chapter 2

Method Development

“Not everything that can be counted counts, and not everything that counts can be counted. “

- Albert Einstein (1879-1955)

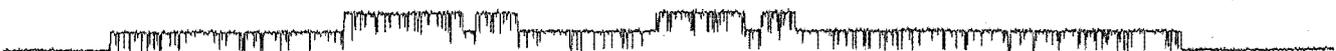
2.1 Patch Clamp Theory

2.1.1 Patch Clamping: An Introduction

“Patch clamp” refers to the experimental technique that electrically isolates a small area, or patch, of membrane and “clamps” the voltage across it in order to observe current flux. The electrical isolation involves sealing a patch of membrane to a clean glass electrode with a high quality giga-seal (Hamill & Sakmann, 1981). With persistence, a patch is obtained with a giga-seal that contains a single ion channel.

2.1.2 “Patch”

Once a giga-seal has been achieved, there are a number of physical manipulations that allow access to both sides of the membrane, and definition of the solutions on both sides of the membrane patch (figure 2.1). The status of the patch when it is first attained is known as cell-attached; the patch is electrically but not physically isolated from the rest of the membrane. A problem with this configuration is that the pipette, or extracellular, solution is the only defined solution. This is remedied by tearing the patch away from the rest of the membrane, taking the single functional channel with it (Hamill *et al.*, 1981; Horn & Patlak, 1980). The defined bath solution is now in contact with the intracellular face of the membrane (i.e. the face that was previously the internal face of the droplet). This configuration is known as an inside-out excised patch. A method has also been developed to place the outer face of the membrane on the bathing solution side (see outside-out excised patch in figure 2.1) therefore allowing access to the extracellular membrane face (Hamill *et al.*, 1981). This method first bursts the patch so that the pipette solution diffused into the tonoplast – whole cell configuration – then withdrawing the electrode from the membrane as for the inside-out configuration attainment. The membrane is then thought to reform across the electrode tip (Hamill *et al.*, 1981). In some cases, the reformed piece of membrane has a single channel present that can be studied.



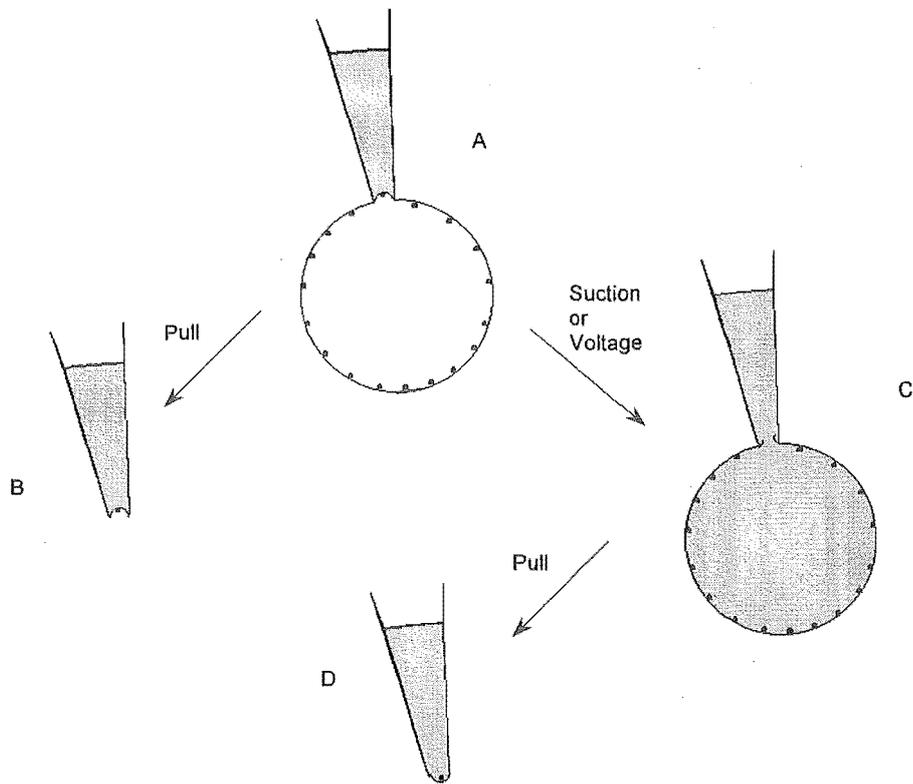


Figure 2.1 Patch clamp configurations. Cell attached (A), Inside-out excised patch (B), Whole cell (C), and Outside-out excised patch (D). The small black circles indicate the inside of membrane. The gray fill represents the solution inside the pipette (patch electrode) which can, in the case of the whole cell configuration, perfuse the cell.

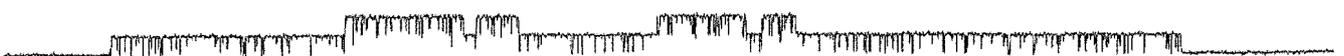


2.1.3 “Clamp”

The electrical potential difference (voltage) can be measured across the patch of membrane. Since current is the flow of charged ions, it can be seen as a change in voltage, but this can only happen when there is an electrochemical gradient to force the movement of ions when the channel opens. Therefore, to measure single channel current the voltage clamp technique must be employed. This is a method where an artificial voltage gradient is enforced across the patched membrane. Using two electrodes and specialised electronic circuitry (Ypey & DeFelice, 1999) any variation in voltage, which is constantly monitored by a feedback circuit, signals a movement of ions, or current. Concurrently, the circuitry keeps the voltage to the clamped value by adjusting the charge on either side of the membrane, and sends this data (how much voltage adjustment is required) to the output device (oscilloscope or PC). The data received by the output device is proportional to the current flux through the open or closed channel and is easily converted to a relevant scale and units (usually picoAmperes (pA)). Thus raw data shows current (pA) plotted against time (for example see figure 3.4).

2.1.4 Analysing Single Channel Recordings

Plotting current (I) against the clamped voltage (V) for raw data at a range of voltages (produced by the step-waveform application of Clampex7's Protocol Editor) gives an IV curve that provides a means of identifying channels. Data were recorded from approximately 1 second after the voltage change. The point at which the curve crosses the x-axis (the reversal potential) is indicative of the ion(s) the channel is selective for (see section 2.1.2). The maximal slope of the curve gives the channel's conductance (found by application of Ohm's law, equation 3.1), where the curve plateaus shows where the channel has become saturated (at maximum flow) or has achieved maximal flux in that solution.



$$V = IR = I/G \quad 3.1$$

$$G = V/I \quad 3.2$$

V = potential difference between two points

I = current

R = resistance

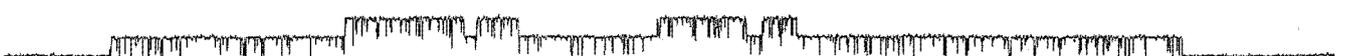
G = conductance (measured in seimens (S))

Also shown is the rearrangement to a form that allows calculation of a channel's conductance from IV data (equation 3.2).

2.1.5 Substates

Since substates have been reported in characean tonoplast membranes it is important to recognise the difference between a channel exhibiting subconductance and multiple active channels. Although the term 'subconductance' seems to explain itself, it is often hard to recognise substates in a patch clamp recording where there often may be more than one channel present in a patch. In an attempt to overcome this difficulty, Fox (1987) outlined the following criteria for identifying substates. These are that:

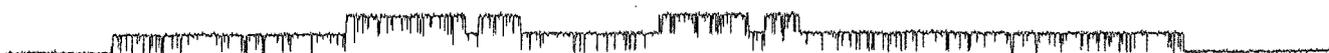
- 1 Conductance transitions should exist between all the conductance states (otherwise the patch may contain multiple channels)
- 2 Substates should only appear in the presence of the main state (again, showing that there are not multiple channels present)
- 3 It must be established that the main state is not the sum of two smaller channels. To ascertain that a 60pS conductance is a subconductance of a 100pS mainstate it should be recorded in the absence of a 40pS conductance.



2.2 Patch Clamp Methodology

The study material, characean algae, was harvested from a relatively stagnant pond in Beckenham, Christchurch, New Zealand (grid reference: S 43° 33.715', E 172° 38.864'). The samples were kept in culture in the laboratory in a glass tank. The culture was exposed to natural lighting with shading from direct light to reduce unwanted growth of other organisms (e.g. other fast growing species of algae). The samples were collected approximately once a month. Pond water was collected at the time of harvest and was used to bathe the culture. Droplets were produced by drying an internodal cell for ~10 minutes before cutting one end off the cell and extruding the cell contents into the bath solution.

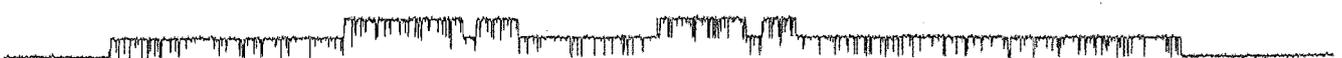
For patch clamping experiments the patch electrode was filled (using suction initially through the tip, then back filled) with the pipette solution and attached to the headstage (CV 203BU, Axon Instruments) via a polycarbonate pipette holder (HL-U series holder, Axon Instruments). The pipette holder served to support the electrode, connect it electrically to the amplifiers of the rig, and to provide an outlet from which suction or pressure could be applied. The electrode was only filled as much as was required (about 1 cm from the tip) to partially cover the chloride-coated silver wire, that is the output to the headstage, since excess solution increased noise. With positive pressure in the patch electrode, it was lowered through the air-water interface. To achieve a correct read out from the voltage clamp, the voltage between the patch electrode and the reference electrode (connected via a 3M KCl-agar salt bridge to the bath solution) was then set to zero. Still with positive pressure in the electrode, it was moved to contact the chosen membrane using a micromanipulator (MX-2 Narishige Direct drive, Narishige Group) and viewed with a standard research microscope (Axioskop 2 FS, Carl Zeiss (NZ) Ltd). A release of the positive and a small amount of negative pressure usually caused a seal between the membrane and the glass of the electrode tip. Seal quality was observed using the seal test function of Clampex7 set to a voltage step of 5mV. Clampex7 is part of the pClamp program suite (version 7.0) which



was used for all acquisition and analysis of data. In this study, when a suitable seal was achieved, the patch was excised to inside-out configuration (see figure 2.1). If the patch contained a single channel, the experiment proceeded and data were sampled at 200 kHz and filtered at 1 kHz using the internal 4-pole bessel filter of the Axopatch 200B amplifier.

The headstage amplified the data from the patch and reference electrodes (figure 2.2) and sent it to the main amplifier. These data were amplified using an Axopatch 200B amplifier and a Digidata 1200B analogue to digital converter (all Axon Instruments, Inc. Foster City CA) allowed the signal to be read by a Pentium computer running the acquisition program, Clampex7 (figure 2.3). Data were archived on 650 MB compact discs. The experiment was conducted inside a Faraday cage that surrounded the microscope, micromanipulators, headstage, and cell preparation. This shielded as much of the normal background electromagnetic radiation as possible from the sensitive amplifiers, thus reducing noise.

For glycation experiments the glycating agent was added in a step-wise manner using a Gilson pipette directly to the bath medium. This method was found to be the quickest and least damaging for the patch, provided sufficient care was taken not to jar the microscope or headstage. Data were collected in Clampex7's gapfree mode for approximately one minute between additions. A 1 mL chamber filled with bath solution was used in each experiment, this was sufficient to contain the experimental material, allow the use of water emersion lenses, and allow sufficient space for the patch electrode to fit into the field of view. This chamber consisted of a plastic microscope slide with a tapered hole (20-16mm) in the centre of it. A coverslip was attached with petroleum jelly to the bottom of this chamber. Analysis was performed using the Fetchan and Pstat programs to produce and fit Gaussian functions to all-points histograms that were used to show the distribution of time spent at various levels of current. The 'open' peak of an all-points histogram can be subtracted from the baseline value or 'closed' peak to calculate the actual current flowing through the open channel.



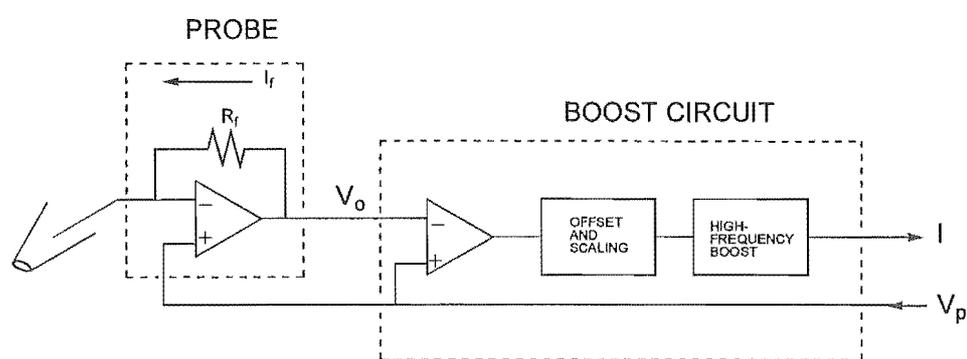
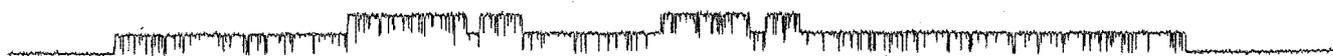


Figure 2.2 Circuitry diagram of the resistive headstage used in all patch clamp experiments. The headstage in patch clamp apparatus is a current to voltage converter. Because the output bandwidth of the probe is low it is passed through a high-frequency boost circuit (Axon instruments Inc, 1997).



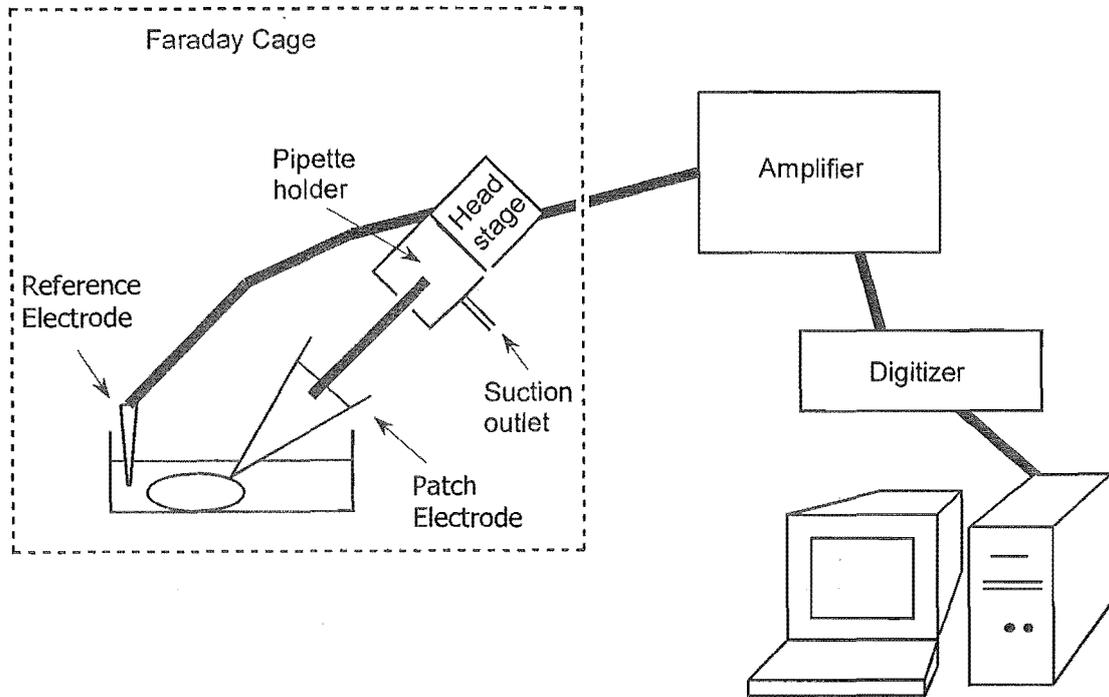
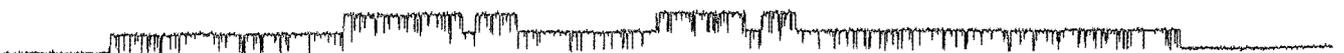


Figure 2.3 Schematic representation of the patch clamp rig used in this thesis. The entire experiment is contained in a Faraday cage to protect the exposed circuitry from electrical noise caused by electromagnetic radiation. All electronics outside the cage are shielded and grounded, again to avoid unwanted electrical noise.



2.3 Solution Development

2.3.1 Solution Development: Theory

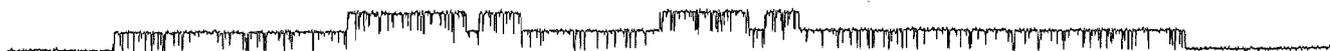
To be able to resolve single channels and identify them, the bathing and electrode filling solutions need to satisfy two criteria (see Ogden (1994) for review):

1. The ionic concentration must be high enough so that when a selective channel opens in an isolated patch of membrane, there are adequate ions present to create a detectable current (i.e. the channel needs to be sufficiently saturated with substrate that its action can be observed). For instance, if the concentration of potassium ions in the solution is very low, a potassium selective channel may be undetectable.
2. The reversal potentials (the voltage at which no ionic current moves between the solutions) of the various ionic components of the solution must be sufficiently separated so that the channel can be confidently identified. That is, channels that are selective for different ions need to be distinguishable, even if resolution is decreased (determined by the amount of electromagnetic and vibrational noise).

The ability of a solution pair (bath and electrode solution) to satisfy the first criteria can only be found by experimentation, since it relies on the characteristics of channel being observed (e.g. the conductance, selectivity, etc).

2.3.2 Calculation of Reversal Potentials

The second point mentioned in section 2.3.1 can be calculated without experimentation and with reasonable accuracy by using the Nernst equation (Nernst, 1888, see Hille, 1992). Rearrangement and partial solving of this equation (equation 2.1) provides equations for each mobile ion in a solution to give their reversal potentials.



$$E_s = E_1 - E_2 = \frac{RT}{z_s F} \ln \frac{[S]_2}{[S]_1} \quad (2.1)$$

E_s = equilibrium potential or the membrane potential difference (i.e. the difference between the electric potential on both sides, denoted 1 & 2, of the membrane; $E_1 - E_2$)

R = gas constant ($8.315 \text{ J K}^{-1} \text{ mol}^{-1}$)

T = temperature (degrees Kelvin)

F = Faraday's constant ($9.648 \times 10^4 \text{ C mol}^{-1}$)

z_s = charge of the ion being studied

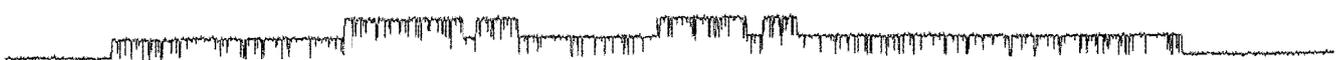
S = the ion (1 and 2 refer to the sides of the membrane as above)

Examples of some biologically relevant ions are shown in equations 2.2a-c.

$$E_K = \frac{RT}{F} \ln \frac{[K]_o}{[K]_i} \quad (2.2a)$$

$$E_{Cl} = \frac{RT}{F} \ln \frac{[Cl]_i}{[Cl]_o} \quad (2.2b)$$

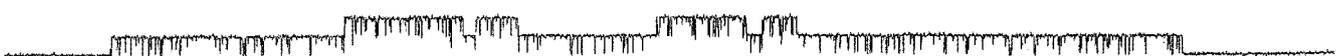
$$E_{Ca} = \frac{RT}{2F} \ln \frac{[Ca]_o}{[Ca]_i} \quad (2.2c)$$



2.3.3 Designing a Solution Pair

Solutions must be designed in pairs (bath and pipette solutions) to allow the calculation of reversal potentials. The bath comes into contact with the extracellular side of the membrane in cell attached, whole cell, or outside-out configuration, while it is in contact with the intracellular side when in the inside-out configuration. There are three objectives that must be taken into account when designing a solution pair:

0. The cell, or tonoplast, must be able to survive. If the bath solution is too far removed from the salinity of the cell's previous environment, the rapid increase or decrease in osmotic pressure may destroy the membrane or alter its normal properties. For the present study previous reports were used as a starting point to fulfil this requirement (Andjus *et al.*, 1999; Djurišić & Andjus, 2000; Klieber & Gradmann, 1993; Laver *et al.*, 1997; Lühring, 1986; Lühring, 1999; Reeves *et al.*, 1985; Theil & Dityatev, 1998; Tyerman & Findlay, 1989; Tyerman *et al.*, 1992; Weise & Gradmann, 2000).
2. Each of the solute's reversal potentials must be sufficiently separate so that a channel can be identified. It is often preferable to have the reversal potentials separated by at least 5 mV especially in the case of a noisy recording where it is hard to pinpoint the exact voltage of current reversal (Hille, 1992).
3. The make-up of a solution pair affects the length (chronologically) and electrical quality of the seals. A seal must last long enough to allow experimentation, and must be of sufficient quality (low noise) to show every required trend (Hille, 1992). It is conceivable that a solution that meets all other criteria won't form seals of any quality or longevity. Some unknown factor (the concentration of a certain ion or an impurity in one or both of the solutions) may be responsible for this.



2.3.4 Junction Potentials

At an interface between two solutions there can be an electrical potential difference due to either different ionic composition, or concentrations (Halliwell *et al.*, 1994). This is known as the junction potential. Each solution pair must have its junction potential calculated (or measured see Ogden & Stanfield; 1994) so that during analysis of the raw trace, the clamped values can be corrected. In the present study junction potentials have been calculated, using Clampex7's junction potential function, and shown in the caption below each table.

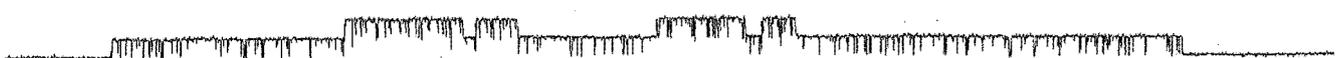
2.3.5 Solution Development: Methodology

Seven solutions were designed and tested for their ability to form and the stability of a gigaseal. All solutions were made to fulfil the three objectives outlined above with respect to the experiments to be performed. For example, all the reversal potentials were positioned so that the ion movements that were of particular interest (i.e. K^+ and Cl^- flux) were distinguishable. The solutions were evaluated with respect to their ability to allow quick seals and to support seals of sufficient quality and longevity that experimentation can be completed. To do this a seal must have a resistance of greater than $5G\Omega$ and last at least 20 minutes.

All chemicals were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) or BDH Laboratory Supplies (Poole, England). All solutions were ultrafiltered with $0.2\ \mu m$ single use, sterile syringe filters prior to use (Sartorius AG, Goettingen, Germany). Displayed in the caption for each solution's description (tables 2.1-2.7) are the junction potential values for the interface at the electrode tip between each solution pair.

2.3.6 Solution Development: Results

When developing the skills necessary to operate the patch clamp rig (apparatus including microscope, oscilloscope, amplifier, micromanipulators, and PC software) a solution developed by A. Garrill (2000), for the purpose of setting up the rig and for use



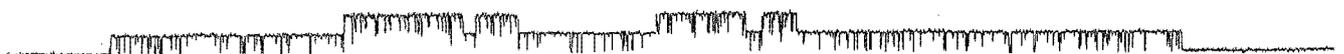
in undergraduate laboratories (dubbed #1 and described in Table 2.1), was used. Use of solution #1 was eventually discontinued since the reversal potentials proved to be too close together.

Salt	Concentrations (mM)	
	Bath	Pipette
KCl	100	45
CaCl ₂	0	10
NaCl	10	20
KNO ₃	0	45
MES	5	5

Ion	E _{rev} (mV)
K ⁺	-3.1
Cl ⁻	6.5
Na ⁺	17.5
Ca ²⁺	-∞

Table 2.1 Description of #1 solution after pH was adjusted with KOH to 5.5. Additional potassium ions were accounted for in E_K. The junction potential for this solution pair is -1.3 mV.

Solution #1 was altered so that the reversal potentials of K⁺ and Cl⁻ which are conducted by the ion channels most commonly observed were placed further apart, allowing easier channel differentiation. The composition details of this solution (#2) can be seen in Table 2.2. Attempts to use this solution pair in experimentation provided no high quality seals (~40 attempts with no success).



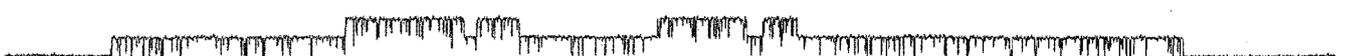
Salt	Concentrations (mM)	
	Bath	Pipette
KCl	100	50
CaCl ₂	0	10
NaCl	10	10
MES	5	5

Ion	E _{rev} (mV)
K ⁺	-17.5
Cl ⁻	8.1
Na ⁺	0
Ca ²⁺	-∞

Table 2.2 Description of solution #2. The pH was adjusted to 5.5 with KOH and E_K was duly altered. The junction potential was -4.6mV .

Solution pair #3 (table 2.3) used ethylene glycol-bis(β -aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA, see figure 2.4), a chelating agent added to remove any calcium ions from the bath solution. Calcium ions were removed as it has been reported that a lack of extracellular calcium ions (i.e. in the bath solution) assisted in the formation of excised patches in the inside-out configuration (Hamill *et al.*, 1981) and that extracellular calcium ions aid seal formation in the cell-attached configuration (Tyerman *et al.*, 1997). Tyerman's laboratory overcame the problem by changing the bath solution after forming a gigaseal (Garrill, 2000). In the present study, calcium ions did not noticeably affect seal formation, therefore the physical disturbance of changing solutions was avoided.

Since EGTA is a tetracarboxylic acid, a large amount of KOH was required to attain a pH of 5.5. The increase in K⁺ ions from the KOH reduced the difference between the two most important junction potentials E_K and E_{Cl} . Thus choline chloride was introduced as a source of chloride ions to offset the effect of these extra potassium ions. This allowed the [KCl] to be lowered while the chloride concentration remained high. Choline is a relatively large inert cation that is not among the group of ions commonly transported via channels in characean algae (figure 2.5). Therefore, choline



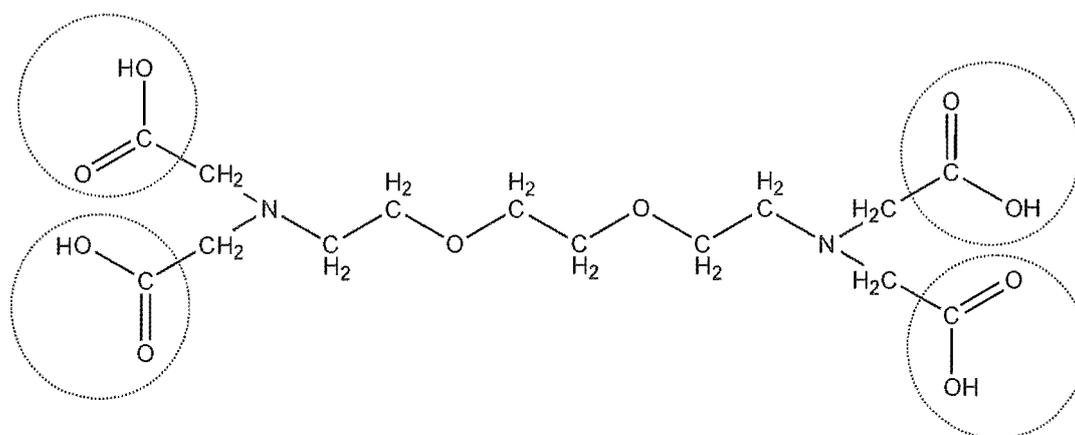


Figure 2.4 Chemical structure of Ethylene glycol-bis(β-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA). The carboxylic acid functional groups, which effect the acidity of solutions containing EGTA, are circled.

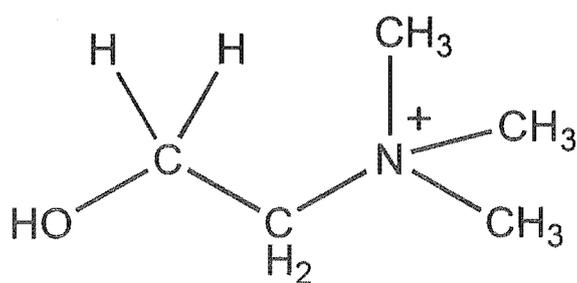


Figure 2.5 The chemical structure of choline ([2-Hydroxyethyl] trimethylammonium). Note the quaternary amine group that is relatively protected from attack by a glycosylating agent because of the steric hindrance of the surrounding methyl groups.

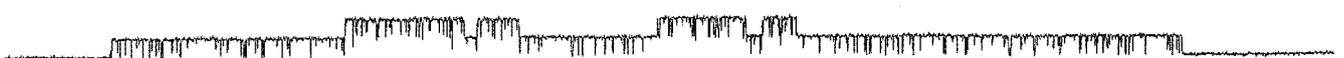
chloride (CholCl) is a good source of chloride ions that does not alter other relevant ionic concentrations. An important consideration with respect to the glycation experiment is that both EGTA and choline contain amino groups. This means that they could theoretically undergo the Maillard reaction and alter the effect of the glycating agent on the channel (section 1.6). However, since both nitrogenous groups are in a tertiary (EGTA) or quaternary (choline) arrangement they are unlikely to react due to the steric hindrance of their nitrogen's side groups. Solution #3 did not produce quality seals ($n \approx 50$ attempts).

Salt	Concentrations (mM)	
	Bath	Pipette
KCl	10	50
EGTA	5	0
CaCl ₂	0	10
NaCl	10	10
CholCl	40	0
MES	5	5

Ion	E_{rev} (mV)
K ⁺	21.3
Cl ⁻	-7.3
Na ⁺	0
Ca ²⁺	$-\infty$

Table 2.3 Description of solution #3. The solutions were adjusted to pH 5.5 and the E_K was adjusted accordingly. The junction potential was 1.4 mV.

Solution pair #4 was designed with reference to solutions made by Findlay and Tyerman at Flinders University in South Australia (Findlay *et al.*, 1994; Tyerman & Findlay, 1989; Tyerman *et al.*, 1997; Tyerman *et al.*, 1992). Solution pair #4 is adjusted to pH 6 rather than 5.5 as in most other solutions trailed. The effect of pH was not studied and the pH of all solutions were adjusted to values used in previous studies (Tyerman & Findlay, 1989) (see Lühring (1999) for the pH effect of characean potassium



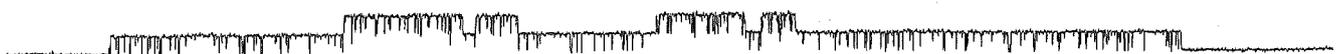
channels). About 20 mV separates the reversal potentials of K^+ and Cl^- (table 2.4). Solution pair #4 failed to produce useable seals ($n \approx 35$ attempts).

Salt	Concentrations (mM)	
	Bath	Pipette
KCl	100	70
EGTA	2	0
CaCl ₂	0	1
NaCl	10	10
CholCl	10	0
MES	5	5

Ion	E_{rev} (mV)
K^+	-9.8
Cl^-	9.6
Na^+	0
Ca^{2+}	$-\infty$

Table 2.4 #4 solution. The solutions were adjusted to a pH of 6 with KOH. The junction potential was 1.3mV.

Solution #5 (defined in table 2.5) was trialed in order to compare the effect of EGTA and CholCl concentrations with those in solution #3. This solution pair produced good quality patch recordings. Several seals that lasted in the order of minutes were achieved and methods for methylglyoxal (MG) addition were trialed.

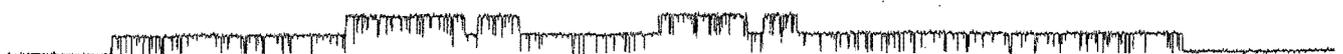


Salt	Concentrations (mM)	
	Bath	Pipette
KCl	10	50
EGTA	10	0
CaCl ₂	0	10
NaCl	10	10
CholCl	50	0
MES	5	5

Ion	E _{rev} (mV)
K ⁺	-4.0
Cl ⁻	-3.4
Na ⁺	0
Ca ²⁺	-∞

Table 2.5 Solution #5. The solutions were adjusted to pH=5.5. The bath solution required a lot more KOH than previous solutions because EGTA is a tetracarboxylic acid. The junction potential was -2.8mV.

Solution #6 (table 2.6) was the same as the previous solution except for the removal of EGTA. This allowed the effect of EGTA to be observed. This solution was successful in achieving gigaseals but with very low frequency ($n \approx 40$ with 4 successes) and was used in the characterisation of four channels through IV data (see section 3.1.3).

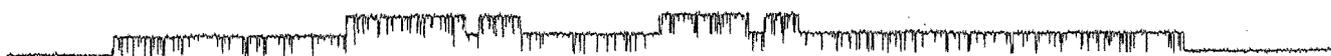


Salt	Concentrations (mM)	
	Bath	Pipette
KCl	10	50
CaCl ₂	0	10
NaCl	10	10
CholCl	50	0
MES	5	5

Ion	E _{rev} (mV)
K ⁺	40.7
Cl ⁻	-3.4
Na ⁺	0
Ca ²⁺	-∞

Table 2.6 Description of solution #6. Adjusted to pH 5.5 with KOH, K⁺ taken into account in E_K. The junction potential is 8mV.

Solution #7 was derived from the work done in the Lühring laboratory which used solutions with high concentrations of potassium chloride (Lühring, 1986; Lühring, 1999). This reflects data collected in Lühring's studies showing that the common potassium channel in *Chara corallina* (a characean alga) becomes saturated at a potassium ion concentration of about 150 mM. The composition of this solution pair is detailed in table 2.7. A notable feature of this solution pair is that its pH is adjusted to 7. #7 solution proved very promising and good quality seals were achieved (n≈20 with 3 successes).



Salt	Concentrations (mM)	
	Bath	Pipette
KCl	150	150
CaCl ₂	0	1
CholCl	0	50
MES	5	5

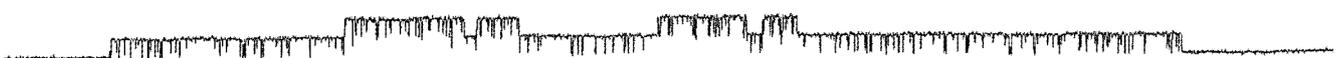
Ion	E _{rev} (mV)
K ⁺	0.02
Cl ⁻	-7.5
Na ⁺	-∞
Ca ²⁺	-∞

Table 2.7 #7 solution. The solutions were adjusted to a pH of 7 with KOH. The junction potential was -7.4 mV.

Solution pair #7 was tested further with reduction of its pH to 6 in an attempt to further stabilise the gigaseals obtained with this solution pair. The pH of 6 was closer to the experimental conditions used in the Australian laboratories (pH=5.5; Tyerman & Findlay, 1989). Solution pair #7 solution was used in all further experimentation.

2.3.7 Solution Development: Conclusions

Solution pair #7 was the best for the purposes of this research after its pH was reduced to 6. While the initial idea was to have a large separation of reversal potential (see design for the #2 and #3 solutions) a separation of 5 mV was found to be sufficient to distinguish between the two most common channel types, chloride and potassium, in low noise recordings. Since allowing a smaller separation increased the chance of achieving a quality seal, the sacrifice of lower separation in reversal potentials was accepted.

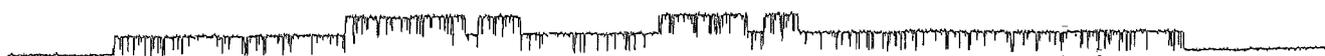


2.4 Electrode Optimisation

2.4.1 Electrode Optimisation: Theory

A critical step in recording single channel events is the manufacture of patch electrodes for use as patch electrodes. These must be of the correct size and shape so that a stable and strong seal can form between the electrode and the membrane (Sakmann & Neher, 1995; Cory & Stevens, 1983; Rae & Levis, 1992; Ogden & Stanfield, 1994; Halliwell *et al.*, 1994; Levis & Rae, 1995). The opening at the mouth of the pipette (pipette tip; figure 2.6) is microscopic (approximately 0.5 to 1 μm ; Hamill *et al.*, 1981), therefore, its characteristics are usually determined from interpretation of the pipette's physical properties. Traditionally, electrical resistance is measured across the tip, between saline solutions in the pipette and a bath using an ohmmeter (Sakmann & Neher, 1995). An alteration of this technique by Cory and Stevens (1983) measures the physical resistance to gas under pressure, giving a quicker result. This method involves applying air pressure to the non-tip end of the patch electrode with a 10 mL syringe while the tip is immersed in 95% ethanol. A reading (bubble number) is taken when air bubbles first appear from the tip. Theoretically, the same properties are measured even though quite different substances are being measured (i.e. pressurised gas compared to voltage-driven ions).

How a seal physically forms has not been formally established (Penner, 1995), but it is suggested that the composition of the membrane lends itself to sealing best to specific pipette characteristics. As the membrane fluidity increases, a patch pipette should require an increasingly smaller diameter tip to achieve the required stability (a larger patch could easily become unstable and break) thus the smaller tips were expected to form the better seals. On the other hand, it is also logical to assume that the larger the inside surface area of the pipette (i.e. the larger the pipette) the larger, and therefore better, the area of the seal might be.



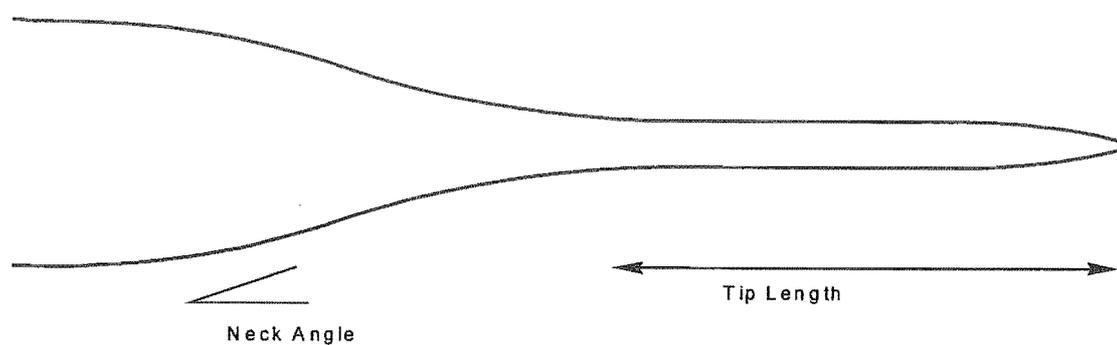
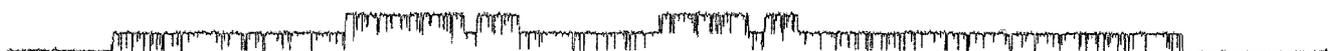


Figure 2.6 The terminology to be used when describing patch electrode shape. This diagram shows the typical pipette shape when pulled using the single pull, non-fire polished method. Electrodes for patch clamping usually have a tip length of around 5mm and a tip diameter of 0.5-1 μ m (Hamill *et al.*, 1981).

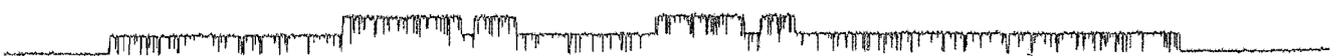


Other factors that have been reported to lead to better quality seals are mainly concerned with keeping the electrode (before and after pulling) free of contamination (Hamill *et al.*, 1981). Grease from fingers is a common problem, since it effects the interaction between the glass of the electrode and lipids of the membrane; therefore physical contact is avoided with the section of glass that would become the tip. When the tip passes through an air-water interface it should always have a slight positive pressure and each pipette should only be used once therefore avoiding contamination (Hamill *et al.*, 1981).

This part of the research entailed a review of currently used techniques and an optimisation of these for conducting patch clamp experiments on *Nitella bookeri*. The techniques investigated include a comparison of the single and two step pulling methods, the use of a silicon elastomer (Sylgard) to electrically insulate the thin-walled tip of the electrode (thus reducing capacitance and related current spikes), and fire polishing (a method of sculpting the microscopic electrode tip). A full range of pipette sizes and shapes were investigated in relation to their ability to form useful seals and the consistency of the result.

2.4.2 Electrode Optimisation: Methodology

Borosilicate glass capillaries (blanks from which the electrodes were made) (GC150T-10), 1.5 mm O.D. x 1.17 mm I.D., from Clark Electromedical Instruments (now Harvard Apparatus in Kent, England) were used for all experiments. A Narishige PC-10 pipette puller (Narishige Group) was used in all pipette manufacture. The pipette puller manufactures small diameter blunt electrodes from the capillary blanks using a vertical drop method. The blank is held at the top by a fixed clamp and at the bottom by a sliding weight. A heating coil is positioned around the blank between the two clamps to heat it to a molten state where the sliding weight can pull the glass into a thin useable electrode.



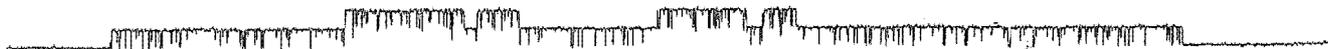
A Narishige MF-830 microforge (Narishige Group) was used for all fire polishing. Fire polishing is a process that performs two functions. It can reduce the electrode's diameter to sizes smaller than is possible using only a pipette puller; it also makes the electrode tip more rounded (for use on delicate membranes, not usually necessary with characean membranes).

Tip resistance was first measured by bubble number using a 12 cc syringe with Luer locking tip (Sherwood Medical Company, St. Louis, Missouri, USA). These were extended to the 10cc mark (filled with air) before the electrode was connected via medical grade polyethylene tubing. The electrode tip was immersed in 90-100% ethanol and the bubble number measured.

Electrical resistance of the tip was also measured using the patch-clamp amplifier's seal test feature. The electrode was positioned as in a patch-clamp experiment and a measurement made when the resistance was stable. The solutions were the same as those used in the patch-clamp experiments (i.e. solution pair #7).

The initial experiment tested the limits of the materials (e.g. what temperature the glass softened at) with the pipette puller set at the single pull method. The resistance of pipettes pulled at the full range of pull temperatures that produced complete pipettes was recorded. Above and below specific limit temperatures the glass capillary blank broke before the pull was complete. Both electrical and physical resistances were recorded so that comparisons could be made.

The two step pulling method, heating the glass blank twice at different temperatures, was then examined to attempt to achieve smaller tip diameter, or noticeably better electrodes. The technique of fire polishing was then investigated. Fire polishing involves placing a pulled electrode tip close to an adjustable heat source and allow melting, and associated shrinkage to take place. As the electrode becomes molten it thickens and contracts, resulting in a thinner, blunter electrode that has been reported to enhance seal formation with some membranes (Hamill *et al.*, 1981).

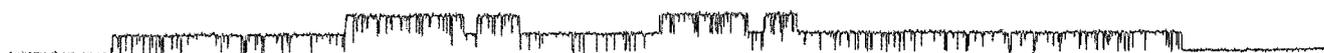


It was observed that the temperature and the distance between the electrode and the heating element during fire polishing (and therefore the speed of the polish) affected the resulting shape of the pipette. Thus, the two different shapes produced by extremes of this practise were briefly tested. Since this method was not used after initial tests concluded that fire polishing was unsuitable, this testing was not extensive. Finally Sylgard (Dow Corning, Michigan, USA), an insulating elastomer used by electrophysiologists to reduce the occurrence of capacitance spikes, due to the very thin walls of the electrode near the tip, was tested for its usefulness in these experiments. Sylgard is 'painted' on to the electrode as close to the tip as possible without breaking it, then air-dried. The clear liquid hardens to form an insulative bead over most of the electrode tip (see figure 2.7).

2.4.3 Electrode Optimisation: Results

The Narishige pipette puller uses arbitrary voltage units (VU) that correspond to the voltage passing through its heating coil as a temperature scale. This makes all values here relevant only to the pipette puller being used in these experiments at the time they were done. Brief experimentation, however, can quickly provide a conversion factor to relate this data to another puller.

Electrode production was possible (given the borosilicate capillary blanks that were used) in the temperature range from 53 to 67 VU. Figures 2.8 and 2.9 show the raw data from this experiment. Both sets of data show a similar trend: as pull temperature increases, the tip resistance increases to a bubble number of ~ 4 or a resistance measurement of $\sim 5.5 \text{ M}\Omega$ (figure 2.10). Table 2.8 shows the relationship of tip resistance to seal quality.



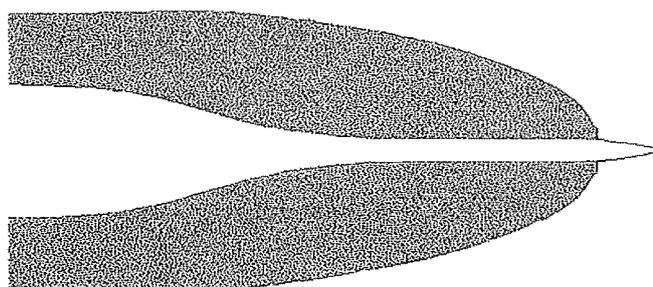
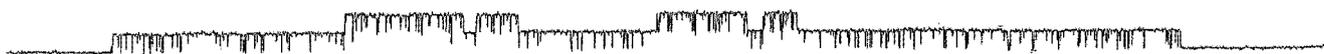


Figure 2.7 Patch electrode showing insulating Sylgard coating (grey).



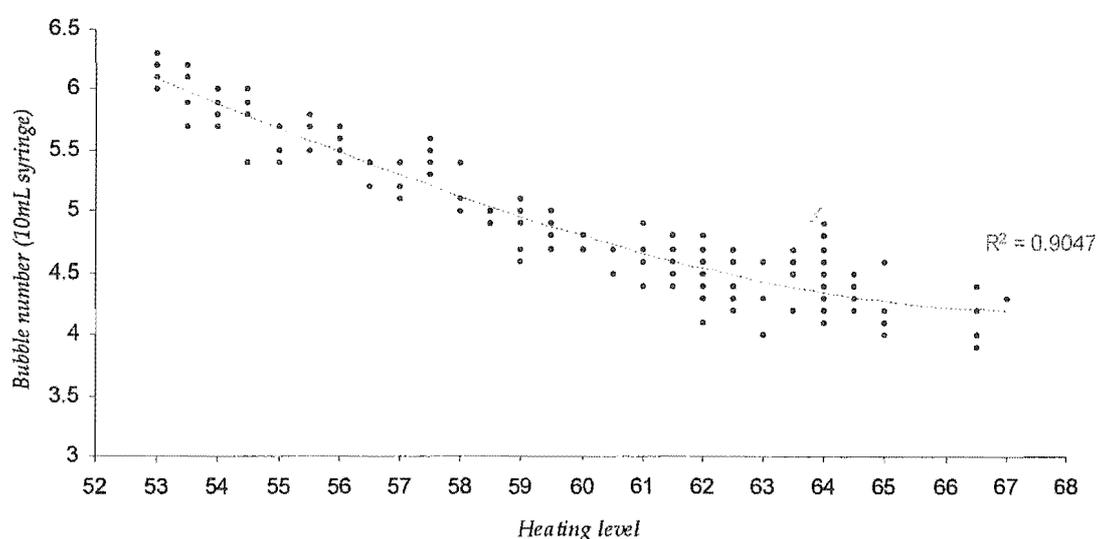
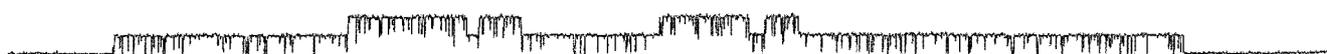


Figure 2.8 Physical resistance compared to pull temperature. Points below a heating level of 53 and above 67 resulted in broken or incompletely pulled pipettes, thus no results are recorded for these values. Note that a decrease in bubble number indicates an increase in resistance (i.e. bubble number is inversely related to resistance). The trendline is fitted to the data as a third order polynomial function. The term 'bubble number' described in section 2.3.1.



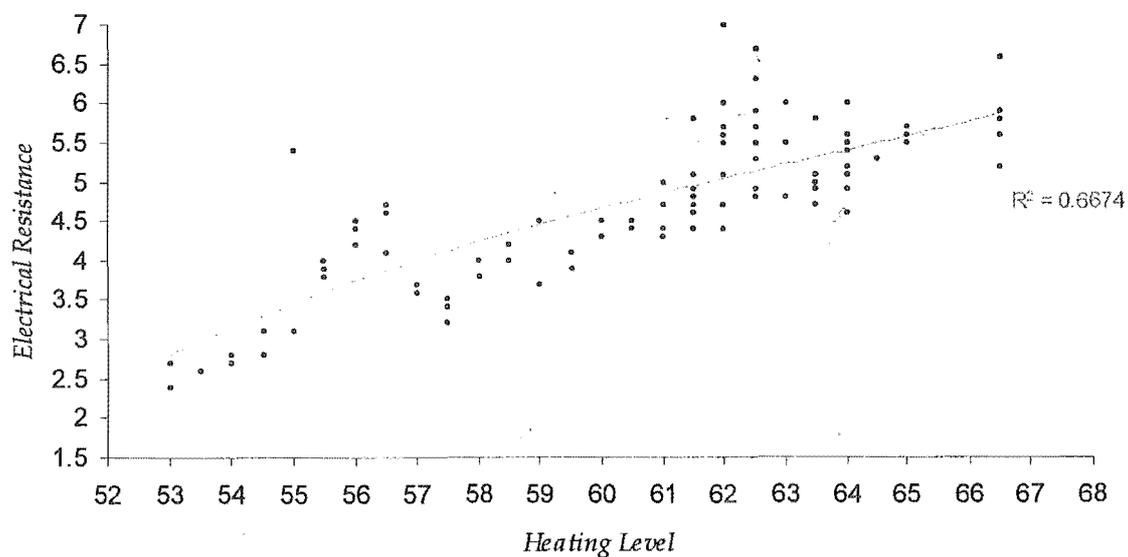


Figure 2.9 Electrical resistance compared to pull temperature. The trendline is fitted to the data as a third order polynomial function.

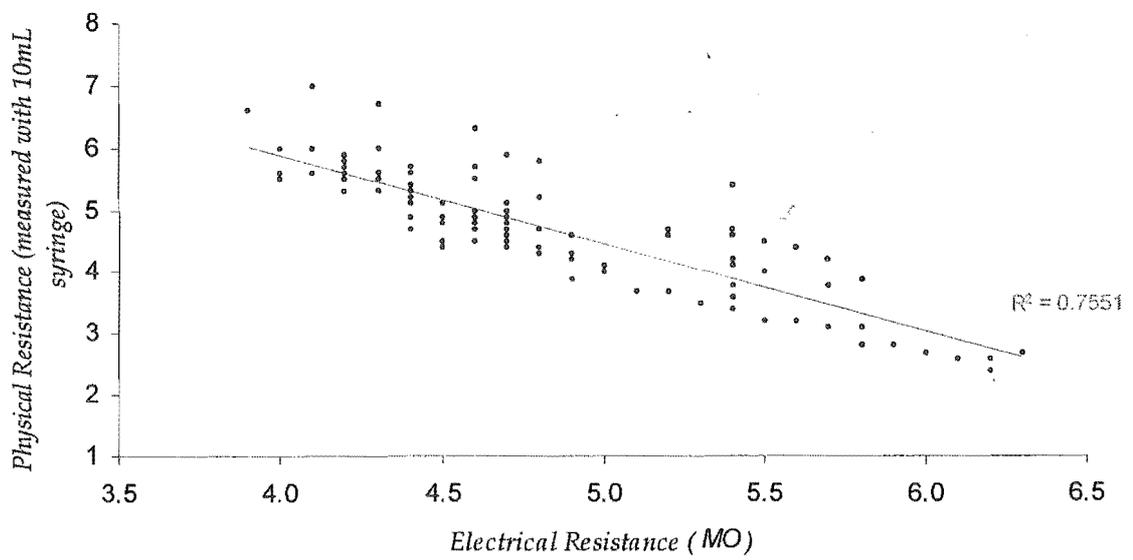
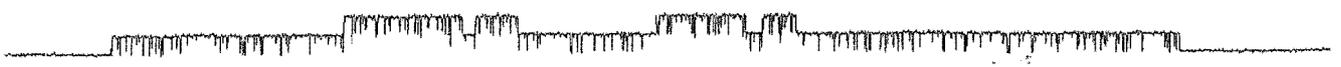


Figure 2.10 Physical resistance plotted against electrical resistance. Note that a straight line signifies correlation.



Bubble Number	Proportion of Seals Achieved	Average Seal Resistance If Sealed was Achieved (G Ω)
4	100%	2.7
4.5	66%	6.0
5	66%	12.8
5.5	33%	21.3
6.2	33%	45.0

Table 2.8 Quality of seal (i.e. resistance) compared with the tip resistance. As bubble number increased, the proportion of seals being achieved decreased while the initial quality of those achieved increased. $n = 3$ for each bubble number.

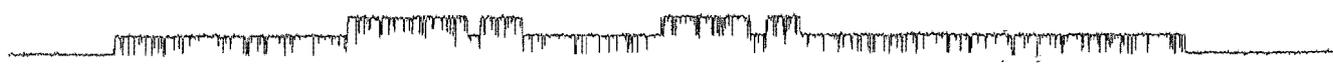
The two step pulling method was investigated only briefly because pipettes could not be manufactured with diameters as small as those made in a single step. Suitably small diameter electrodes (to achieve single channel membrane patches) could not be produced by this method; however, these pipettes were good blanks for the fire polishing experiments. The neck angle and length of the pipette tip were often significantly steeper and shorter making them much easier to fill from the non-tip end. Fire polishing, using pipettes pulled via the two step method, made pipettes with tips of smaller diameter than possible just using the pipette puller. Fire polished electrodes in the Tyerman and Findlay laboratory (Garrill, 2000; Tyerman & Findlay, 1989) and in the initial experiments of this project did not allow quality seal formation ($n \sim 30$ with no success), hence fire polishing was not studied further.

Observations were made, during fire polishing, of the effect that distance between the electrode tip and the heating element had on the final shape of the electrode tip (figure 2.11). The relative speed that each method polished pipettes was most noticeable with the closer proximity producing the fastest polish. The two extremes were investigated but did not differ significantly (figure 2.12).

Sylgard was trialed to reduce capacitance spikes but proved more detrimental than useful. The application method (brushed on manually to a point very close to the tip; figure 2.7) requires that completed electrodes be handled for several minutes, thus increasing the breakage rate. The benefits gained by its use can be almost completely overcome with changes in technique during patch clamp experiments, for instance, taking advantage of the insulating properties of the air to replace Sylgard (Laver *et al.*, 1997).

2.4.4 Electrode Optimisation: Conclusions

Figures 2.8, 2.9, 2.10, combined with table 2.8 showed that the optimum heating level for the single pull method was around 64–65 VU. At this level, the tip reliably showed optimal physical and electrical resistance. At higher temperatures, electrodes with consistent shape and size were not always produced, and at lower temperatures the tip diameter became too big to isolate a single channel. Another problem with the higher temperatures that have not been analysed is the ease of filling the electrode tip. At higher temperatures the tip length (see figure 2.6 for definition) becomes longer so that filling it with solution before a patch clamp experiment takes an increasing length of time. This increased handling time increases both the chance of destroying the electrode and the likelihood that the tip will come into contact with an air- or water-borne contaminant. As the pull temperature gets closer to the upper limit, an increase in the variability was noticed. This increase is presumably representative of an increase in temperature accentuating the effect glass structural irregularities have on the final tip diameter.



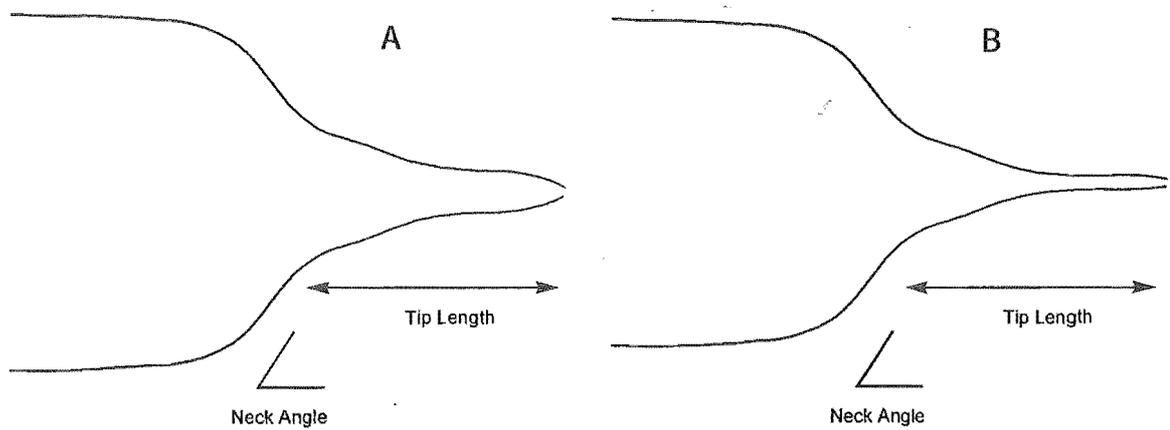
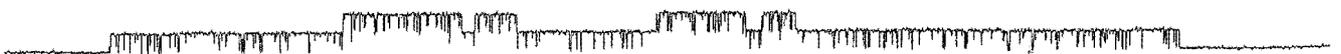


Figure 2.11 Comparison between observed shape of 'fast' (A) and 'slow' (B) polishing methods. Note the longer parallel section at the tip of the 'slow' pipette.



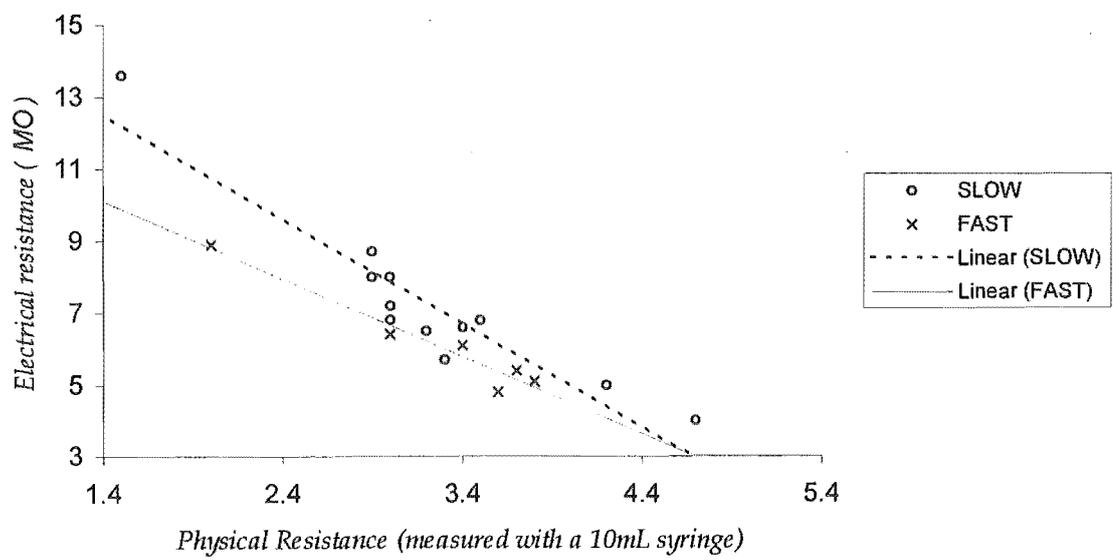


Figure 2.12 Relationship between methods of fire polishing. n=18.

The two step pulling method, combined with fire polishing, has proven very successful in other laboratories when studying various membranes (Levis & Rae, 1995; Sakmann & Neher, 1995; Sontheimer, 1995) With characean membranes, the single pull method has been most commonly used (Lühring, 1986; Tyerman & Findlay, 1989). This leads to the conclusion that membrane properties can differ between organism, or organelle.

However, the change in pipette neck shape that the two-pull technique induces was useful in the fire polishing experiments. Fire polishing is traditionally performed to smooth the edges of the pulled pipette, and to further reduce the diameter of the electrode opening (Levis & Rae, 1995; Penner, 1995). In the 'fast vs slow' fire polishing experiment (figures 2.11 and 2.12) error is expected since the technique is manual and therefore slight technique variation plays a part in the resulting electrode. It is not unexpected that the resistance relationships are similar since resistance is dependent on tip diameter not shape. Any effect that a difference in the shape has is most likely to be observable in the quality of seal attained during the course of a patch experiment. It was theorised that the glass at the tip of 'slow' polished electrodes would soften further back toward the electrode neck during polishing, thus giving a thinner tip (figure 2.11) and providing greater surface area for the membrane to seal with (figure 2.13). However, since characean membranes do not seal well to fire polished electrodes (Tyerman & Findlay, 1989), further investigation, other than observing the characteristics of the pipettes and conducting brief trials was considered unnecessary. As discussed above, the increased handling time decreases the success rate of each electrode and adds to the success of the over all experiment.

The optimum electrode for patch clamping *Nitella hookeri* tonoplast membranes was found to be pulled at 64-65 VU in a single pull then used without further treatment, and handled as little as possible. Electrodes prepared this way were used in all subsequent experiments in this laboratory.

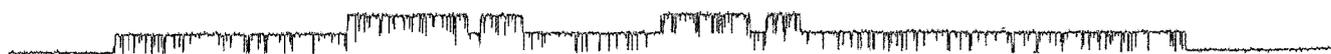




Figure 2.13 Theoretical difference in sealing ability in the two types of fire polishing. (A) shows a microscopic view of the tip of an electrode having undergone 'fast' polishing. Note the relatively small surface area available for seal formation compared with 'slow' polishing (B). Both tips are opening to the right and are at approximately the same scale (i.e. $\text{Ø} = 0.5\text{-}1\text{mm}$).

Chapter 3

Results

'When in doubt, tell the truth'

Mark Twain 1835-1910

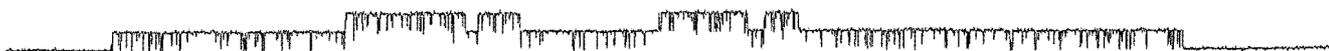
3.1 *Nitella hookeri* Channels Observed

3.1.1 Channels observed in *Nitella*: Introduction

Through the course of this research, a number of different channels were observed in *N. hookeri*. A cell requires a large degree of control over the movement of various ions into and out of its cytoplasm and vacuoles and organelles, therefore, it is no surprise that several types of channel were observed. This allows an organism to adapt at a cellular level to environmental changes, such as a shift in salinity.

3.1.2 *Nitella* Channels

Because of the high turnover rate of membrane channels (see section 1.1) and the speed at which they react to environmental change, there may be a low abundance of each channel type in a given membrane as only a few channels of each type may be required to play a significant role in a cell's physiology (Stryer, 1995). Because of this, a number of the channels described have only been observed once (i.e. $n \approx 1$ for each IV curve). In a published work identifying ion channels in the plasma membrane of the halophytic angiosperm, *Zostera muelleri*, seven types of ion channel were characterised and published (Garrill *et al.*, 1994). Approximately twice that number were actually found but these were not published as they were only ever seen in one or two experiments (Garrill, 2001). All data were collected while in the inside-out excised patch configuration.

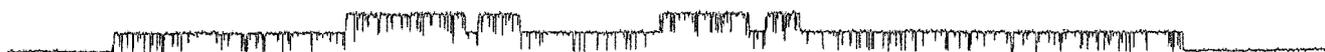


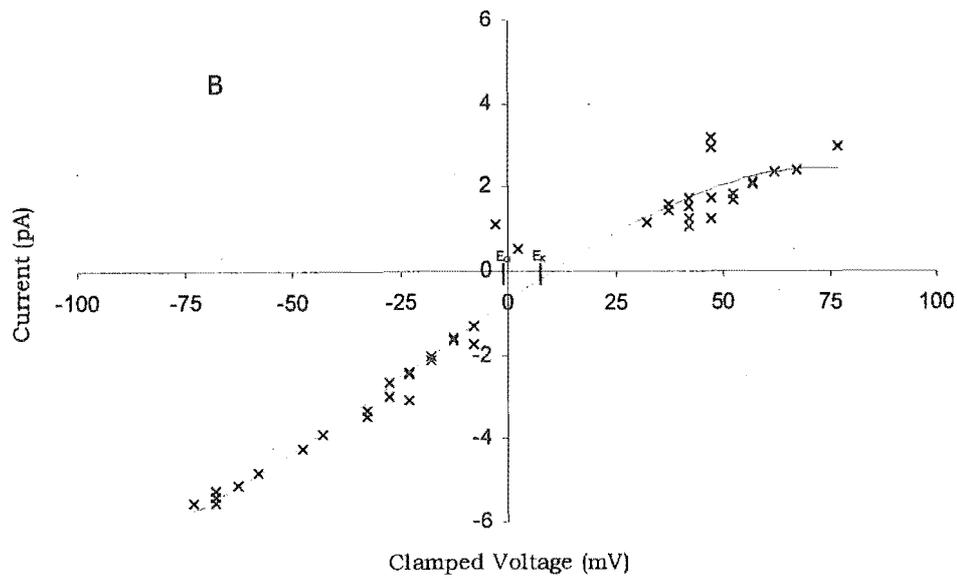
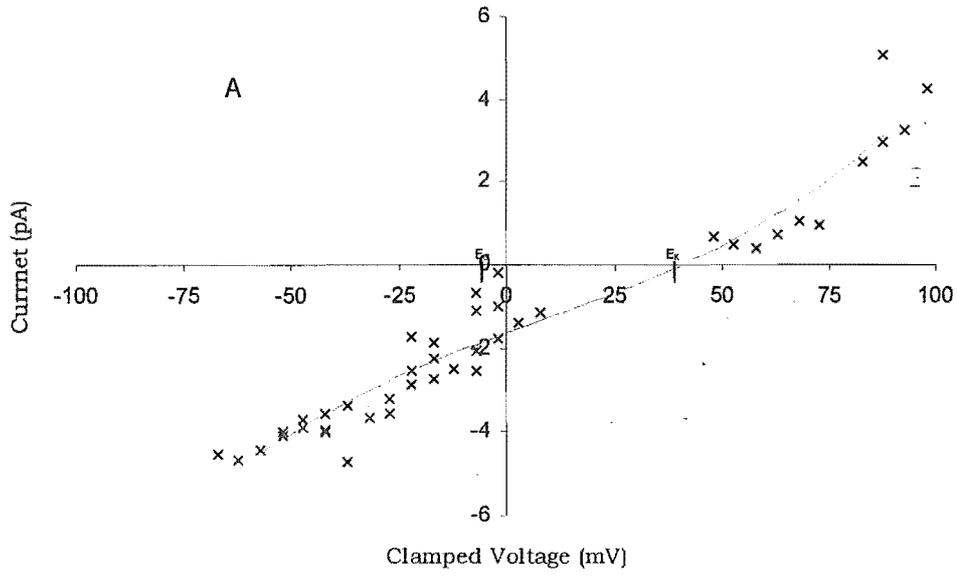
Potassium selective channels with conductances of ~ 43 , ~ 62 , ~ 20 , and ~ 80 pS respectively are described by the IV curves in figure 3.1 a, b, c, and d. Likewise channels selective for chloride conducting at ~ 78 , ~ 100 , and ~ 23 pS are shown in figure 3.2 a, b, and c. Since the conductance can be dependant on solution composition (Lühring, 1986; Lühring, 1999) it is important to note that it is not completely certain that these are all separate channels (table 3.1).

Channel Selectivity	Conductance (pS)	Solution #
Potassium Ions	43	6
Potassium Ions	62	5
Potassium Ions	20	7
Potassium Ions	80	5
Chloride Ions	78	6
Chloride Ions	100	7
Chloride Ions	23	6
Unknown	92	1
Unknown	75	6
Unknown	15	7

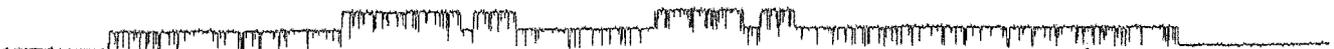
Table 3.1 Summary of channel types found in *Nitella hookeri*.

The IV curves shown in figure 3.1b, d and 3.2c are examples of current rectification. In the first two examples inward rectification is observed while in the latter case rectification is outward. The channels featured in the other seven IV curves probably exhibit ohmic characteristics, that is, display a symmetric curve, as the IV curve shown in figure 3.1b typifies. The majority of curves however cannot be distinguished absolutely in this way since data was not collected over a sufficient range of voltages (due to patch breakdown).





See overleaf



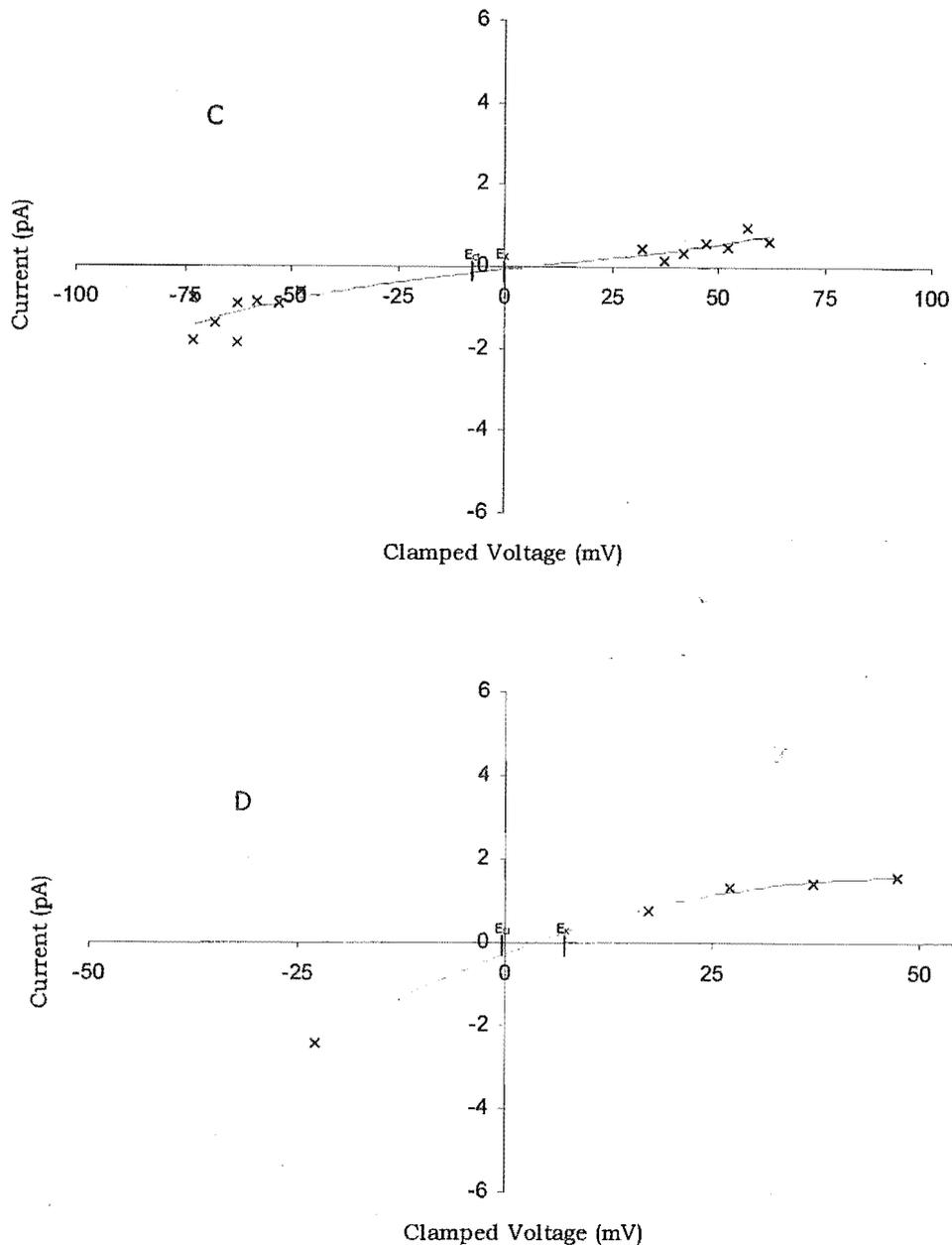
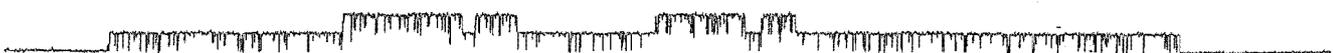
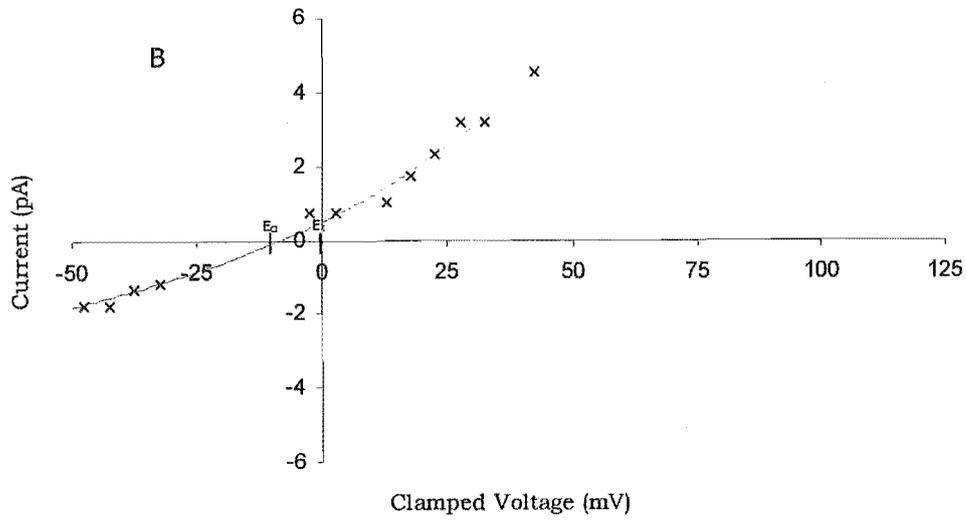
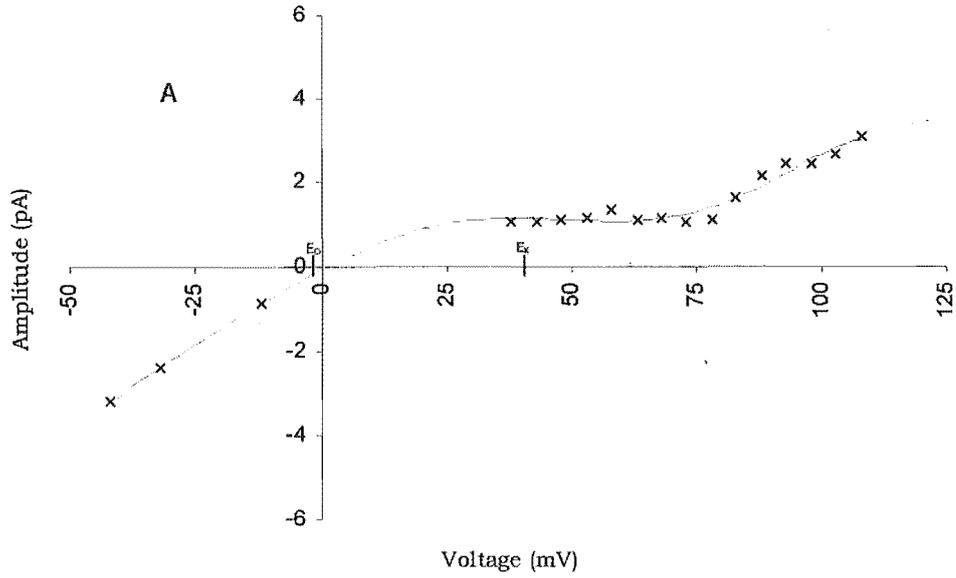
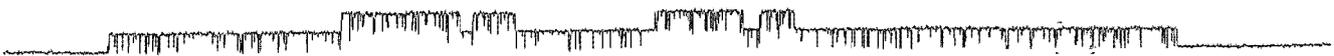


Figure 3.1 A selection of potassium channel IV curves. A ~ 43 pS channel record provided IV curve A which was obtained using #6 solution (see table 2.6) has a junction potential adjustment of 8mV. E_K is 40.66mV, and E_{Cl} is -3.37 mV. (B) Shows IV curve 5 which used #5 solution to record data from a ~ 62 pS channel, and is adjusted to a junction potential of -2.8 mV. E_K is 9.12mV, and E_{Cl} is -3.37 mV. IV curve 6 (C) had a conductance of ~ 20 pS. It is adjusted to compensate for a junction potential of -7.4 mV. The solution used in this experiment is #7. E_K is 0.02mV, and E_{Cl} is -7.5 mV. IV curve 7 (D) which used #5 solution, is adjusted to a junction potential of -2.8 mV. It shows data collected from a ~ 80 pS channel. E_K is 9.12mV, and E_{Cl} is -3.37 mV.





See overleaf



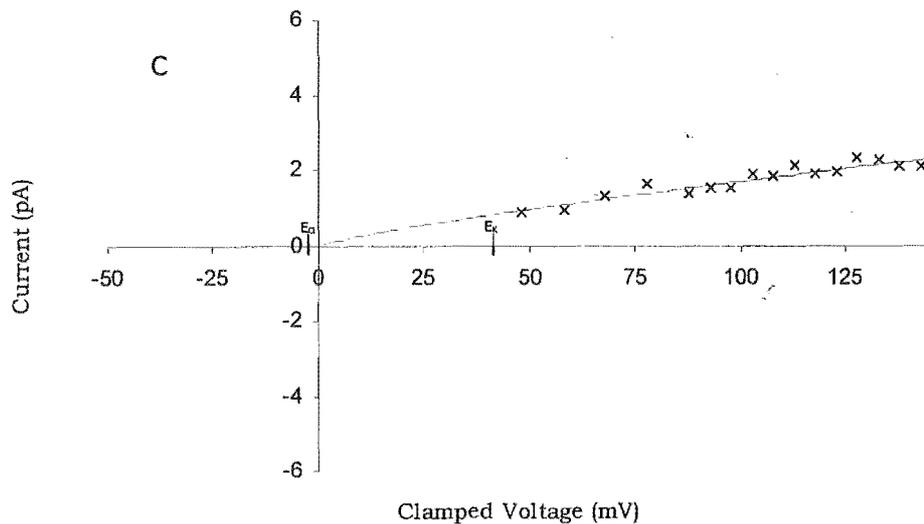


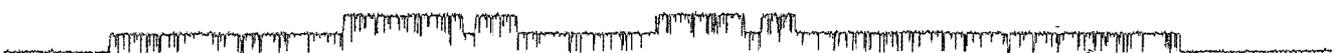
Figure 3.2 A selection of IV curves describing chloride selective channels. IV curve 8 (A) was the result of an experiment on a ~ 78 pS channel using #6 solution. It is adjusted for a junction potential of 8mV. E_K is 40.66mV, and E_{Cl} is -3.37 mV. (B) Shows an IV curve of a ~ 100 pS channel, which was ascertained from an experiment using #7 solution which has an E_K of 0.02mV, and an E_{Cl} of -7.5 mV. It was adjusted to a junction potential of -7.4 mV. IV curve C was adjusted for a junction potential of 8mV. #6 solution was used in to observe this ~ 23 pS channel. E_K is 40.66mV, and E_{Cl} is -3.37 mV.

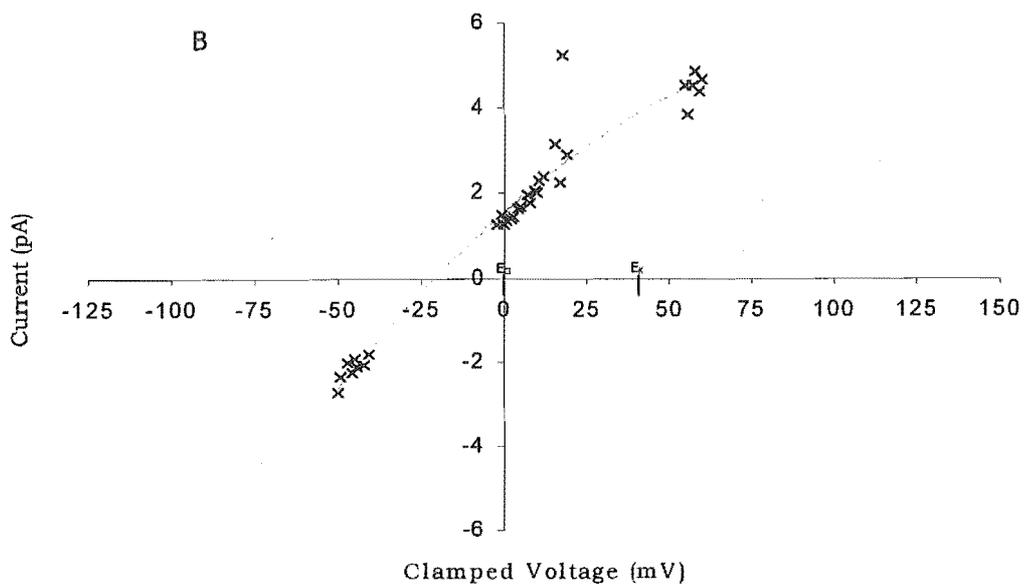
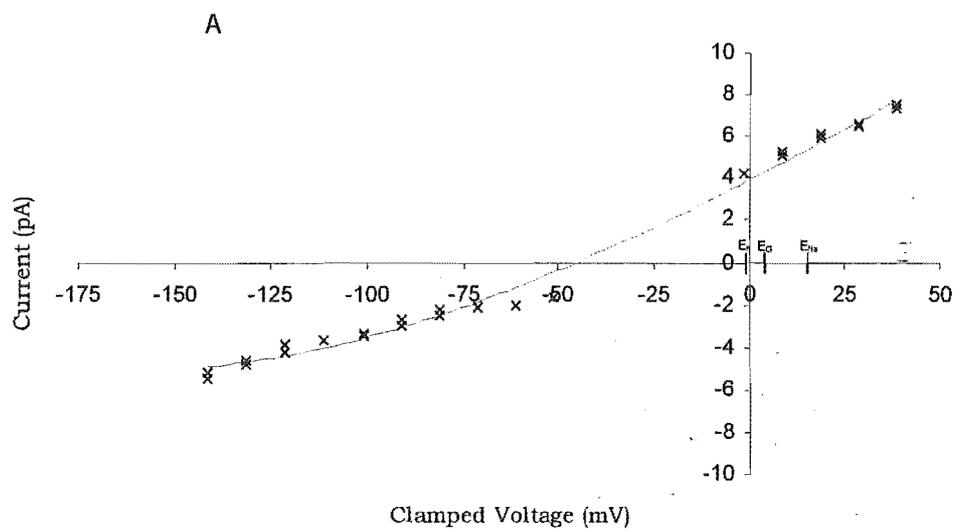
On occasion (~30%), channels were observed which were unidentifiable under the experimental conditions utilised. An IV curve of a ~92 pS channel, constructed from an experiment using solution pair #1 is shown in figure 3.3, it shows a curve that fails to bisect any of the reversal potentials (but see section 3.2.3). Two other unidentifiable IV curves were observed using solution pairs #6 and #7 with conductances of ~75pS and ~15pS consecutively (figure 3.2a and b).

There were a number of other channels observed but which did not allow an IV curve to be produced. The main reason for this is that the channel opened rarely, reducing the chance of it opening at every clamped voltage, or it appeared to be open all the time and thus the channel amplitude could not be measured. An example of a rare-opening channel is shown in figure 3.4; this channel opened once over a period of several minutes. Figure 3.5 is a trace showing a channel that is open for the majority of the time, this partial trace includes the majority (>80%) of the rare closed states observed over a period greater than three minutes at a range of voltages.

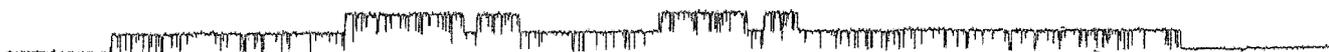
The vast majority of membrane patches (>90%, $n \approx 900$) were found to contain multiple channels. When acting simultaneously, openings of several different or similar channels become difficult to analyse. Figure 3.6 shows the most common example of this while figure 3.7 shows a milder case. A problem with multiple active channels becomes apparent when attempting to discern between multiple channels and substates. Figure 3.8 shows the confusion this can cause. It can be seen that, at the shorter time scale (figure 3.8b) the trace shown is probably made up of current recordings from at least two channels. Recordings that are unable to be analysed, such as this, were discarded.

Subconductance states in characean tonoplast channels were not obvious (i.e. time spent in subconductance states was generally short) as in some published works (see Vaca, 1999) but they did occur with high frequency when observed (figure 3.9 shows ~900 subconductance transitions per second).





See overleaf



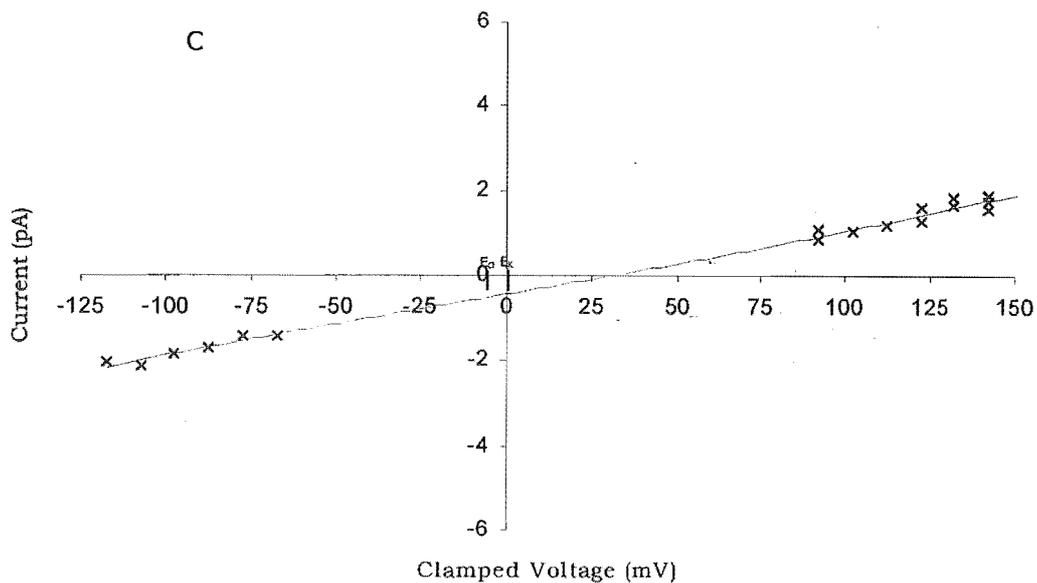
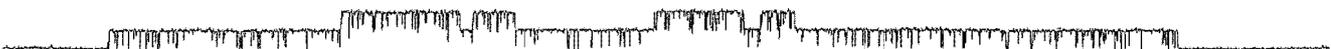


Figure 3.3 IV curves of channels that were unidentifiable in those conditions in which the experiments were conducted (solution composition, seal quality, etc.). The IV curve marked A shows data collected from a ~ 92 pS channel. Adjusted to compensate for a junction potential of -1.3 mV. The solution used for this experiment is solution #1 (see table 2.1). E_K is -3.01 mV, E_{Cl} is 6.51 mV, and E_{Na} is 17.51 mV. Channel data from a ~ 75 pS channel provided IV curve shown in B. It was obtained from an experiment using #6 solution (see table 3.1.3). It is adjusted to a junction potential of 8 mV; E_K is 40.66 mV, and E_{Cl} is -3.36 . The IV curve in C was adjusted for a junction potential of -7.4 mV. #7 solution was used for this experiment (see table 3.1.6). E_K is 0.02 mV, and E_{Cl} is -7.5 mV. Note the low conductance (~ 15 pS) of this channel.



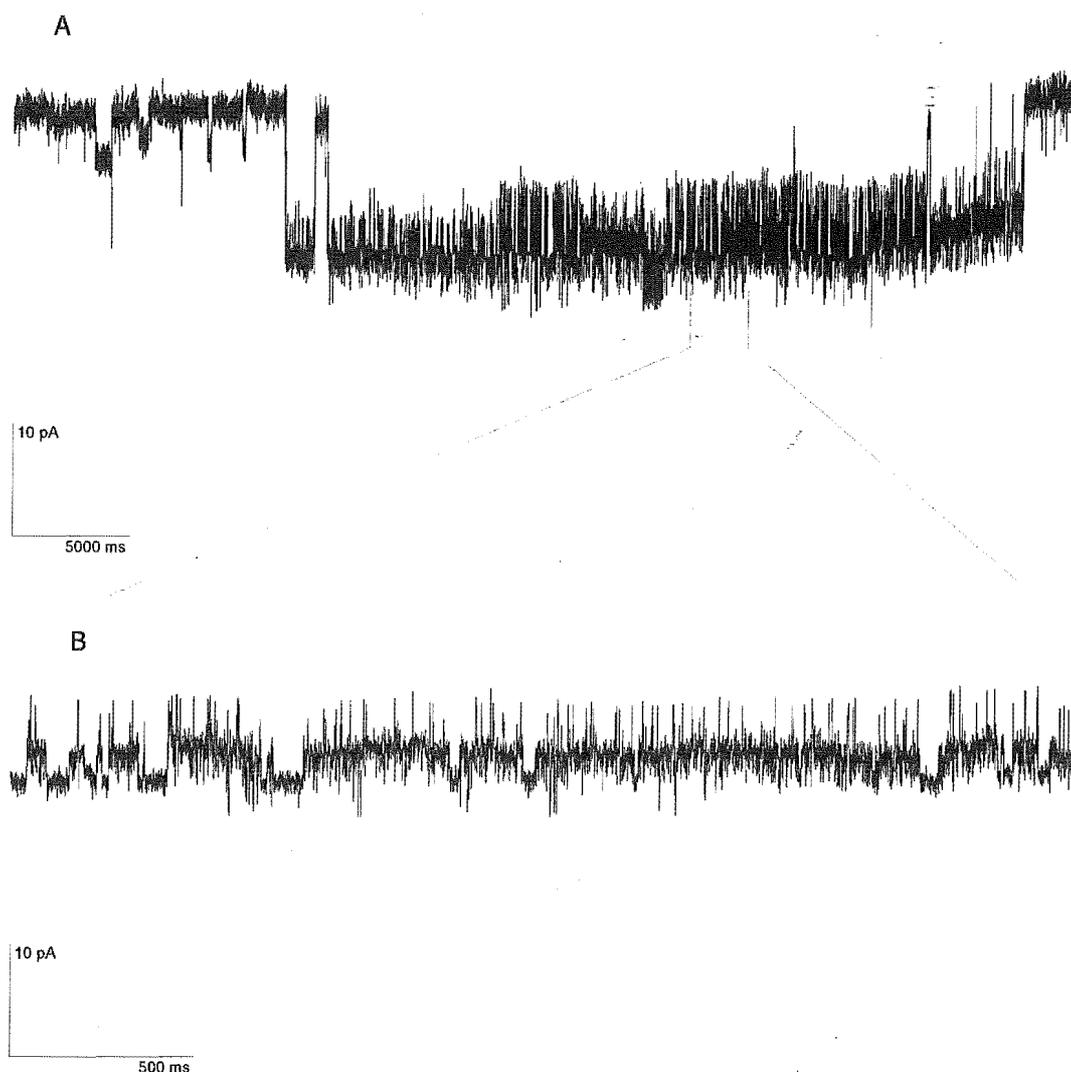
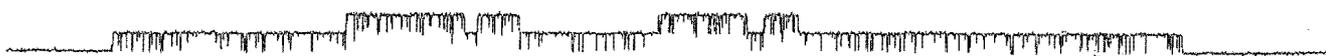


Figure 3.4 Trace of a large conductance, infrequently opening channel that was observed on three occasions. The rarity of this channel's openings meant that it was not possible to record an IV curve. This recording was made while the patch was clamped at 130mV therefore the large down steps occur when the channel closes. (A) Shows a full closing step. (B) Reveals the quickly oscillating subconductance states present when the channel was closed.



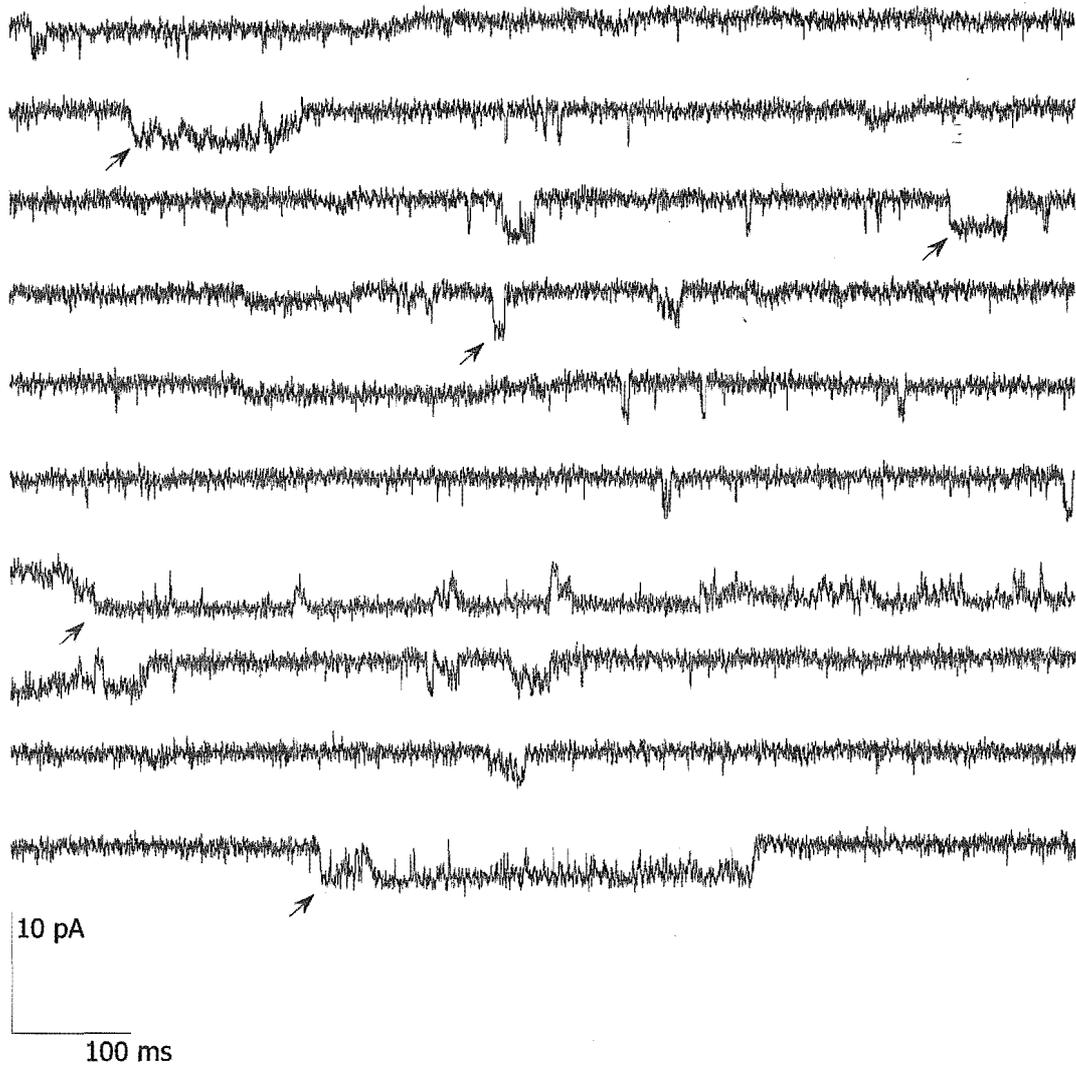
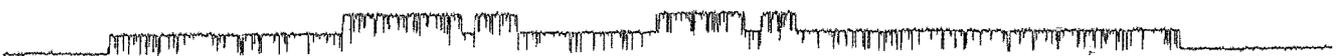


Figure 3.5 Data trace of a channel that spent a larger than normal amount of time (~90-95%) in its full conductance state. This trace was recorded at a clamped voltage of 70mV. Arrows indicate examples of closed state amplitude.



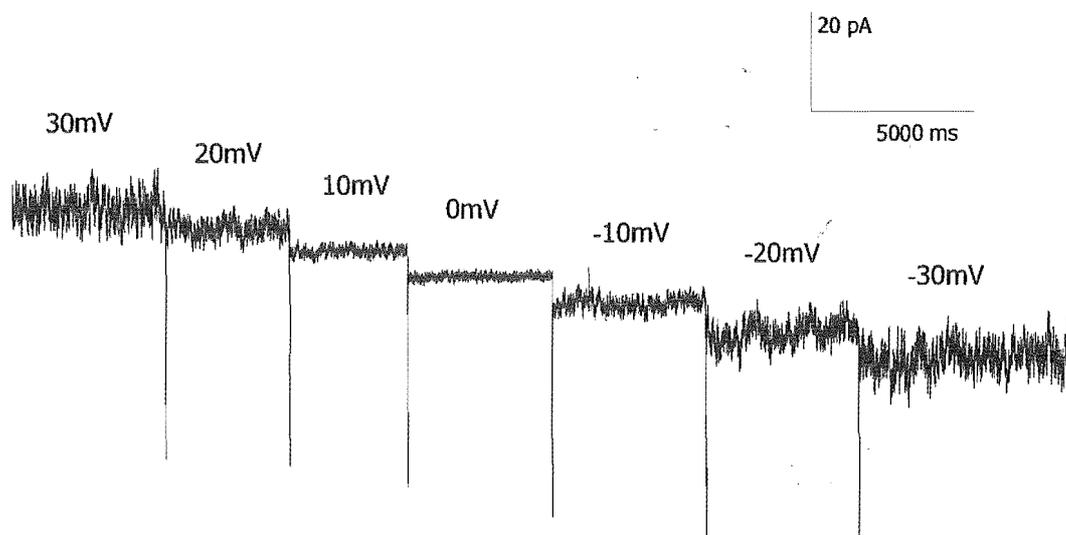
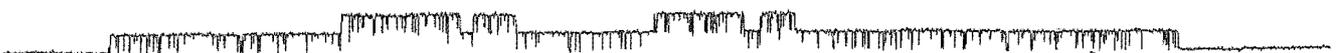


Figure 3.6 Example of a stepped trace (as used in collection of IV data) where multiple channels are present in the isolated patch of membrane. This is distinguished from electrical noise because external artefacts would be unaffected by the voltage changes.



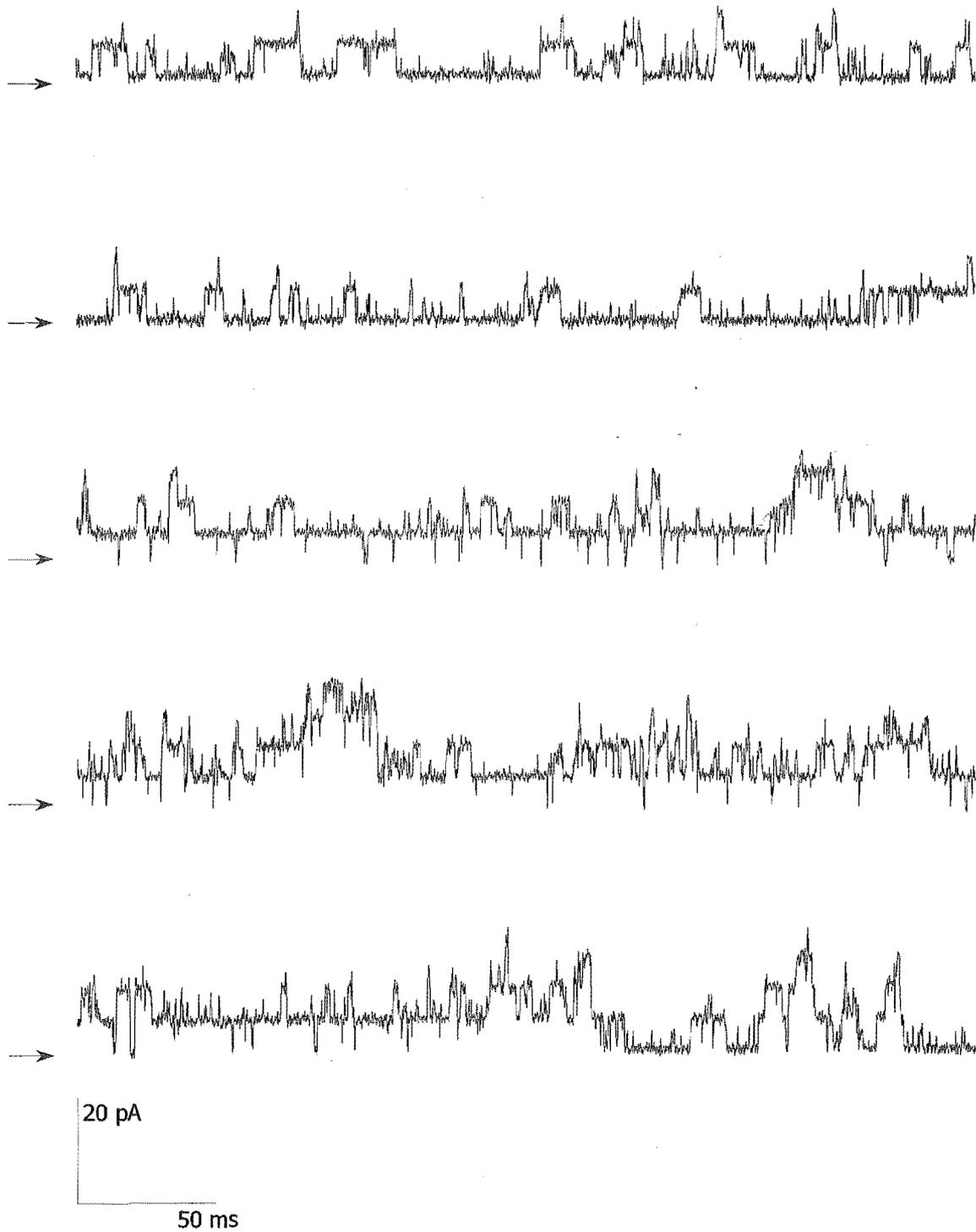
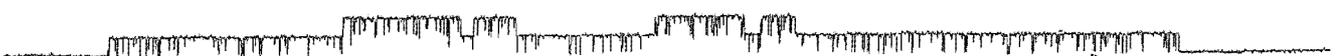


Figure 3.7 Data trace showing multiple channel activity at a clamped voltage of 70mV. Arrows indicate the amplitude of the closed state of all channels present.



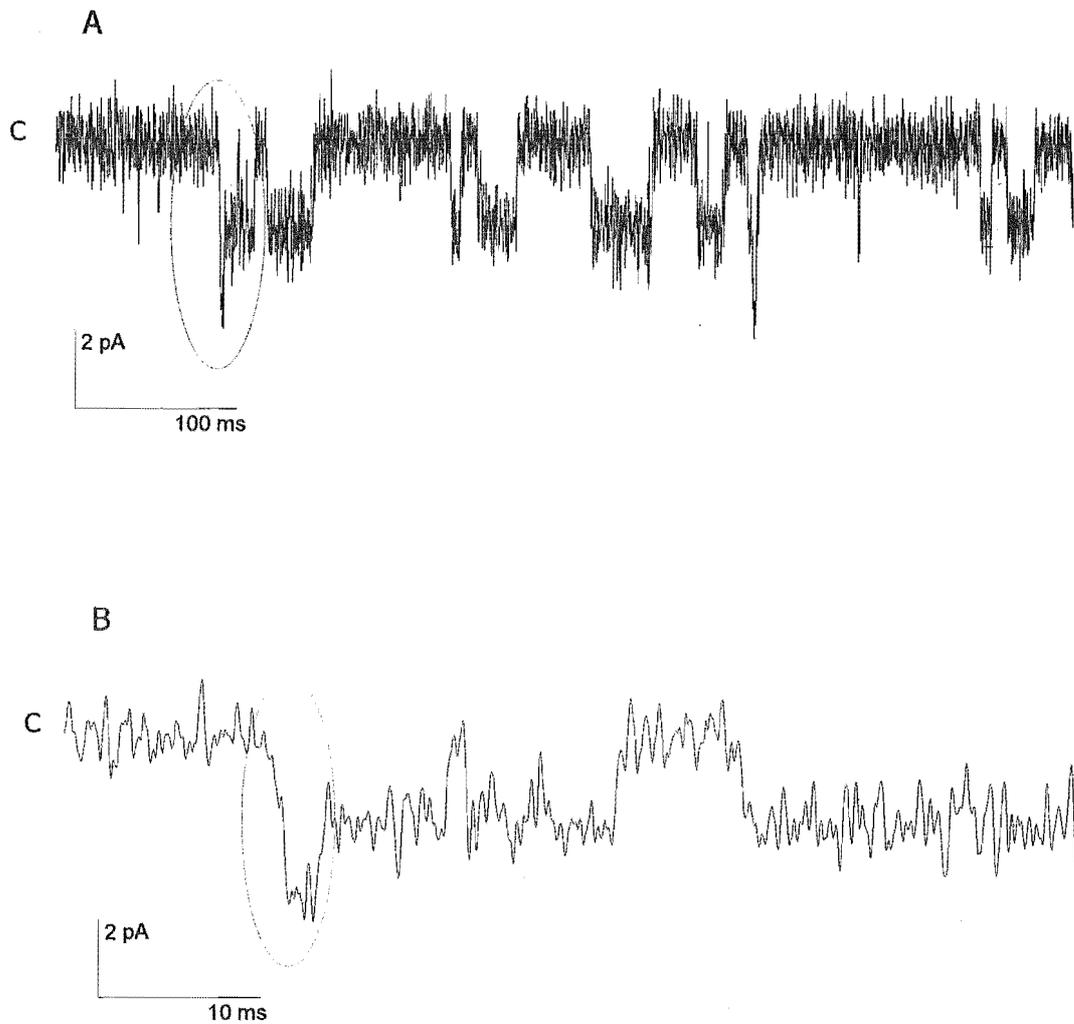


Figure 3.8 A trace recorded at -40mV which illustrates the importance of careful interpretation of recordings. The top trace shows what could be a transition between the closed conductance state (C) and a full conductance state (A). Closer inspection however, (B) shows that there is a brief period of half conductance indicating that at least two of the conductance states shown in A are due to two channels in the same membrane patch.



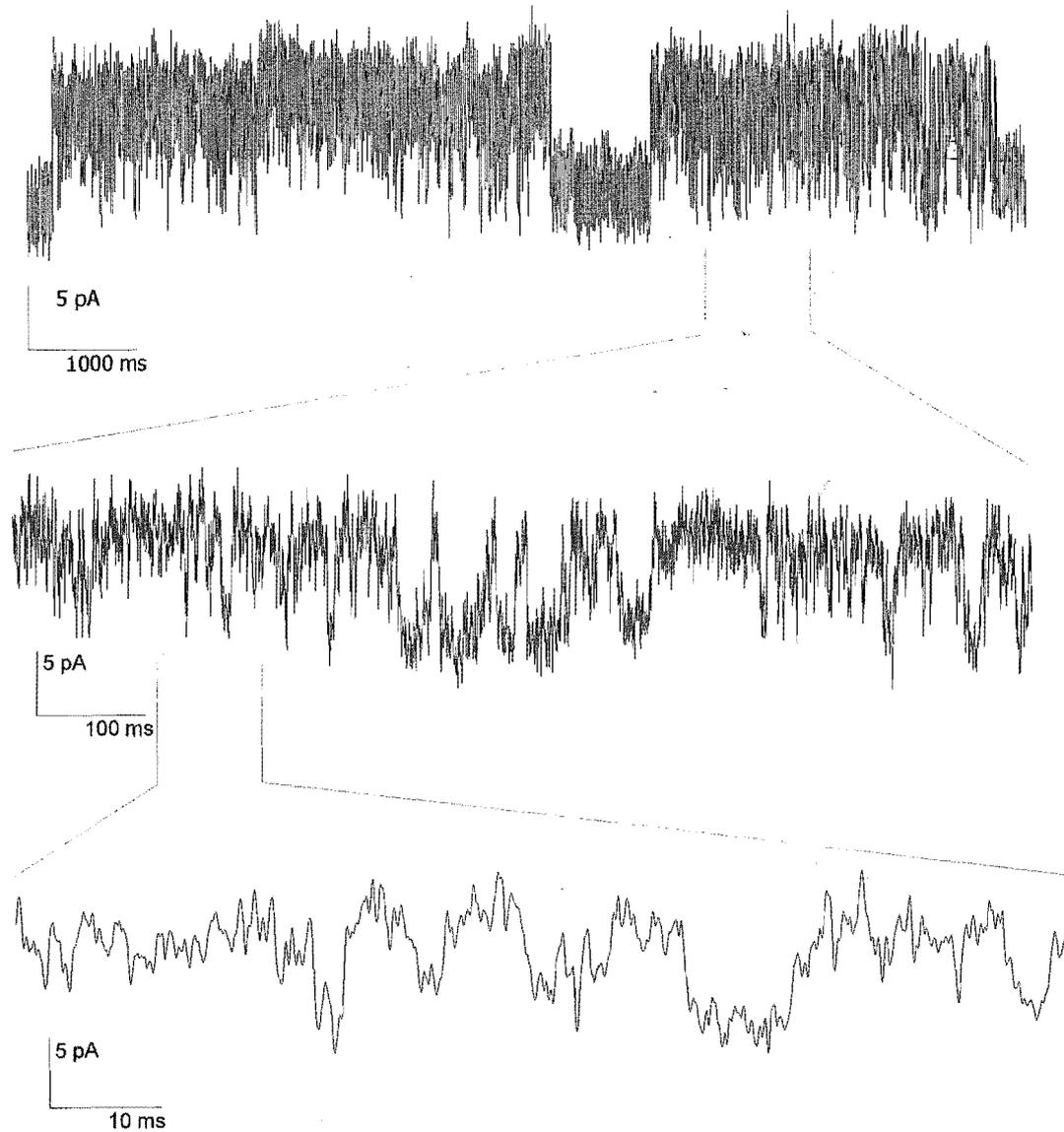
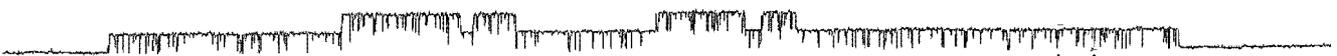


Figure 3.9 A raw trace of a Multi-K⁺ channel shown at three time scales (x-axis). This data was recorded at a clamped voltage of 90mV.

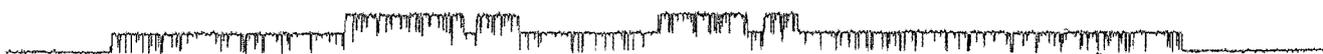


3.1.3 Channel Identification Discussion

Figure 3.1 shows the variety of potassium ion selective channels found. The IV curves shown in 3.1a and b are thought to be the same channel type due to conductance similarity (the maxi-K⁺ channel reported in Lühring, 1986) however it should be noted that they were obtained in different solutions. Although the curves may be aligned (their reversal potentials placed on top of one another irrespective of the clamped voltage values) and declared the same, this is not necessarily the case. One or both data sets may be influenced by ionic saturation, giving the appearance that it conducts less than its pore size actually permits. To confirm the similarity of these channels they would need to be examined in identical solutions. Another reason that any two channels may appear different is the presence of channel agonists or antagonists in the solutions or preparations. Although the utmost care was taken not to introduce anything other than the components of the experiment (i.e. the salts defined in the solutions, the cytoplasmic droplets and the glycyating agent), the potential action of any biochemical in the internodal cell before the tonoplast was produced could not be removed. Thus it is likely that, depending on the conditions that the internodal cell was subjected to before tonoplast production, any number of gating factors, agonists, and antagonists, might be affecting channel activity.

The IV curve shown in figure 3.2a may result from a patch containing two active channels that open at different voltages. A representation of this prediction is shown in figure 3.10. The two channels are likely to be a chloride selective channel and a potassium selective channel, with the potassium channel being voltage-gated (triggered to open only at voltages above 80 mV). Figure 3.2b shows the IV curve of the largest conductance chloride channel found (~100 pS).

Due to the fact that the most common characean ion channels are K⁺ and Cl⁻ selective (Garrill, 2000; Tyerman & Findlay, 1989) the solutions were designed with priority towards identifying those types of ion channel. This may be the reason a number of IV curves (figures 3.3) did not pass through, or near, the reversal potential of



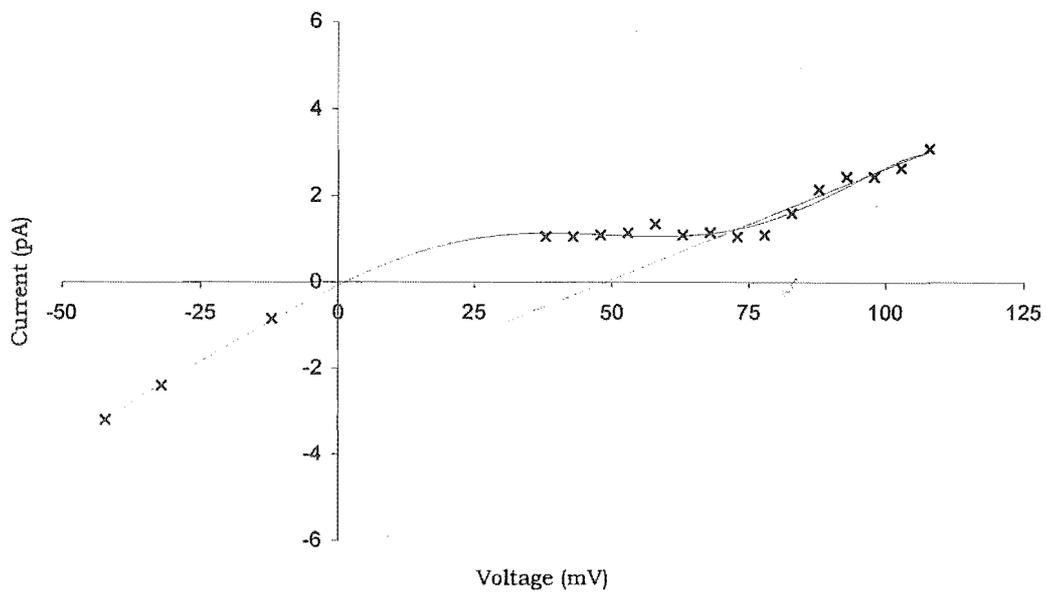


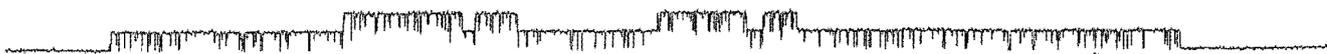
Figure 3.10 The IV curve shown in 3.2a with overlaid line suggesting the possibility that the data is taken from the activity of two channels, one of which might have been selective for potassium ions. The straight line is a linear function of the data points above a clamped voltage of 80mV. The reversal potentials are: E_K 40.66mV, E_{Cl} -3.37mV; the data points were adjusted for a junction potential of 8mV.



any ion in the solution (i.e these channels were allowing the flux of another ion). There is always the chance that the patched channel may have been conducting another unknown ion. Another possibility is that these patches contained more than one channel and one opened preferentially at positive potentials while another opened at a negative clamped voltage. Figure 3.11 is an estimation of what the two IV curves of these alleged channels might look like (the data points from figure 3.3a have fitted to separate trendlines).

Of the two other unidentifiable IV curves, figure 3.3b could also fit the above theoretical conjecture (figure 3.11). In the case of the IV curve shown in figure 3.3c, it is the low conductance of the channel ($\sim 15\text{pS}$) that makes identification difficult. The fact that the channel is not observable above the background noise until the voltage is raised to more than 90mV (or lowered to less than -60mV) means that accurate determination of the x-axis intercept is difficult. An experiment could be conducted where the reversal potentials were spread further apart to ascertain the identity of this channel, however this channel type has not been isolated in subsequent patches. Alternatively the channel may have been unsaturated (with ions) in this solution pair (#7) therefore making it hard to compare with other identified channels.

The IV curve shown in figure 3.1c is a low conducting channel, as is that show in figure 3.3c ($\sim 20\text{pS}$), making it hard to be sure of its identity. It has been placed with the potassium selective channels because channel events were recorded closer to zero voltage and the fitted curve passes through the x-axis close to the reversal potential of potassium. Figure 3.1d shows an IV curve that was included, even though only a small number of points were obtained, because there have been no similar channels recorded. The shape of this IV curve may be due to the presence of more than one channel (similar to figure 3.11) but the lack of data points makes it impossible to be certain.



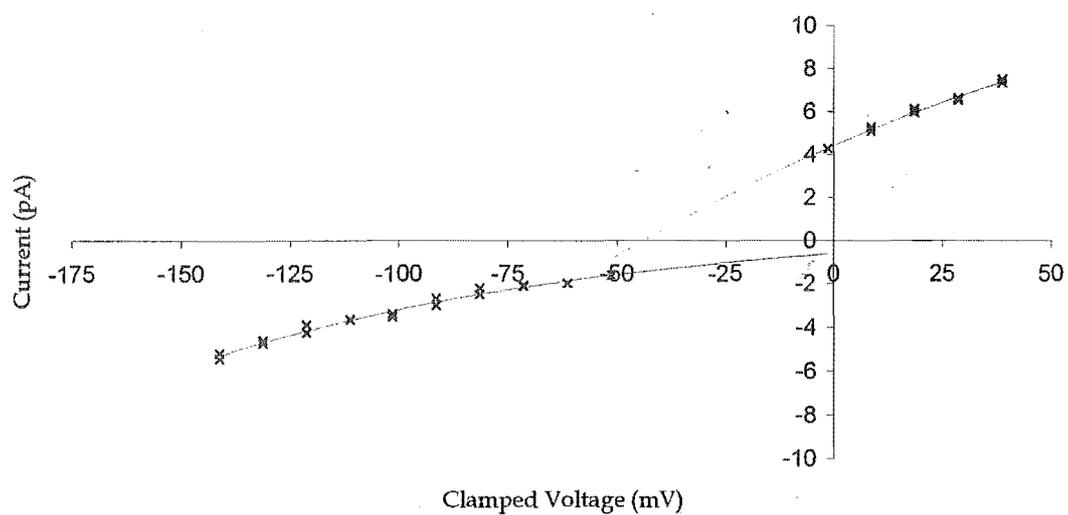
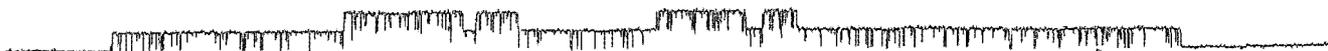


Figure 3.11 A theoretical IV curve based on the possibility that the IV curve shown in figure 3.3a was produced from data collected from a membrane patch that included two channels.



The IV curve in figure 3.2c shows a channel that is voltage gated near -40 mV; no channel events were recorded at voltages above -40 mV. Because the concentration gradient of chloride ions was close to zero (bath 70 mM to pipette 80 mM) the possibility that the effect was caused by an unfavourable gradient (i.e. a chemical gradient countering the voltage pressure applied by the voltage clamp) can be discarded.

It is predicted that more channels exist than observed in this study (see previous reference to the Garrill *et al.* study of *Zostera muelleri* (Garrill *et al.*, 1994). The majority of these channels are predicted to be of low conductance or infrequently found in their open state. These channels are thought to be involved in the 'fine tuning' of the membrane potential, or in response to rare, or infrequent, environmental events. This is suggested because, as a result of the frequent opening and large conductance of the large potassium channel, the resting membrane potential of the characean tonoplast may be approximately clamped to the potassium equilibrium potential (Lühring, 1986; Reeves *et al.*, 1985).



3.2 Protein Channel Glycation

3.2.1 Observations Made during Patch Clamping

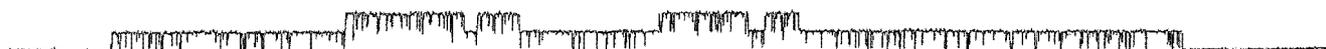
All glycation experiments were conducted on potassium channels because of their predominance in the characean tonoplast membrane.

An example of a full glycation experiment is outlined in figure 3.12. Shown are representative traces from each section of the experiment, that is, a trace showing the channel activity after each addition of glutaraldehyde (glycating agent) and an example of the disturbance associated with the manual addition of a glycation agent (figure 3.13a). This experiment took place at a clamped voltage of 175 mV in an attempt to maximally separate the conductance states for clarity. This means that the steps are much more pronounced than they were during other glycation experiments, (conducted at 70mV).

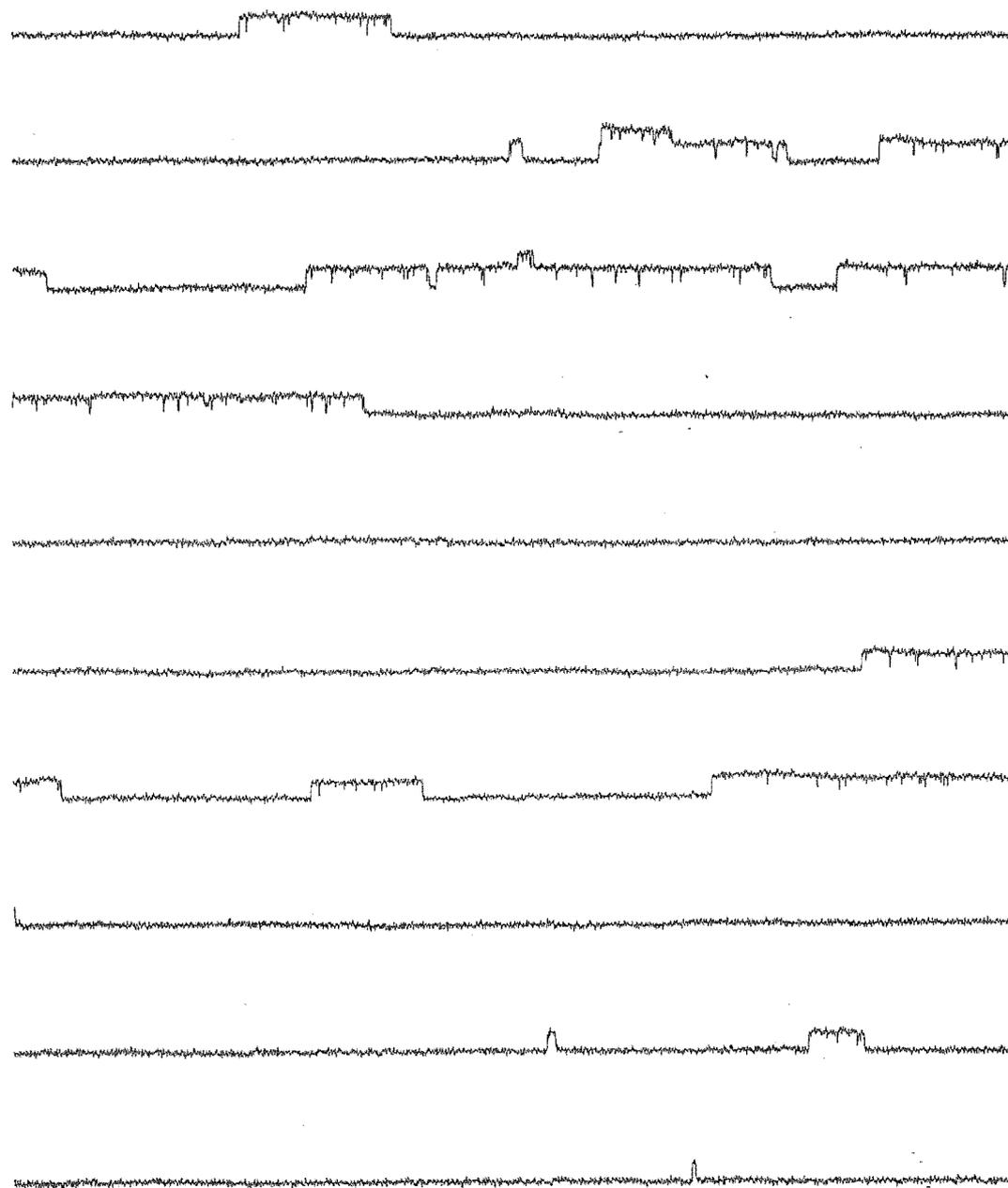
Figure 3.12d shows a “jump” in the baseline and an increase in activity immediately after glycating agent addition (figure 3.13b). There was increased channel amplitude (figure 3.12e) that could be taken as a result of glycation of the channel. However, this is an example of a phenomena observed on several occasions where a “jump” in baseline amplitude is linked to a dramatic increase in activity, usually followed by the breakdown of the seal (figure 3.12d see arrow). Other examples of a “jump” in baseline are shown in control and methylglyoxal addition experiments (figure 3.14). Figure 3.15 suggests that the baseline “jump” was not, however, always linked to an increase in activity. It is suggested that this phenomenon may be correlated with a drop in seal quality although how this results in an increase in channel activity in most cases is uncertain.

3.2.2 Channel Glycation Results

It was only possible to manually count transitions between conductance states with very low noise recordings. One occasion allowed this analysis technique to be used during a methylglyoxal addition experiment (figure 3.16). There is software available for



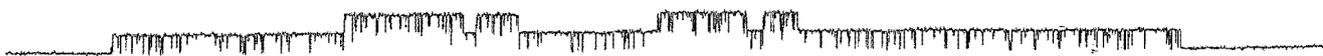
A



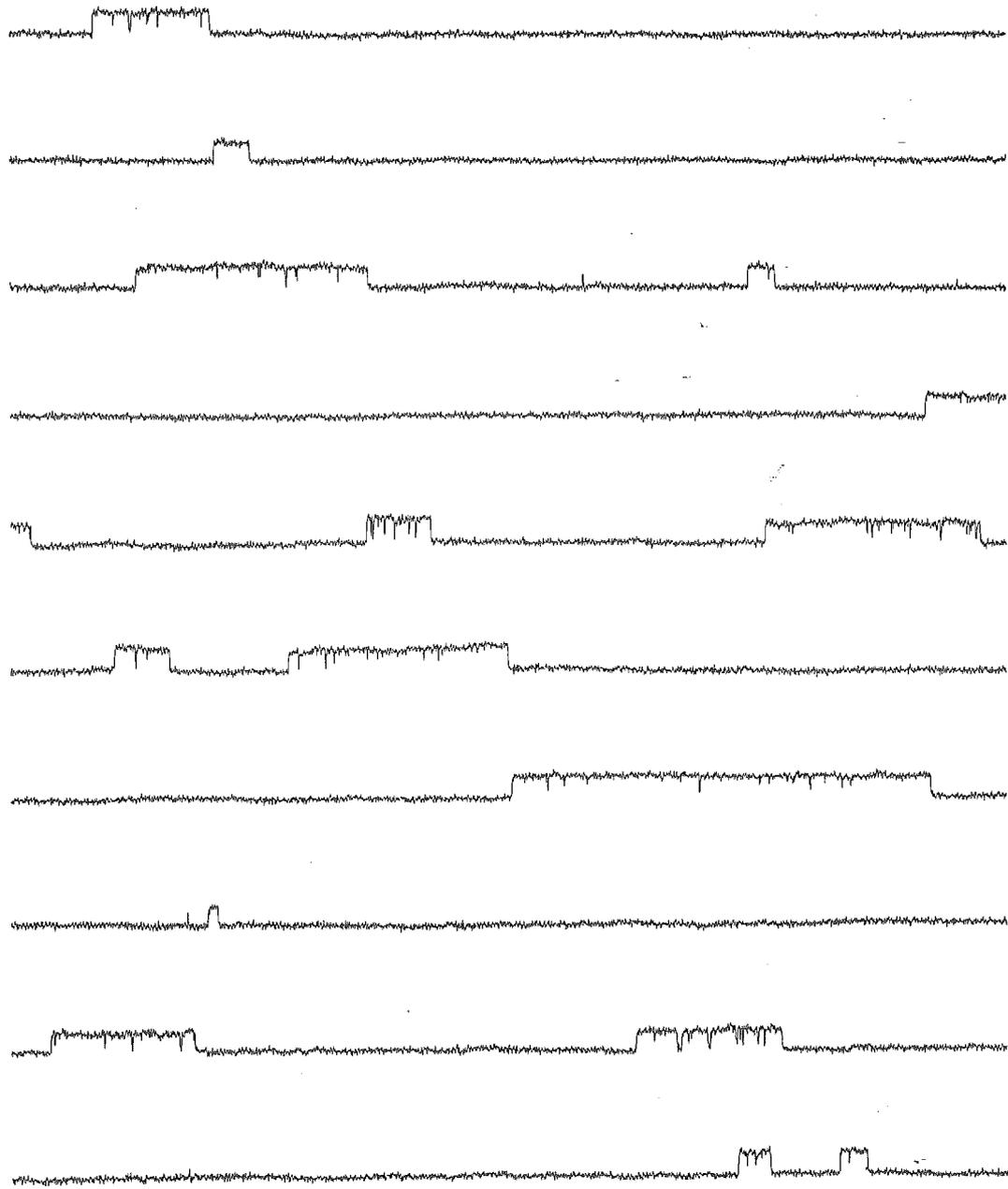
10 pA

200 ms

See overleaf

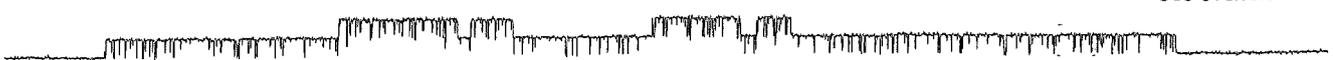


B

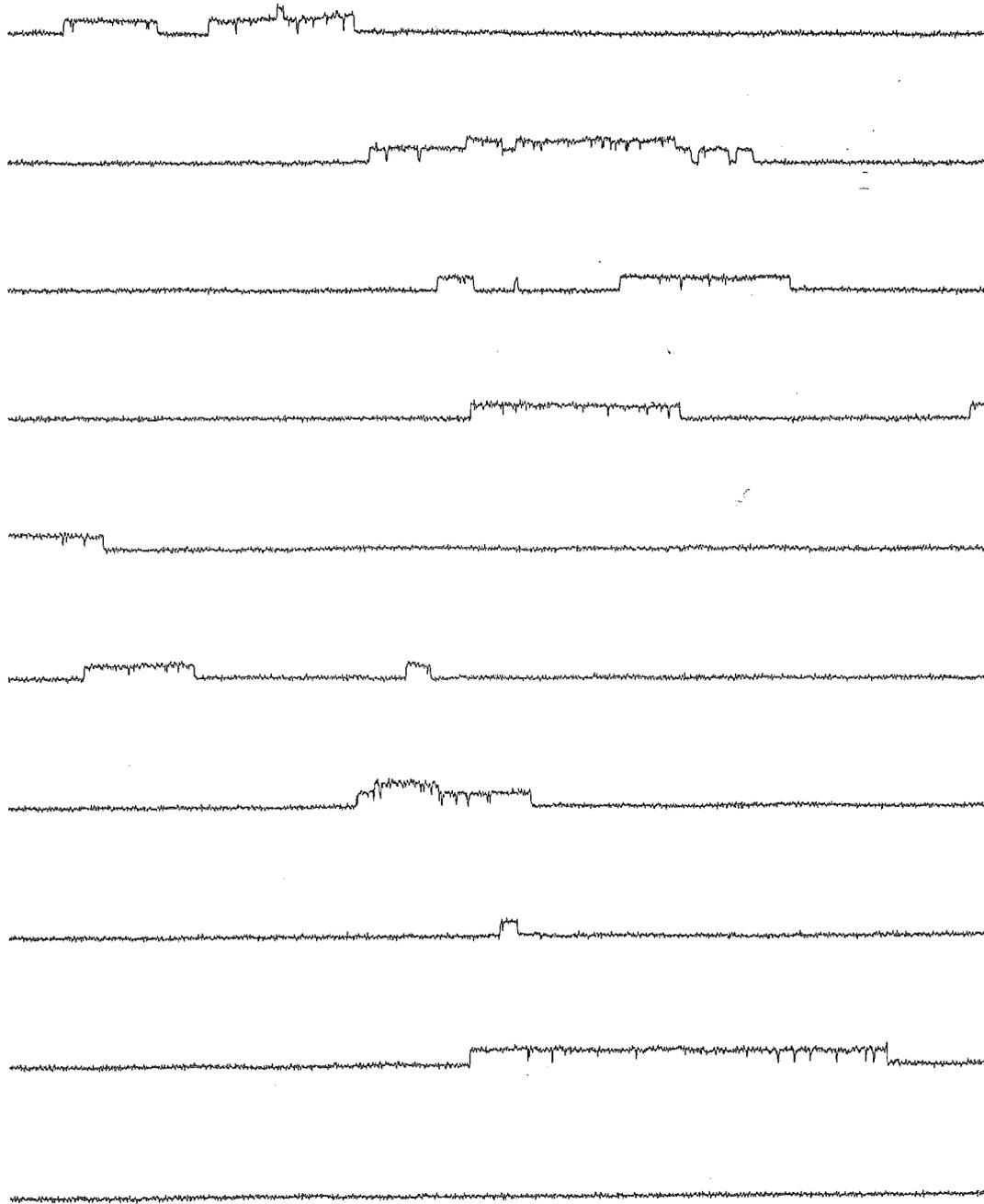


10 pA
200 ms

See overleaf

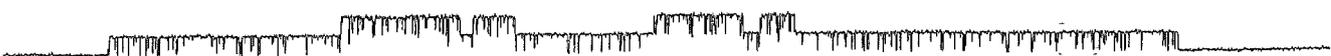


C

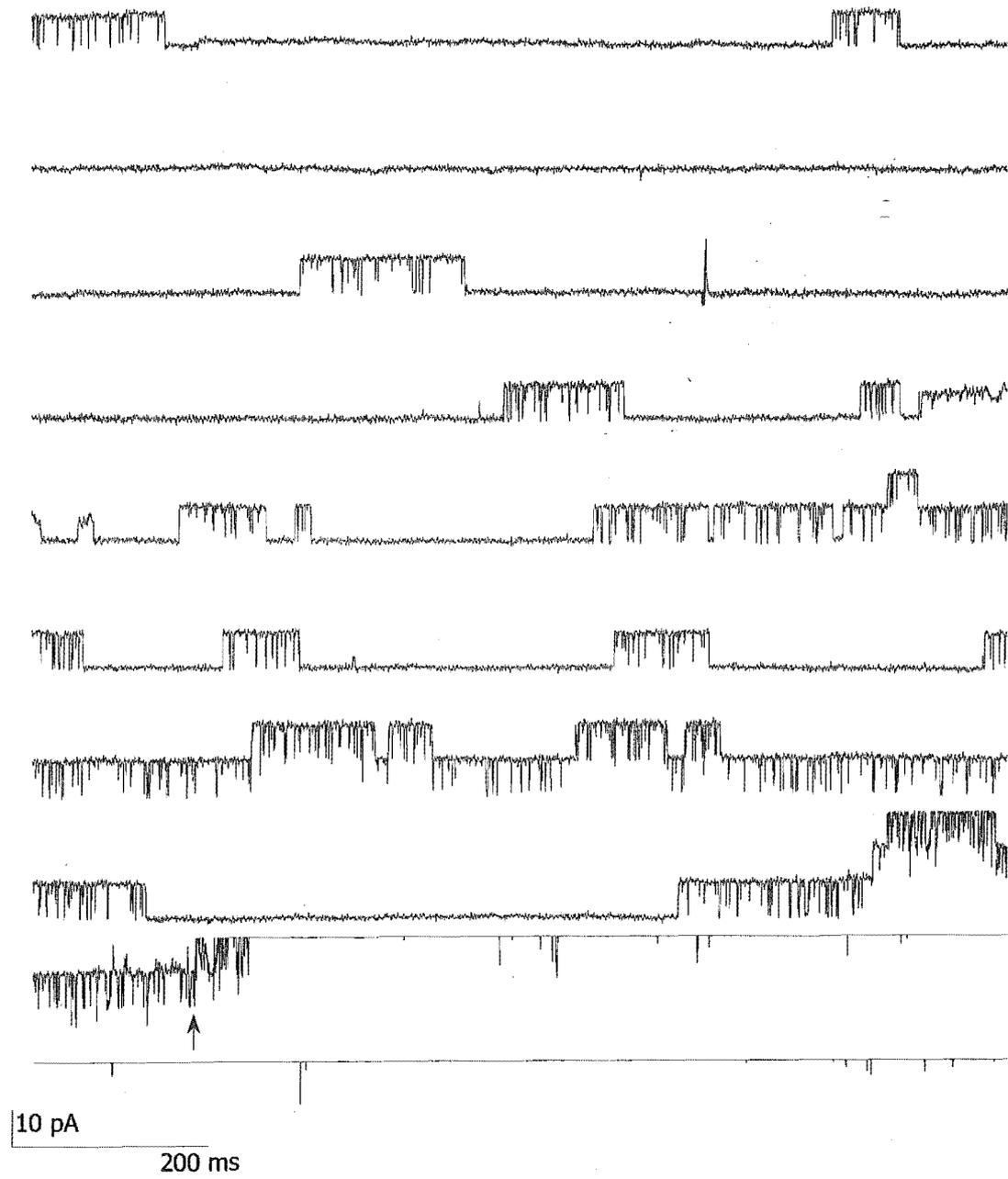


10 pA
200 ms

See overleaf



D



See overleaf



E

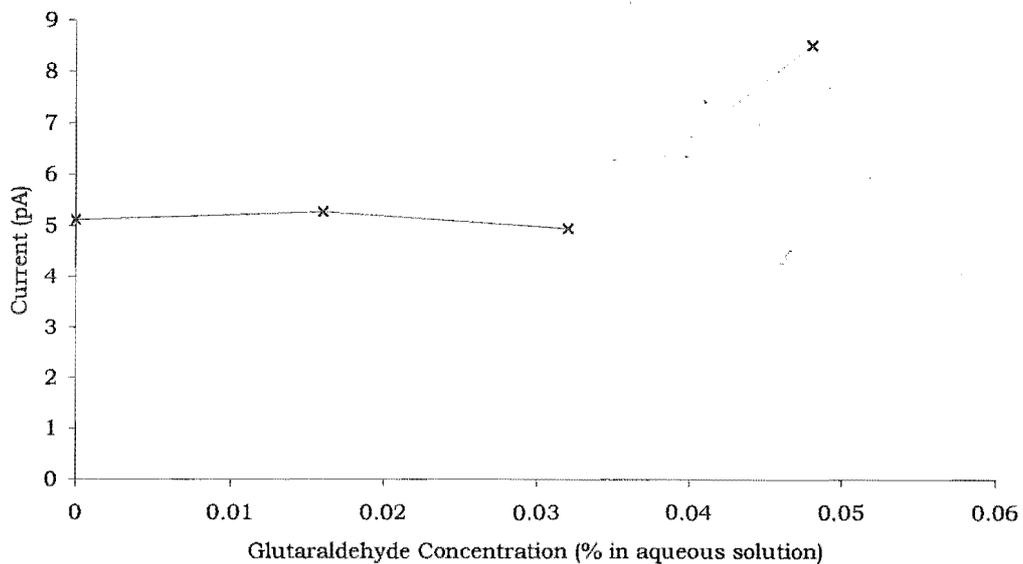
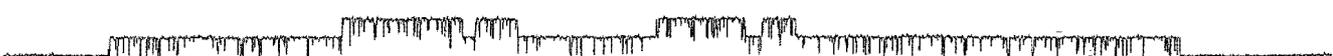


Figure 3.12 Typical traces taken during a glycation agent-addition patch clamp experiment. Traces are taken from approximately 2 seconds after glycation agent addition to avoid noise generated by the addition process. The traces represent 10 second recordings after the addition of 0, 20, 40, and 60 μ L (A, B, C, and D respectively). The arrow in D shows the approximate point when the seal burst (sudden loss in seal quality). This channel was voltage-gated at approximately 150mV so this data was recorded at 175mV where channel resolution was best (this data was not used in the 'glycation of channels' section because it wasn't recorded at 70mV). (E) Shows the effect that the glycation agent had on the current passing through the channel.



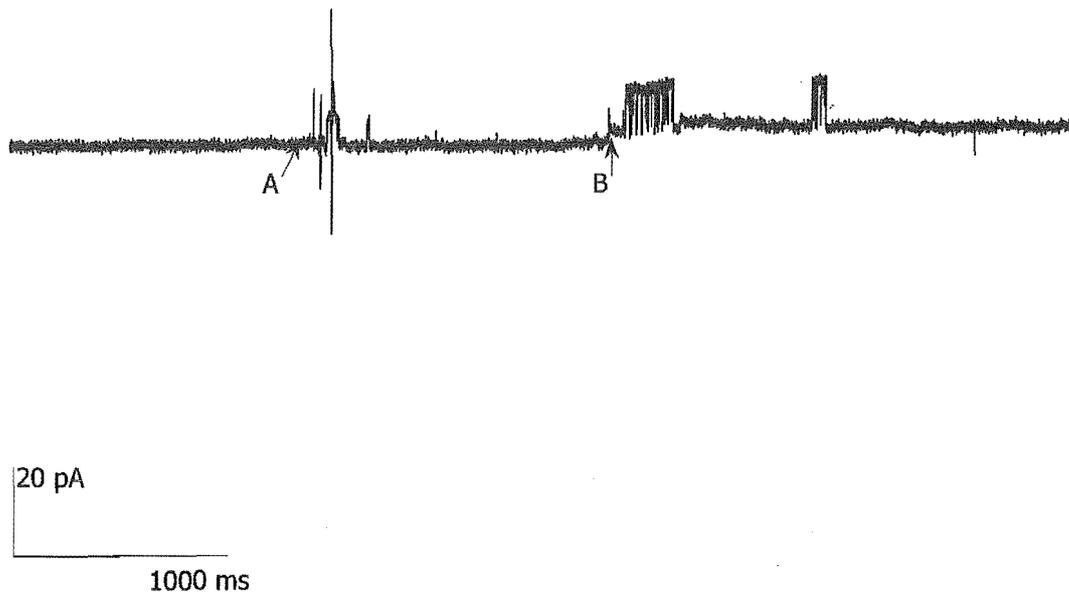
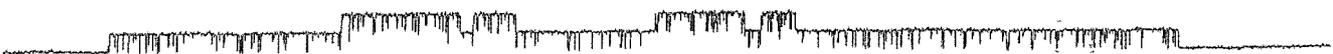
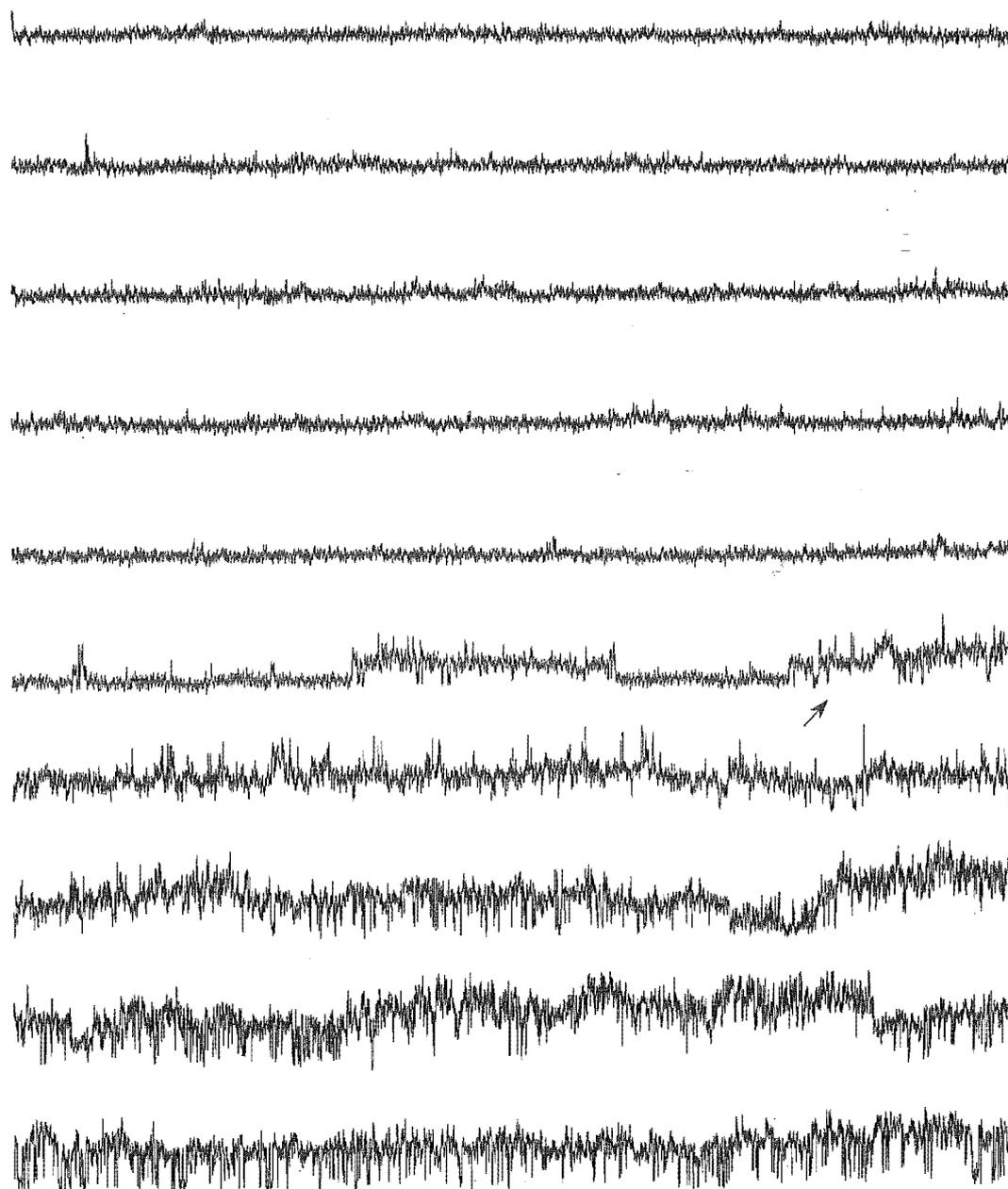


Figure 3.13 Data trace showing the brief burst of noise associated with manual addition of glycating agent (A). Also shown (B) is the "jump" which usually precedes a dramatic increase in multiple channel activity that leads to seal breakdown.

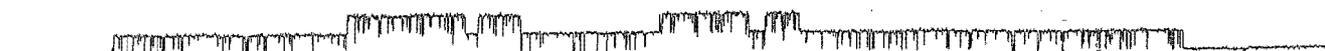


A



50 pA
1000 ms

See overleaf



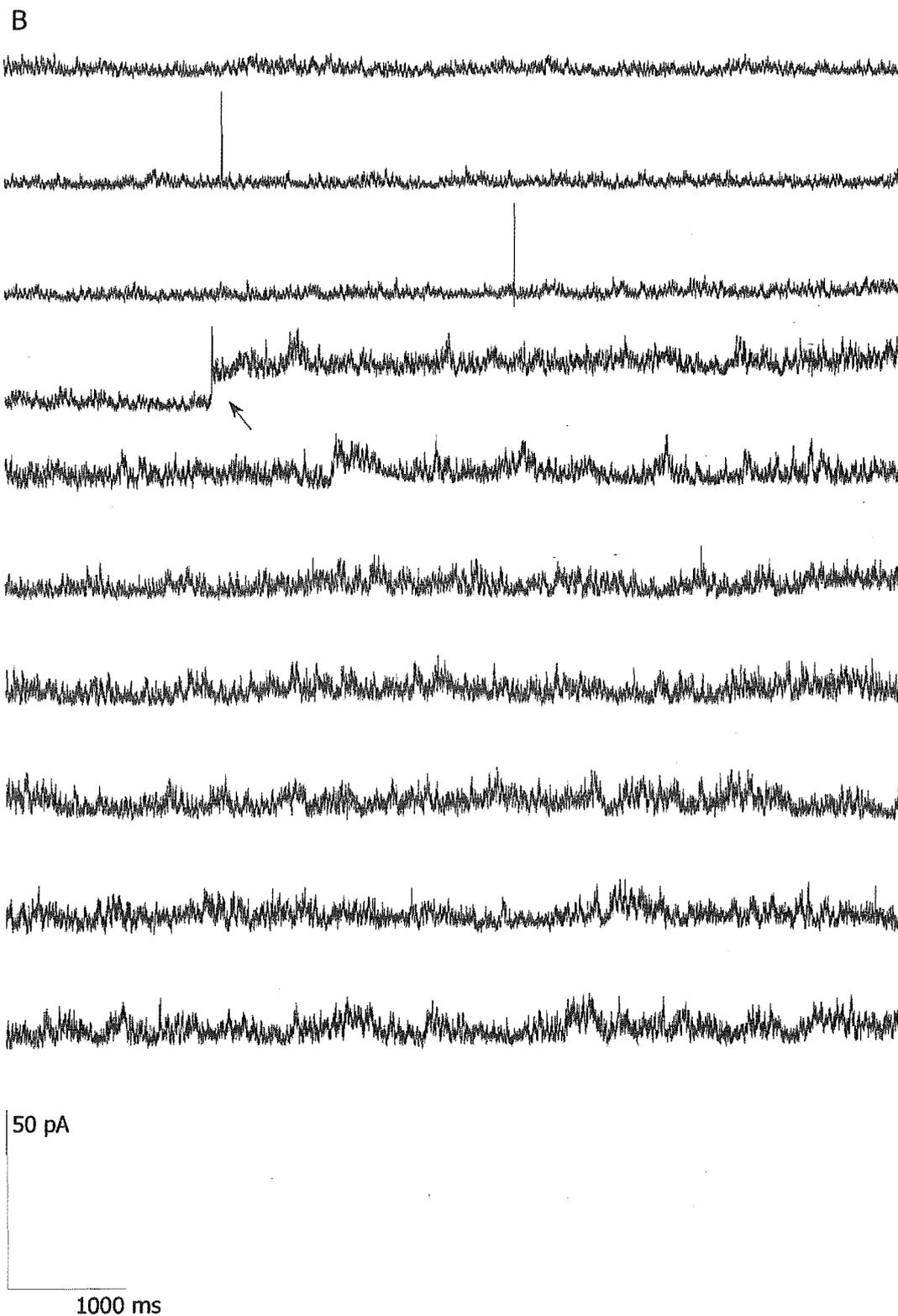
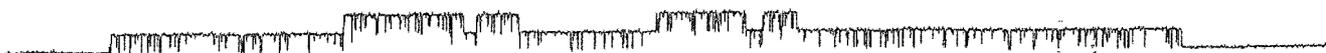


Figure 3.14 Trace showing “jumps” in baseline with associated increase in activity. (A) Shows a control experiment (no glyating agent was added) at 70mV (baseline “jump” is not obvious). (B) Was recorded at 40mV shortly after an addition of methylglyoxal. Arrows indicate the “jump”.



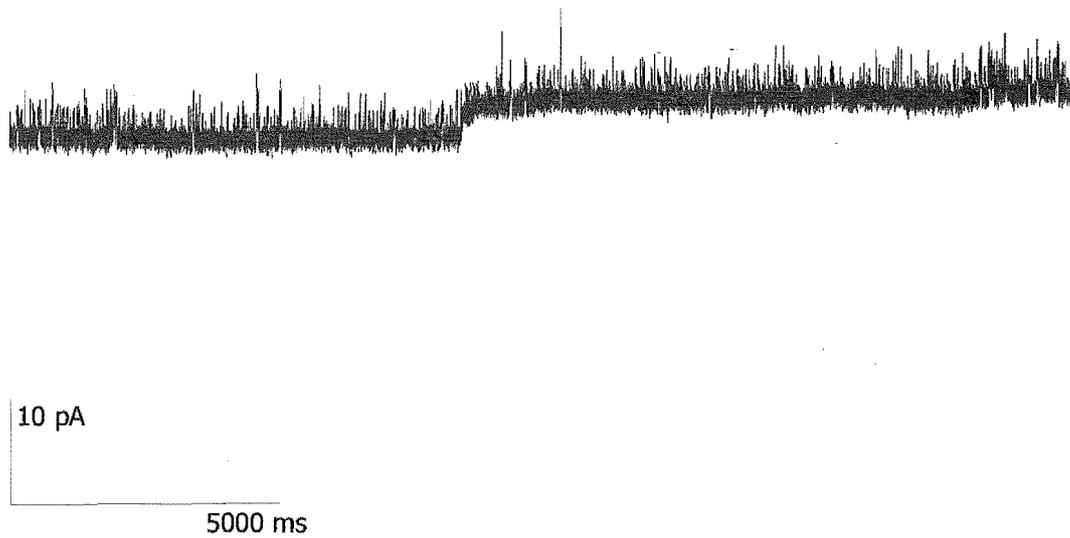
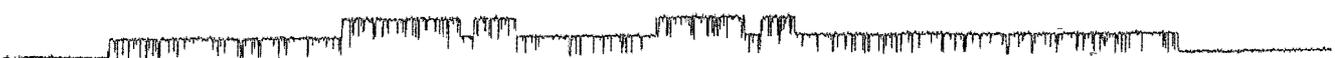


Figure 3.15 Trace showing a "jump" without an obvious increase in activity. Recorded at a clamped voltage of 40mV, no glycating agent had been added.



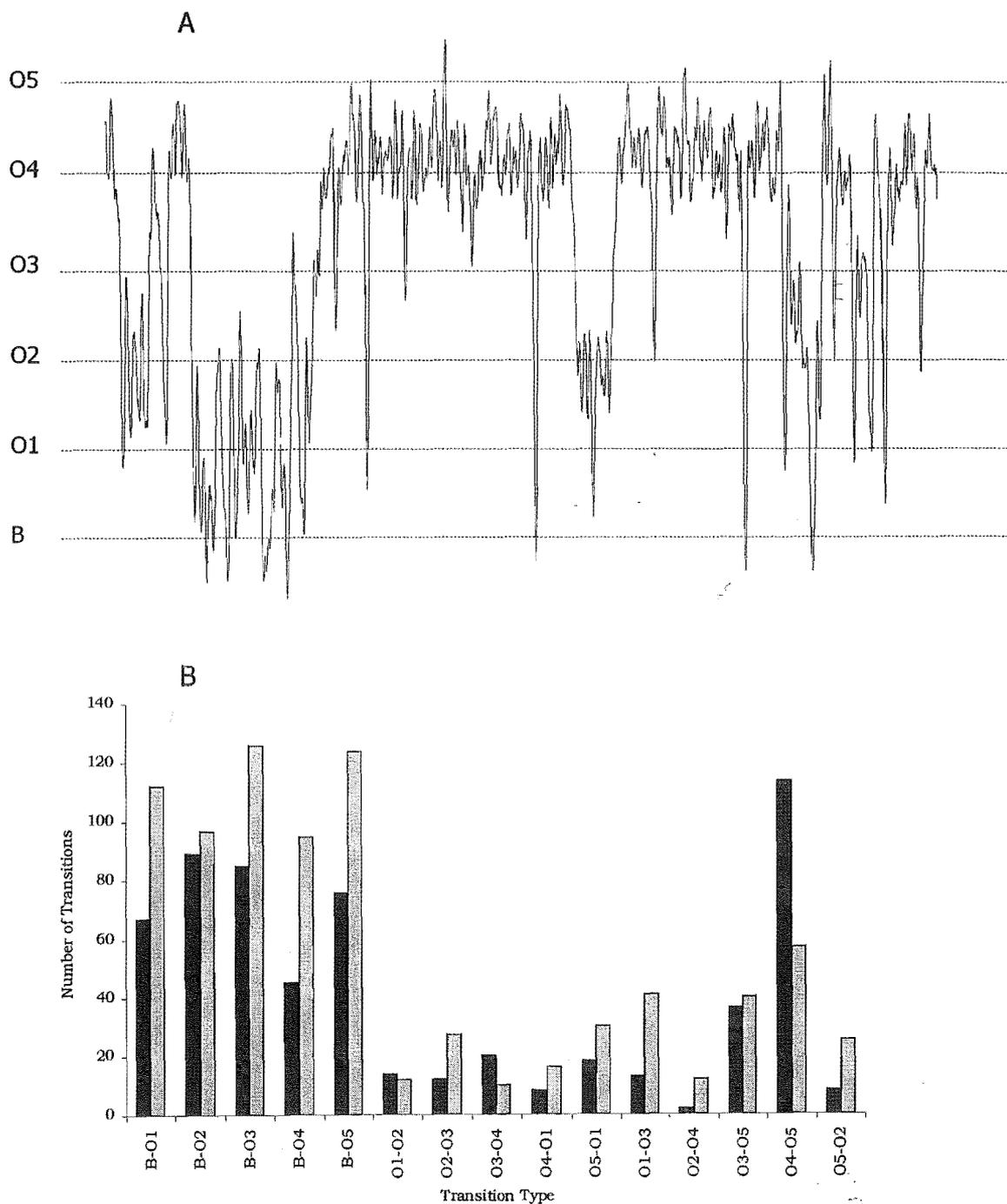


Figure 3.16 Transition analysis of subconductance states. Example of conductance states observed (A) B=baseline, O1 \approx 2pA, O2 \approx 4pA, O3 \approx 6pA, O4 \approx 8pA, O5 \approx 10pA. (B) shows the frequency of transitions between conductance states of a characean potassium channel before (dark fill) and after (grey fill) glycation agent (methylglyoxal) addition. Each set of transition frequency data was taken over a one second period. Total transitions = 1431.

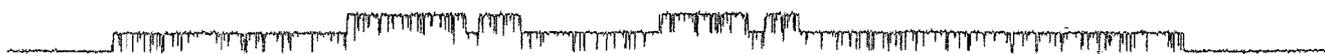
the analysis of transitions between conductance states (used for the study of substates; Tyerman *et al.*, 1992) however the data traces collected with Clampex7 are, as yet, incompatible with this program (Tyerman, 2000).

Figure 3.17 is an example of the transitions observed in this experiment. There is a distinct increase in activity between conductance states after methylglyoxal addition.

The majority of channels in patch clamp experiments unfortunately did not produce data that was distinct enough to extract transition analysis data. The amplitude of the main conductance state was observed with respect to glyating agent addition. The methylglyoxal additions (figure 3.18, n=4) all show a decrease in current amplitude with glyating agent addition. Control experiments with no addition show a constant current over time (figure 3.19, n=4). Preliminary results from glutaraldehyde experiments suggest the same trend occurred, but a higher concentration of the glyating agent was required (figure 3.20).

3.2.3 Channel Glycation Discussion

There was an over all increase in transition frequency of 35.7% that suggests that the glycation agent had some effect. The fact that the transitions from the baseline (i.e. B-O1, ... , B-O5) had higher transition rates both before and after glyating agent addition suggests that there may have been 5 separate channels present in the patch. However, since it does not meet the criteria set out in section 2.1.4 (which defines the distinction between substates and multiple channels) it is suggested that this recording shows changes in conductance of a single, multistate channel. The trend of increasing activity with methylglyoxal addition, is also disparaged by the concurrent increase in the 6pA transition (B-O5), i.e. the opening to, or closing from, the full conductance state. The expected result, if methylglyoxal were to have an effect, would be to see either a change in transition frequency between substates relative to the frequency of B-O5, or to see a change in the time the channel spends in a substate relative to the fully open state (O5). Although the latter of these was not quantified, figure 3.18 shows that this is probably not the case. The result of this experiment showed the need for adequate



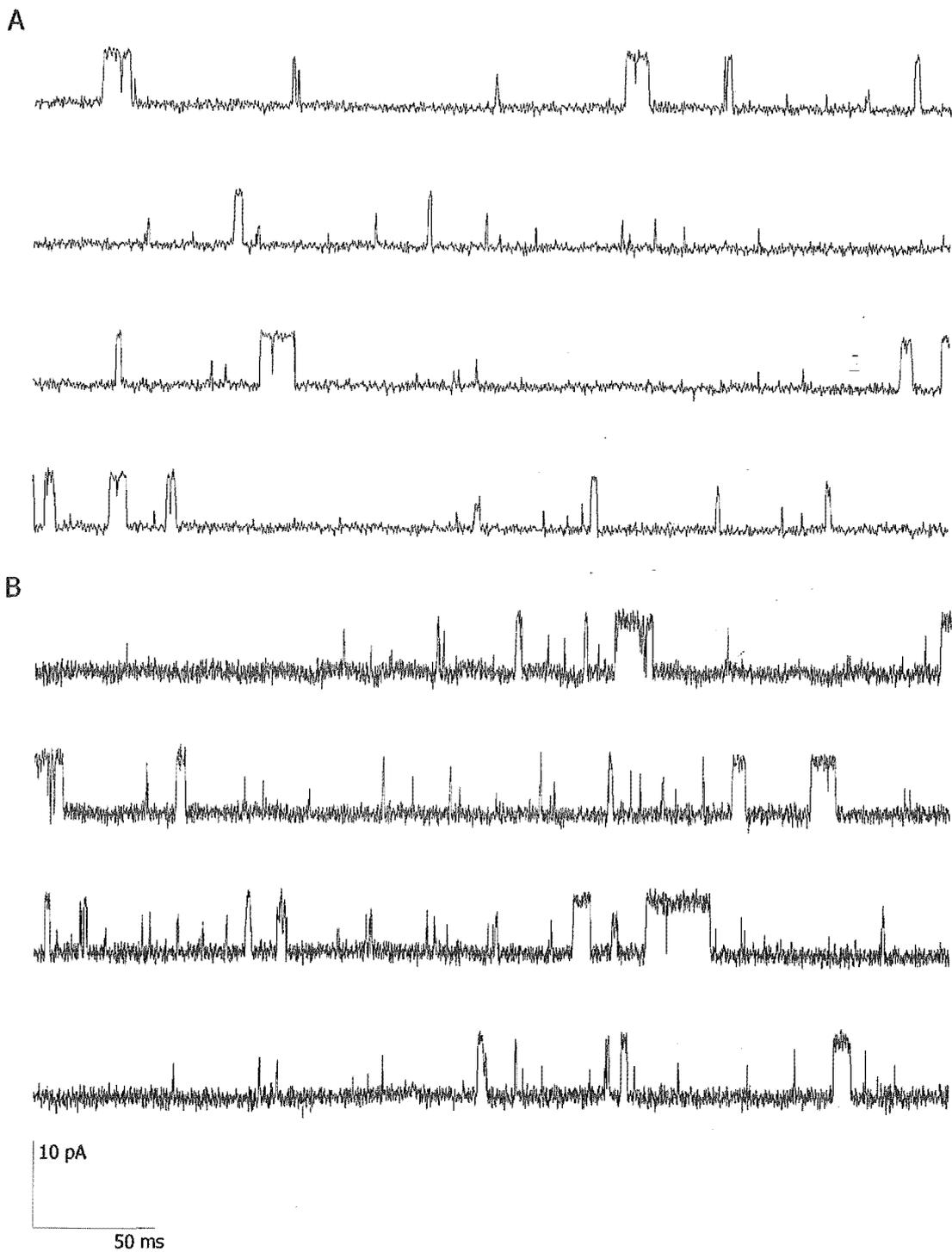
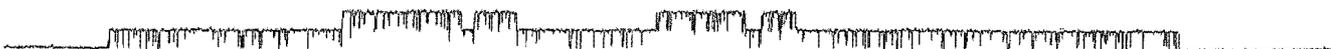


Figure 3.17 Traces of data used in transition analysis before (A) and after (B) methylglyoxal addition. Note that two channels were active after methylglyoxal was added; transition data was taken only from where single channel were distinguishable within the trace. Both traces were recorded at a clamped voltage of 70mV.



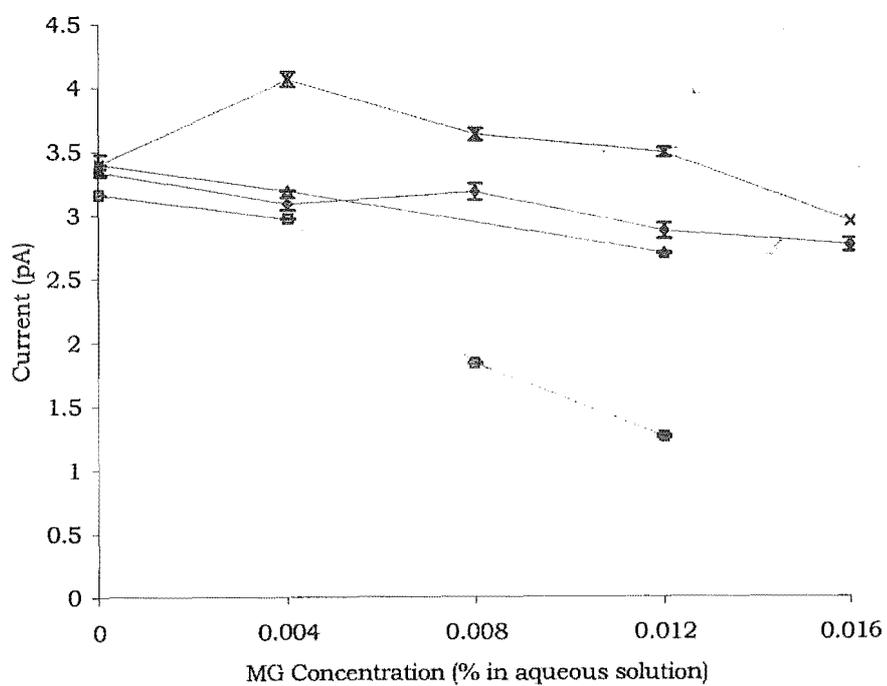
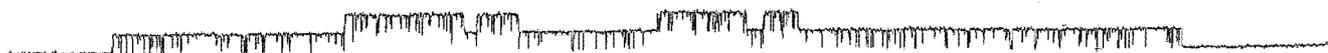


Figure 3.18 Does open channel current with time? Amplitude is displayed from four methylglyoxal addition experiments.



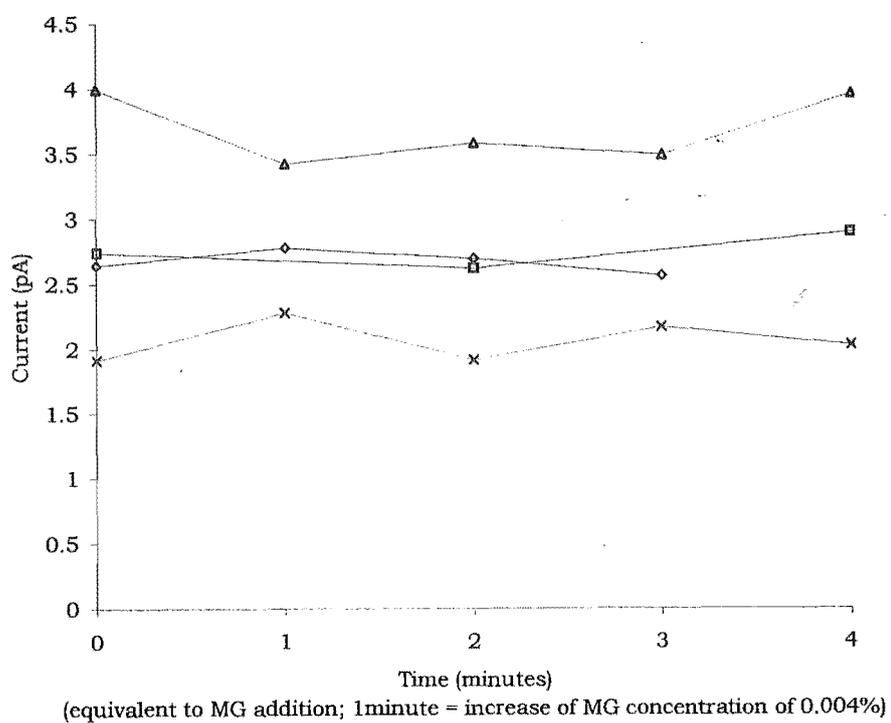
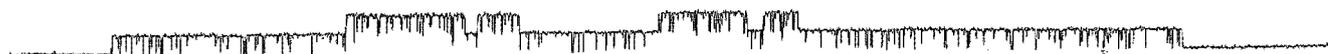
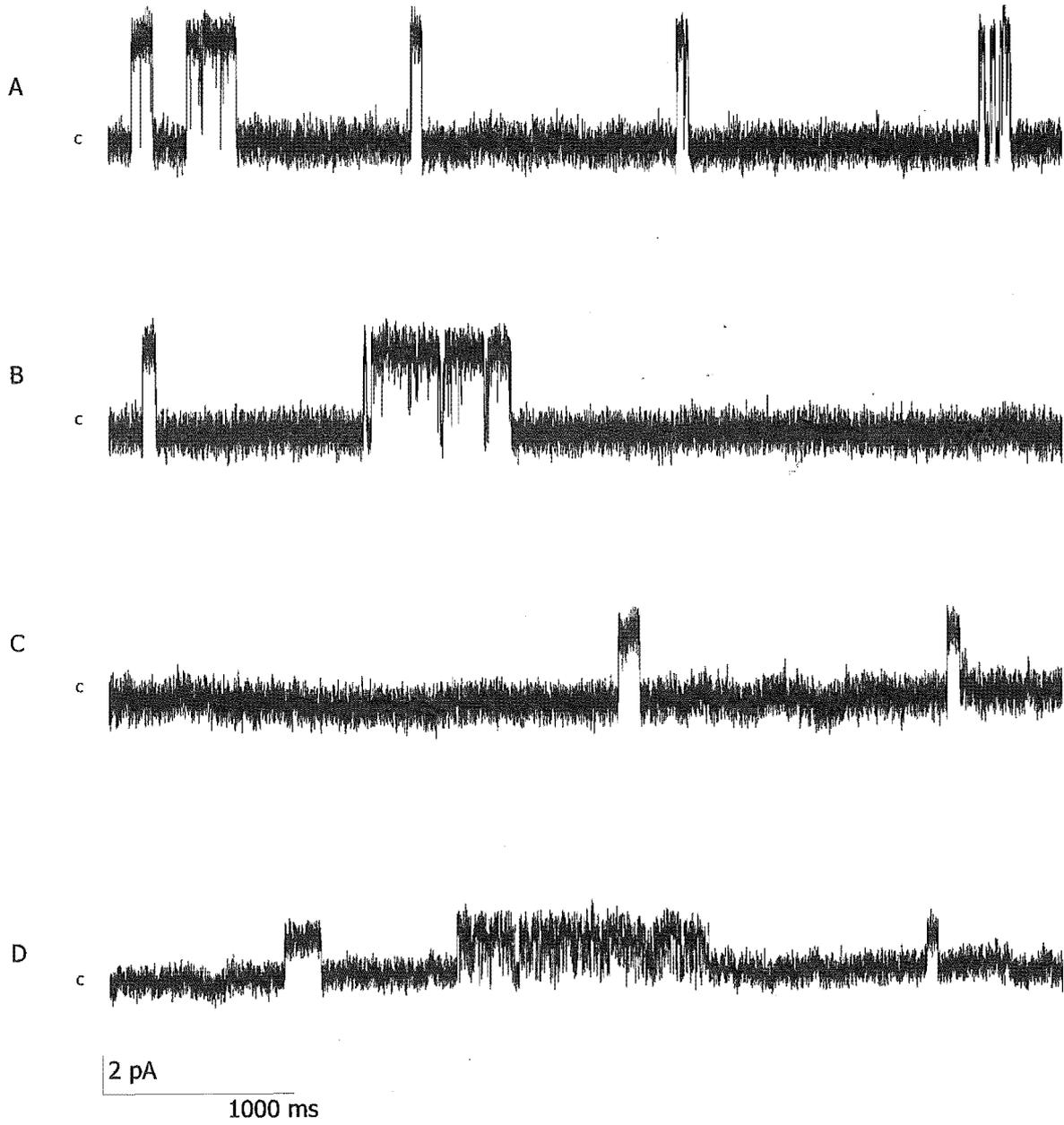


Figure 3.19 Does a channel's amplitude naturally decrease over time? A time control for the glycation addition experiments.





See overleaf



E

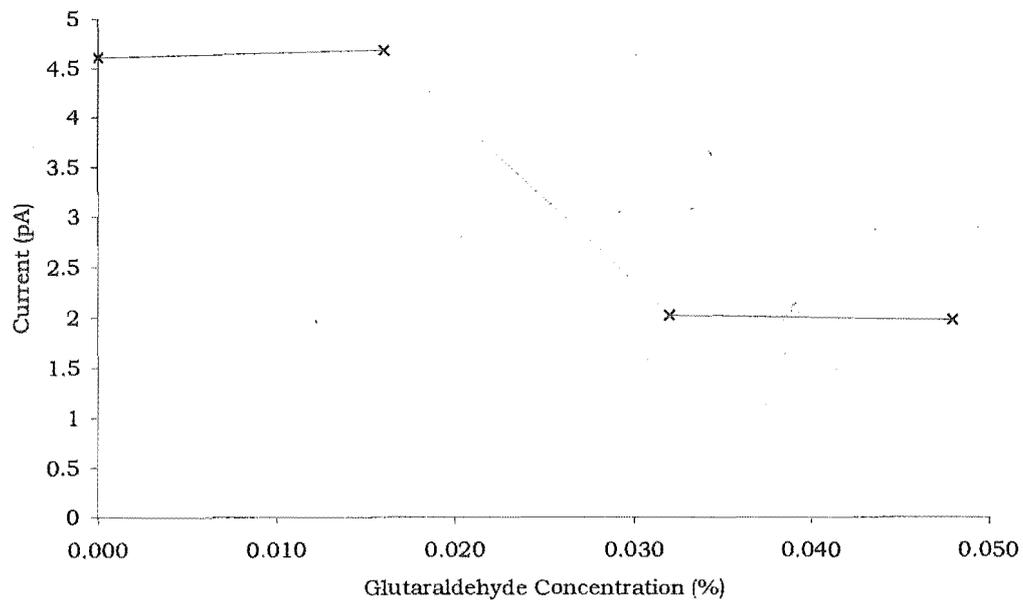
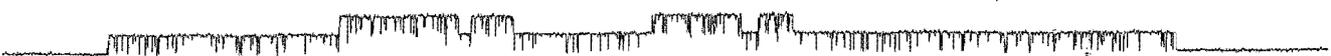


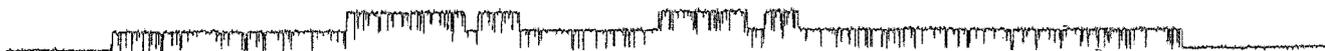
Figure 3.20 Data collected during the addition of glutaraldehyde to an inside-out patch at 70mV. Traces show various concentrations of Glutaraldehyde in the bath solution. (A) 0%, (B) 0.016%, (C) 0.032%, (D) 0.048% (with closed state indicated by a 'c'). Also shown is a plot showing the effect of glutaraldehyde on channel current (E).



controls to ascertain whether or not the observed increase in transition rate was due to the glycation agent addition or if it was part of the normal membrane patch aging process. Further patch clamp experience suggested that activity usually does increase with the age of a membrane patch (figure 3.21).

The Maillard chemistry was thought to potentially cross-link, or covalently modify, the channel and affect its conductance. Figure 3.19 shows this effect as glycation agent is added. While there was an apparent trend for a decrease in amplitude as glycation agent concentration increases, this is found to be not statistically significant ($P > 0.05$ using the one-step ANOVA function of Statistix7 (Analytical Software)). The probable reason for this is the high speed at which the glycation agent reacts, and the range of molecules with which it can react (Degenhardt *et al.*, 1998; Leoncini *et al.*, 1981; Lo *et al.*, 1994; Miller *et al.*, 1980; Oya *et al.*, 1999; Riviere *et al.*, 1998), coupled with the variable amount of cellular material in the bath. It is proposed that the glycation agent had, to some degree, covalently bound to various molecules before it had diffused from the point of addition to the membrane patch at the tip of the recording electrode (~5mm). Thus the size of the decrease in amplitude, and time at which it occurs, is variable. The control for this experiment showed no dramatic change in current amplitude (figure 3.19) confirming that the effect was related to the addition of the glycation agent.

A repeat of this experiment with glutaraldehyde initially shows a similar trend though at a higher concentration of glycation agent. Observations in other protein glycation studies show that glutaraldehyde is a more potent reactor, and reacts faster than methylglyoxal (Fayle *et al.*, 2000). However, with this experiment, a higher concentration than methylglyoxal was required to achieve the same effect. This is likely to be due to the increased reactivity of glutaraldehyde increasing its chance of reacting before diffusing to the membrane patch.



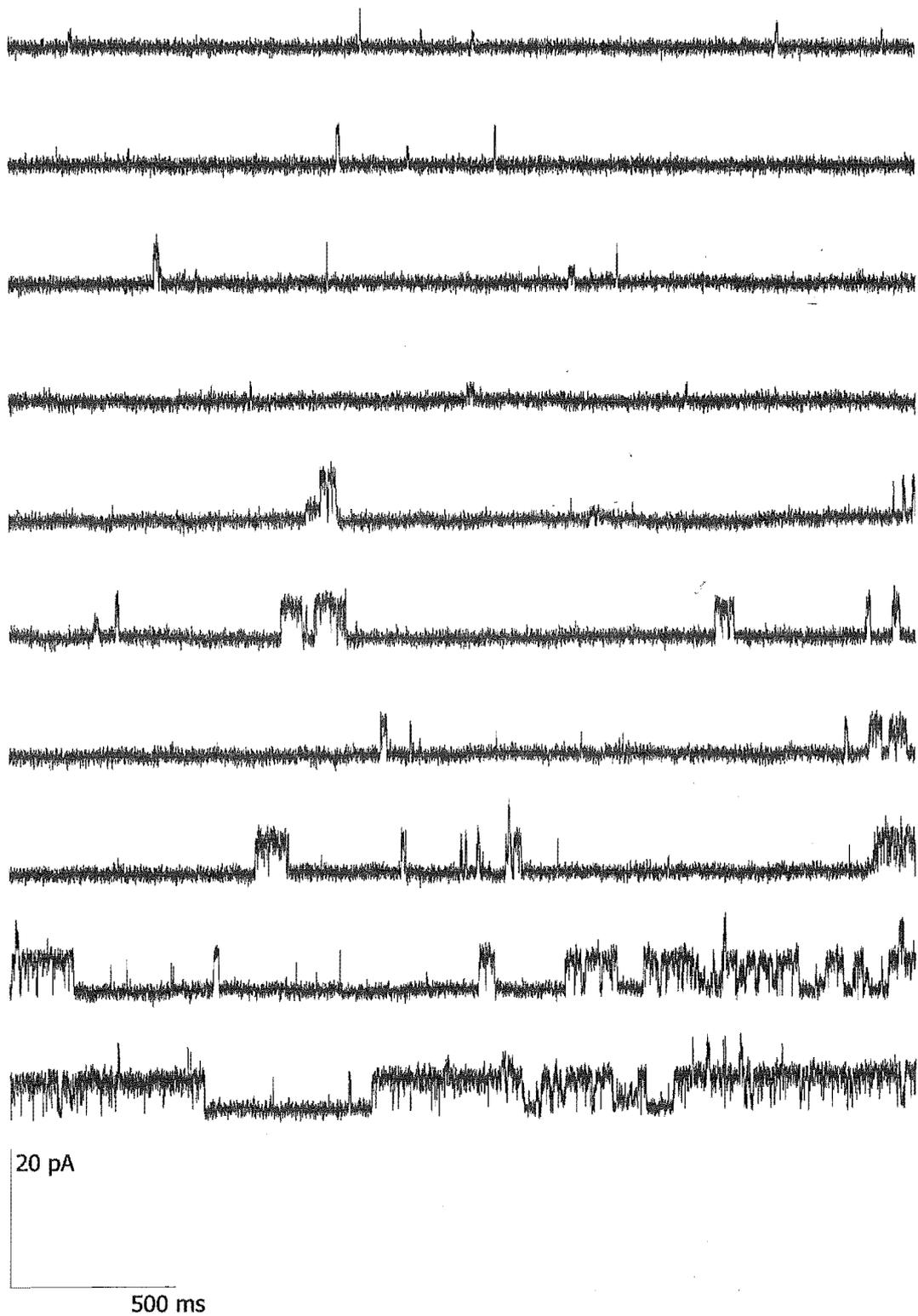
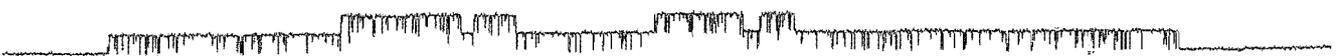


Figure 3.21 Commonly observed trend of increasing activity over time. This ~30second trace was recorded at a clamped voltage of 70mV while in inside-out configuration.



3.3 Future Research

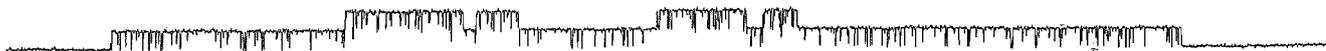
There is much scope for continuation of these experiments. Further replicates of those experiments described in this thesis should first be performed to achieve significant data. Once this is achieved a range of experimental techniques could be employed to confirm and extend the channel protein glycation information gathered thus far, such as:

- Glycating agents of different sizes and reactive properties may be tested for similarities in action, effect of size, shape, etc.
- Microinjection of glycating agents close to the membrane patch to reduce the effect of the residual glyoxalase system from the algal cell (Brownlee, 1994; Mobbs *et al.*, 1994).
- Use of solution changers for the introduction of new bath and pipette solutions during the course of an experiment (Nichols *et al.*, 1995; Tang *et al.*, 1995).
- Use of ion-selective microelectrodes (Shabala *et al.*, 1997; Voipio *et al.*, 1994).
- Use of fluorescent indicators to measure intracellular ion concentrations (Cannell & Thomas, 1994).
- Use of the pressure probe technique that detects changes in the turgor pressure of a cell (Brownlee, 1994; Garrill, 2001).
- Reconstitution of a cloned channel into an artificial membrane system (Williams, 1994).

A cloned channel could be purified and glycated *en masse* allowing hydrolysis experiments to pinpoint positions in the protein's primary structure that are susceptible to glycation. Then, assuming the glycation occurs at specific positions within the protein, the affected residues could be subjected to mutational studies. These may then be able to propose why the drop in amplitude occurred. For example, if a lysine residue were found to be glycated it would be useful to observe the effect that the substitution of a similar amino acid would have on the channel's action. Arginine could be that

replacement (figure 3.22a), or one of the uncommon amino acids such as ϵ -*N*-methyllysine (found in some muscle proteins, figure 3.22b) or *N*-methylarginine (from histone proteins, figure 3.22c).

To explore the application of these studies to medical science, diabetic conditions could be observed on human channels. Human channels could take the place of the characean algae or they could be incubated in glucose to stimulate the Maillard reaction. The characean algae could be incubated in glucose to observe the effect of a “diabetic” environment on a plant cell and to allow correlation of the plant cell studies to animal channel research. Glycating agents have yet to be observed in patch clamp studies of other plant cells. A survey could be conducted on the effect of glycating agents on a range of plant membranes.



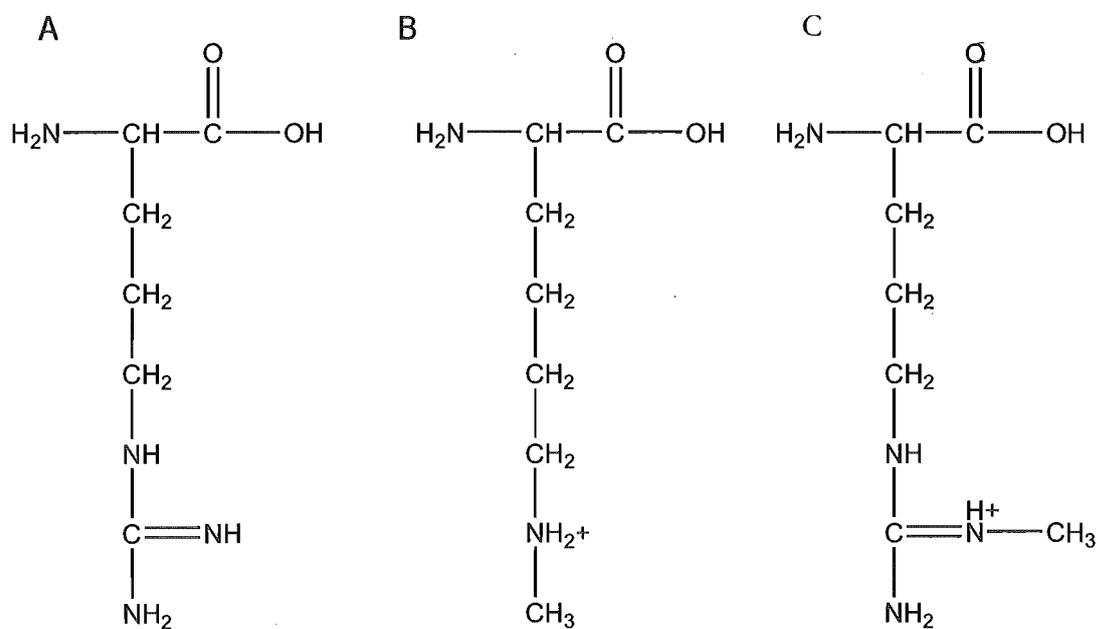


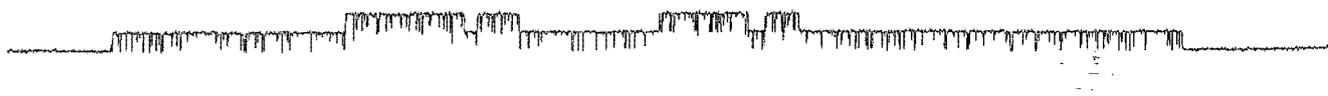
Figure 3.22 Chemical structures of amino acids. (A) Arginine. (B) ϵ -*N*-methyllysine. (C) *N*-Methylarginine.

3.4 Conclusion

After the development of a successful solution pair, and optimising electrode manufacture, up to ten different ion channels were observed in *Nitella bookeri*. These comprised three that were unidentifiable, four that were selective for potassium ions, and three selective for chloride ions.

Also examined was the action that glycating agents – methylglyoxal, and glutaraldehyde – have on characean algae tonoplast membrane channels. The trends observed suggest that the glycation is occurring. Maillard chemistry may have resulted in cross-links within the channel or between the channel and another molecule, or produced an AGE which acts on the channel in an antagonistic way, perhaps an analogue of a natural signalling molecule (second messenger).

Although the results are not yet definitive, the framework for a more detailed study has been put in place. Time restraints reduced the number of glycating agents to two. In a comprehensive study, this should be expanded to include glycating agents that range in size (to form cross-links between different channel protein residues) and activity (to what degree does reactivity matter physiologically when the molecule is long-lived and might have many chances to react). Another useful study would look at a variety of human cell membranes in hyperglycaemic conditions (as occurs in diabetes mellitus).



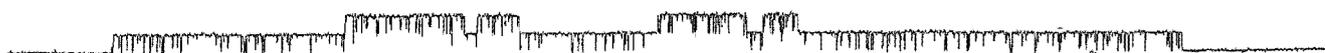
Acknowledgments

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Finally I would like to thank Jamie Gaskill, to whom this thesis is dedicated, for changing the way I critically think more than 6 years of scientific study ever did.



References

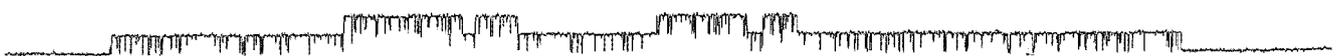
*“Now just tell me, in your own words,
exactly what happened.”*

-A policeman

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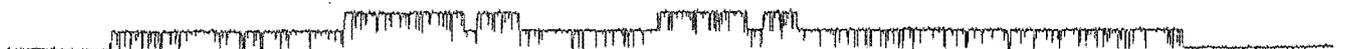
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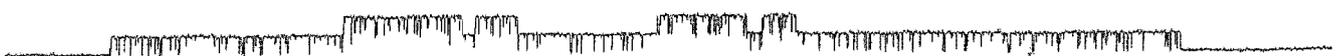


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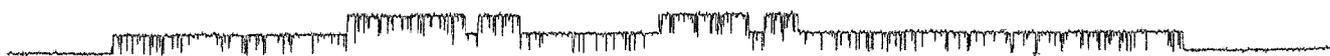
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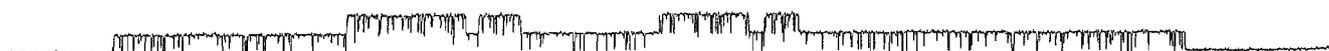
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"Don't let it end like this.

Tell them I said something."

- last words of Pancho Villa (1877-1923)