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MECHANOSENSITIVE TREK-1 CHANNELS IN THE HEART

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STATEMENT

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

Experimental results and observations reported in this thesis were obtained solely through my own effort unless acknowledgement is made. Parts of the results obtained from my work have been published as journal articles during the duration of my postgraduate studies.

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Joy Hui Chieh Tan

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LIST OF ABBREVIATIONS

ANP: atrial natriuretic peptide

APD: action potential duration

4-AP: 4-aminopyridine

ATP: adenosine triphosphate

BK channel: large calcium activated potassium channel

cAMP: cyclic adenosine monophosphate

DHA: docosahexaenoic acid

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

K_{ACh} channel: muscarinic potassium channel

K_{ATP} channel: ATP sensitive channel

MAP: monophasic action potential

MEF: mechanoelectric feedback

mRNA: messenger ribonucleic acid

PCR: polymerase chain reaction

PLA_2: phospholipase A_2

P_o: open probability

RSA: respiratory sinus arrhythmia

SACs: stretch-activated channels

TASK: acid sensitive two-pore K^+ channel

TEA: tetraethylammonium chloride

TES: n-TRIS [Hydroxymethyl] methyl-2-aminoethanesulfonic acid - biological buffer

4TM2P: four transmembrane domains, 2 pore domains

TREK-1: TWIK related potassium channel

TWIK: two pore weakly inward rectifying K^+ channel

ABSTRACT

Stretch-activated ion channels have been reported in many cell types eg. cardiac and intestinal smooth muscle cells (Kim, 1992; Farrugia, et. al., 1999; Kawakubo, et. al., 1999). They are thought to be important for transducing mechanical stretch into electrical signals. Patch-clamp techniques were used in this study to characterise a large stretch-sensitive channel found in rat ventricular myocytes. Experiments were performed at 20-24^{0}C room temperature. In cell-attached and ripped-off patches, suction applied to the pipette activated a potassium-selective ion channel. Open probability (P_o) of the channel was enhanced with increasing amounts of suction applied ($P_o \approx$ 0.005 with no pressure; $\approx$ 0.328 with 90 cm.H_2O: membrane holding potential (V_m) = 40 mV, pH_i = 7.2, n = 5). Lowering the intracellular pH also raised channel activity (P_o = 0.005 at pH 7.2; P_o = 0.16 at pH 5.5: no suction, V_m held at 40 mV). These channels have characteristics of the TREK-1 channel, a member of the recently cloned two pore, four transmembrane domain family of potassium channels (Maingret, et. al., 1999). Using RT-PCR, TREK-1 was found to be expressed in the rat heart reinforcing the suggestion that the channels being investigated are TREK-1. However, a characteristic of these channels that have not been reported before is its activation by intracellular ATP which occurs at a more acidic intracellular pH. At a pH of 6 with no ATP at the intracellular membrane face, P_o was 0.048 $\pm$ 0.023. P_o increased to 0.348 $\pm$ 0.13 with 3 mM ATP (no suction applied). The channel was however not sensitive to ATP at a physiological intracellular pH of 7.2 TREK-1

expression and whole-cell current were also found to be lower in epi- and endocardial cells of rat ventricles. The expression of TREK-1 relative to GAPDH was found to be 0.34 ± 0.14 in endocardial cells compared to 0.02 ± 0.02 in epicardial cells ($p \leq 0.05$). TREK-1 normalised current in epi- and endocardial cells were 0.21 ± 0.06 and 0.8 ± 0.27 respectively ($p < 0.05$). Cardiac potassium-selective stretch-activated channels might have an important role in hyperpolarising the membrane potential in order to prevent excess Ca^{2+} influx which can be deleterious for the cell (Maingret, et. al., 1999). These channels might also be involved in shortening the action potential duration when the heart is stretched. A heterogenous expression of TREK-1 channels in the ventricle might correspond to the different amounts of stretch experienced by muscle cells across the ventricular wall during diastole.

1: GENERAL INTRODUCTION

1.1 BACKGROUND

1.1.1 Observations of stretch-induced cardiac rhythm changes

It has been long known that the electrical activity of the heart drives its mechanical activity. However, it has also been shown that mechanical stimuli can cause electrical and contractile changes in the heart. This phenomenon is referred to as mechano-electric feedback. As early as 1915, Bainbridge reported that heart rate of anaesthetised dogs could be increased when the right atrium was distended by injecting fluid into the jugular vein. This was considered as a potentially important feedback mechanism which adjusted heart rate to changes in venous return. Years later, Blinks (1956) as well as Lange and co-workers (1966) reported that a stretch-induced increase in beating rate could be evoked in isolated cardiac preparations. As stretch-induced responses can occur in isolated cardiac tissue preparations, they cannot be mediated by reflex loops but rather they must be a property of the muscles themselves.

There have also been observations of cardiac rhythm disturbances and sudden death caused by non-penetrating mechanical impacts to the chest published more than one hundred years ago (Nélaton,1876; Meola, 1879). For

example, Nélaton (1876) reported a case of instant death of a manual labourer due to impact to the chest. Post-mortem examination showed no signs of internal structural damage. This condition is referred to as Commotio cordis. Following these observations in humans, experimental work on Commotio cordis had been performed by various researchers on animals. For example, Schlomka and colleagues (Schlomka and Hinrichs, 1932; Schlomka and Schmitz, 1932) used wooden hammers of varying weights and diameters to apply impacts to the precordial region of anaesthetised rabbits. They reported that such impacts caused mainly cardiac dysrhythmias, which were almost always induced by extra-systoles. Ventricular fibrillation which usually caused sudden death occurred in 20% of cases. The smaller the impact zone and the swifter the impacts, the higher the chances of severe rhythm disturbances. Conversely, a thump given to the chest can also effectively revert ventricular tachycardia. A thump in the chest can return heart rhythm to normal by evoking a premature beat that interrupts the tachycardia-sustaining mechanism.

1.1.2 Observations of stretch-induced changes in electrical activity in the heart

More recent experiments performed in whole hearts as well as excised cardiac tissues (Boland and Troquet, 1980; Dean and Lab, 1989; Franz, et. al., 1992; Hansen, 1993), showed that mechanical stretch shortens the action potential and causes depolarisation of the cell membrane during diastole. These depolarisations sometimes appear to cause extrasystoles. An example

of stretch-induced effects in the whole heart is shown in Figure 1. In this experiment, a small balloon was inserted into the left ventricle of an isolated rabbit heart. Transient stretch pulses were given to the ventricle during diastole by inflating the balloon which induced depolarisations. The size of the depolarisations increased with the amount of stretch given until a threshold is reached above which an action potential is triggered with each stretch pulse.

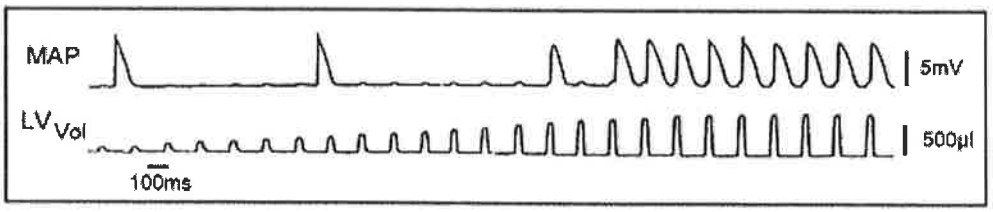


Figure 1: *Stretch-induced effects on the whole heart*
Diagram shows a monophasic action potential recording from the epicardial surface of a rabbit ventricle in response to volume pulses (change in volume is shown in lower trace) applied to a balloon inserted into the left ventricle. Volume pulses induced a transient membrane depolarization which increased in amplitude parallel to increases in volume pulse amplitude. Above a certain volume, the transient depolarisations produced premature ventricular excitations. The isolated heart was Langendorff perfused with Tyrode solution at 37°C. Diagram taken from Franz, et. al., 1992.

There have also been many reports of changes in action potential duration following myocardial stretch. For instance, Taggart and co-workers (1992) who measured monophasic action potentials (MAP) from the left ventricular epicardium of patients undergoing cardiac surgery, reported that an aortic occlusion for 1-3 beats resulted in shortening of action potential duration (APD). When the aorta is occluded, the ventricular volume is not ejected and is thus increased by further filling during diastole. An example of stretch-induced shortening of action potentials in rabbit atria is shown in Figure 2

below. Stretch-activated channel activation is believed to be involved in stretch-induced APD shortening as well as the triggering of extra action potentials in myocardia subjected to pathological amounts of stretch.

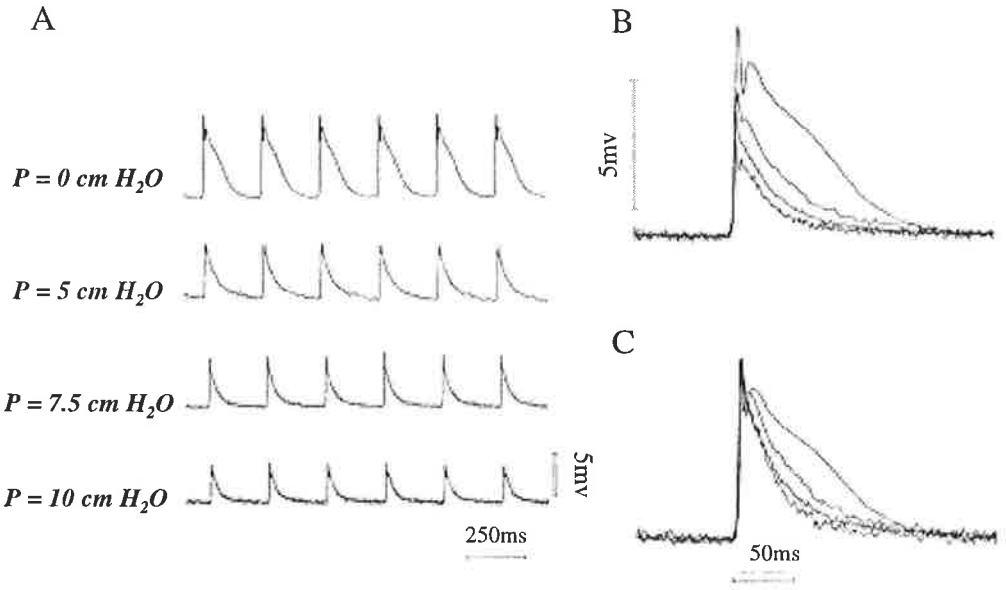


Figure 2: *Effects of stretch on monophasic action potential in the right atrium of rabbit.*

Monophasic action potentials (MAPs) recorded from the right atrium during increases in atrial pressure (Panel A). Panels B and C show MAPs at different pressures which are superimposed, both in absolute voltages (B) and after their amplitude was normalized (C). Rise in atrial pressure greatly altered MAP time course by shortening its duration and lowering its amplitude. P represents intra-atrial pressure. Diagram taken from Ravelli and Allessie, 1997.

1.2 STRETCH-ACTIVATED CHANNELS IN THE HEART

Electrical responses of the heart to mechanical stress can be observed at the cellular level, indicating that there must exist some stretch-sensitive mechanism in the cardiac cells themselves. Stretch-activated ion channels (SACs) were first detected in chick skeletal muscles by Guharay and Sachs in 1984 using single channel patch-clamp techniques with suction applied to the

recording pipette for mechanical stimulation. Open probabilities of these channels were found to increase with increasing amounts of suction. SACs have been proposed to be anchored to the cytoskeleton (Guharay and Sachs, 1984) and possibly to the extracellular matrix (Liu, et. al., 1996). The effects of stretch on the activity of SACs is demonstrated in Figure 3. The diagram shows single-channel recordings of mechanosensitive channels found in chick ventricular cells. The channels were activated when suction was applied to the patch pipette.

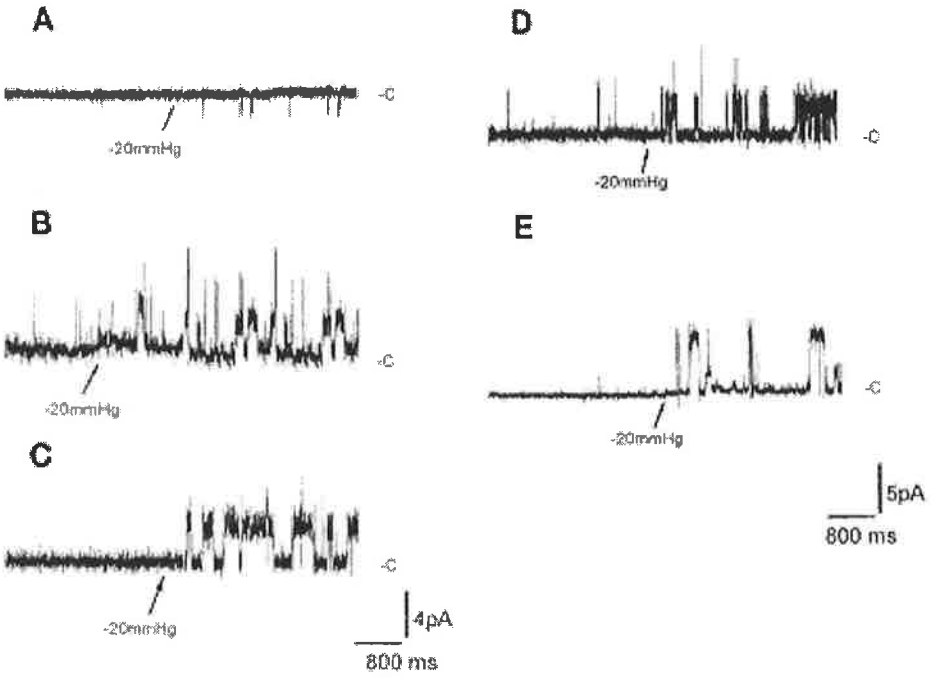


Figure 3: *Single-channel current recordings of 5 different types of SACs in chick ventricular myocytes.*

A: 25-pS nonselective SAC at 80 mV. -C, closed level of channel. B: 25-pS K^+ selective SAC at +120 mV. C: 50-pS K^+ selective SAC at +80 mV. D: 100-pS K^+ selective SAC at +40 mV. E: 200-pS K^+ selective SAC at +30 mV. All recordings shown were obtained from inside-out patches with symmetrical 145 mM KCl solutions except for the trace in A, where the pipette contained 145 mM NaCl and the bath contained 145 mM KCl. Application time of suction is shown by arrows at baselines. Diagram obtained from Kawakubo, et. al., 1999.

SACs refer to ion channels that are directly gated by mechanical stimulation and consequently, it is still not clear whether some volume-activated currents should be considered as SAC currents as they have been shown to be activated by secondary mechanisms such as changes in intracellular Ca^{2+}, ATP or cAMP (Hu and Sachs, 1997). For a while, swelling the cell by hypo-osmotic solution was thought to be an easy substitute for studying the effects of stretch on membrane currents. However, whole-cell currents activated by hypotonic swelling may not be the same as those activated by mechanical strain. For example, Sasaki, et. al., (1992) reported that in guinea-pig ventricular cells, cell-swelling increased the delayed rectifier current while application of direct stretch on the cell induced a time-independent non-selective cationic current. Axial stretch (stretch applied in one direction) caused an increase in the length of the cell whereas cell swelling did not (Roos, 1986). Therefore, this must correspond to different situations. Biaxial stretch occurs during ventricular filling while cell swelling would occur after ischeamia (Cazorla, et. al., 1999; Vandenberg, et. al., 1996).

It is thought that SACs have gates that are normally compressed, blocking the cytosolic side of the pore; but these gates can be distended by stretch, allowing ions to pass through more easily (Morris, 1990). Figure 4 shows how stretch may influence the gating of SACs.

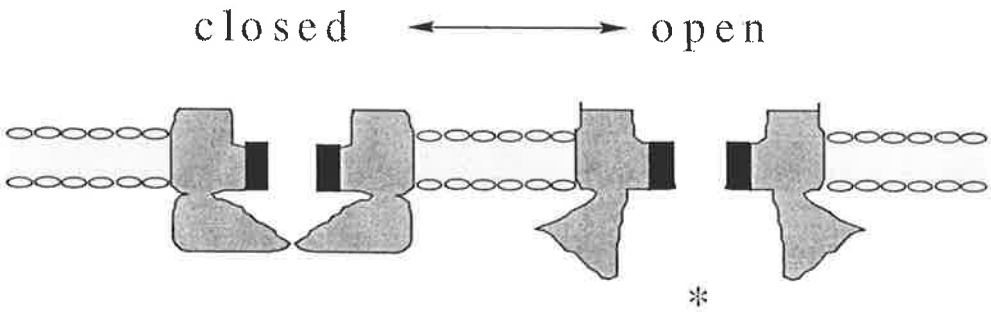


***Figure 4**: A proposed model of the activation of stretch-activated channels by mechanical stimuli.*
The gates of the stretch-activated channel are normally compressed in the absence of stretch, preventing ions from passing through the pore; but when the membrane is stretched, these gates can be distended, allowing ions to pass through more easily. The selectivity filter (black region) is unaffected by membrane tension. Diagram obtained from Morris, 1990.

1.2.1 Types of SACs found in the heart

So far, SACs found in the heart are all cation-selective. Most of these channels are weakly selective among monovalent cations and allow the passage of divalent cations like Ca^{2+} and Ba^{2+}. The other cardiac SACs are K^+ selective. More than one type of SAC have been found to be present in a certain tissue. For example, in cultured chick ventricular cells, five types of SACs have been detected (Ruknudin, et. al., 1993; Kawakubo, et. al., 1999). Table 1 shows a summary of the different types of SACs found in cardiac cells.

Cell type	Conductance (pS)	Reversal potential (mV)	Selectivity	Gadolinium block	Source
Chick ventricle (cultured)	25 25R* 50 100 200	-50 10 -3 -70 -40	CAT CAT CAT K^+ K^+	y y y y y	Ruknudin *et al.*, 1993
Chick ventricle	21 90	-2 -70	CAT K^+	y y	Hu & Sachs, 1996
Rat atrium	52	-	K_{ATP}	n	Van Wagoner, 1993
Rat atrium	64 –94†	-	K^+	y	Kim, 1992
Rat atrium	21	—	CAT	n	Kim, 1993
Rat atrium	36‡	—	CAT	—	Kim & Fu, 1993
Rat ventricle (cultured)	46	—	CAT	y	Sadoshima *et al.*, 1992
Rat ventricle (freshly-isolated)	120	$V_{resting}$+31	CAT	—	Craelius *et al.*, 1988
Guinea-pig	—	—	CAT	y	Bustamante *et al.*, 1991
Porcine (right atrium)	32(mono-) 13(di-valent)	-	CAT	-	Hoyer *et al.*, 1994
Mollusc ventricle	33	-70	K^+	—	Sigurdson *et al.*, 1987
Chick ventricle	25 50		K^+ K^+	–	Kawakubo, et. al., 1999

***Table 1**: Stretch activated ion channels found in the heart.*
*g, conductance (most values in the table were measured with 140 mM symmetrical K^+ saline; CAT, cation selective (weakly selective among monovalent cations and permeable to certain divalent cations); * inward rectifier channel; † the conductance refers to values at -60 mV and +60 mV respectively, ‡ channel activity induced by hypotonic swelling. Table was modified from Hu and Sachs, 1997.*

Potassium-selective SACs have different effects on cellular electrophysiology from the cation non-selective SACs. The reversal potentials for cation non-selective SACs is ranges between 0 and -50 mV (Craelius, et. al., 1988; Bustamante et. al., 1991; Ruknudin et. al., 1993). Thus, the cation non-selective SACs would induce depolarisation during diastole (Franz, et. al, 1992), while systolic stretch-induced activation will bring about repolarisation in the more positive plateau of the action potential and an ionic influx at the late section of the action potential (Kohl, et. al., 1999). The reversal potential

of potassium in the intact heart is around -90 mV (Kim, 1992) and thus opening of stretch-activated potassium channels should cause potassium ions to passively diffuse out of the cell. Activation of this channel during systole would be more effective in repolarising the cell while activation during diastole would have less impact on the resting membrane potential. Generally, stretch applied to the myocardium during diastole induces depolarisation (Zabel, et. al., 1996; Sasaki, et. al., 1992; Franz, et. al., 1992) whereas systolic stretch reduces the amplitude of the action potential plateau and either shortens the action potential duration (Dean and Lab, 1989b; Taggart, et. al., 1992; Hu and Sachs, 1997) or causes cross-over of the repolarisation curve ie. shortening of the early part of repolarisation and prolongation of its late section (Nilius and Boldt, 1980; Franz, 1995). The varying electrical effects observed could be due to the activation of different types of cardiac SACs. This report is mainly founded on TREK-1, a potassium-selective SAC found in cardiac cells. This channel will be discussed in the next section.

1.2.2 Theories on the involvement of SACs in the mechanics of the heart in physiological and pathological conditions

Mechano-electric feedback is thought to be important for the beat-to-beat regulation of the heart. The heart responds to increases in mechanical stress by increasing the force of contraction and heart rate (Kohl, et. al., 1999). These compensatory mechanisms enable the heart to cope with an increase in venous return. An increase in myocardial stretch occurs during physiological events such as inspiration. When breathing in, intrathoracic pressure decreases while

abdominal pressure increases (excursion of the chest and contraction of the diaphragm). This causes a change in the thoraco-abdominal pressure difference and consequently, there is an increase in venous return from the more pressurised lower part of the body towards the right atrium. Kohl, et. al., (1999) proposed that the raised right-atrial preload during inspiration promotes diastolic stretching of the thin-walled right atrium and sino-atrial node (SAN) causing a positive chronotropic effect known as respiratory sinus arrhythmia (RSA). SACs located within the sino-atrial node (SAN), are proposed to be involved in this stretch-induced response (Kohl, et. al., 1999). RSA generation would increase during exercise as there would be an augmentation in respiratory effort which would further increase venous return. RSA was found to be abolished or reduced when the SAN was mechanically fixed in the anaesthetised pig demonstrating that stretching the SAN is involved in RSA generation (Horner, et. al., 1996).

Lab (1999) proposed that a drop in venous return can also trigger an increase in cardiac contractility. Possible mechano-electric feedback mechanisms involving cardiac SACs during such an event are demonstrated in Figure 5. In the diagram, an action potential is shown to produce contraction in the cardiac muscle. If the next electrical event is associated with a reduction in cardiac load (eg. a drop in blood pressure), activation of SACs would be reduced as the myocardium is under less stretch. Consequently, the subsequent action potential is prolonged and this tends to increase the force of contraction over the next few beats, causing an increase in venous return.

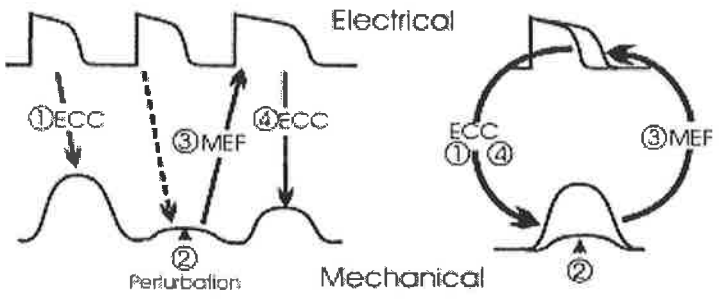


***Figure 5**: Beat-to-beat regulation by mechano-electric feedback.*

Panel A: top trace shows action potential and bottom trace shows the myocardial mechanical change. After the electrical event, normal excitation contraction coupling (solid and dashed arrows at (1) ECC) produces a myocardial mechanical change (bottom trace). Associated with the next ECC (dashed line), there is a perturbation at (2). In this illustration, it is a load reduction, e.g. drop in venous return or blood pressure. Consequently the ventricle is under less stretch and thus activation of SACs is decreased. This feeds back ((3) MEF) to prolong the action potential. This prolongation tends to increase the contraction force ((4) ECC). Panel B: Mechano-electric feedback loop. Diagram from Lab, 1999.

Another possible function of SACs is to prevent arrhythmias from occuring when the myocardium is contracting. During normal contraction of the intact ventricle, myocardial segments are reasonably synchronous in their electrical and mechanical activity. Different segments of the normal ventricle contract at about the same time. However, in cardiac pathology, these interactions can be seriously disrupted. In certain cardiac conditions, there is delay in conduction in some cardiac segments. Consequently, there is a delay in the firing of action potentials between a normal segment and a segment that has slower conduction. Fig. 6 illustrates an example of the possible regulation of action potentials in an intact heart. Segment (a), a normal segment, is stimulated first and contracts. Segment (b), which has delayed conduction and

therefore delayed mechanical activation is stretched. Stretch induces activation of SACs and thus APD in segment (b) is shortened. The shortening of action potential duration (APD) in segment (b) causes a reduction in the dispersion of repolarisation between the two segments. Dispersion in repolarisation is the difference in duration between the APD of one segment with the APD of the adjacent segment. However, if there is no mechano-electric feedback from SACs in segment (b), there will be a larger dispersion of repolarisation and this would increase the susceptibility of the heart to arrhythmias.

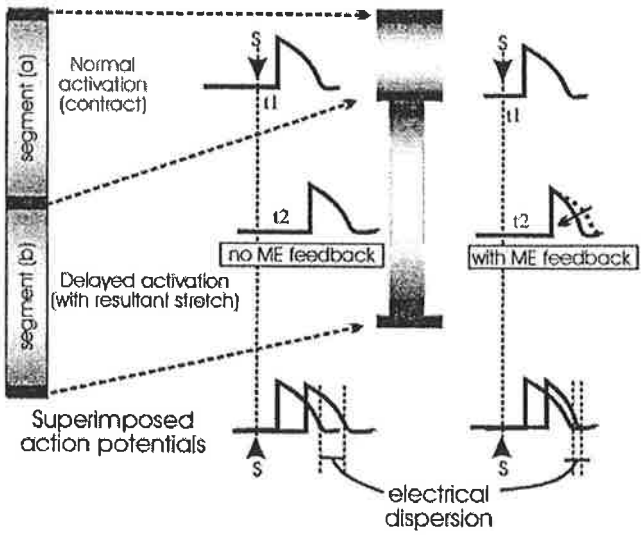


Figure 6: *Intact heart regulation involving mechano-electric feedback.*

Diagram shows action potentials of two segments in series during mechanical interaction, with one segment contracting abnormally. Segment (a) stimulated at (S), produces an action potential after a delay of t1 ms (top action potential). It contracts normally. Segment (b), for some reason, has a conduction delay (t2). This means a later action potential and later mechanical activation, so segment (a) stretches segment (b). Superimpositions of action potentials show the repolarisations of the action potentials with a wide time difference between them- large electric dispersion. If, however, the stretched segment involves mechano-electric feedback, stretched segment (b)'s action potential shortens, and this reduces the potentially dangerous electrical dispersion. Diagram obtained from Lab, 1999.

Besides having a benevolent role in the heart, mechano-sensitive channels also have the potential of generating hazardous cardiac rhythm disturbances in certain conditions. Several studies have shown that activation of SACs by passive stretch could generate electrical disturbances that would possibly cause arrhythmias to occur in the heart. For example, Akay and Craelius (1993) reported that activation of a small number of SACs in a patch can provide sufficient current to directly trigger an action potential in cardiac cells of neonatal rats. In freshly isolated chick ventricular cells, Hu and Sachs (1996) demonstrated that suction applied to the cell membrane through a pipette that contained potassium or sodium ions, stimulated cell beating in a dose-dependent manner. The effect of membrane stretch on beat rate could be emulated by the application of current to the pipette without applying suction. These results suggest that membrane stretch could induce current flow, which in turn affects the beat rate. Hence, acute mechanical stimulation (eg. sudden impacts to the chest) can change beat rate or trigger ectopic excitation which can induce cardiac arrhythmias, which in certain cases, can cause death (Kohl, et. al., 1999).

Sustained cardiac stretch is a common cause of fatality as cardiac tissues can become more susceptible to both the induction and the maintenance of disturbances in cardiac rhythm. There are many reports of sudden death in patients with regionally or globally impaired ventricular function (Taggart, 1996). This is thought to be related to regional stress forces. For example, in acute ischaemia, contractility in the ischaemic

segment of the ventricular wall is reduced. (Tyberg, et. al., 1974). During systole, when the heart contracts, blood is forced into the less contractile ischaemic region (Fig.6). As a result, this segment bulges out and is stretched. The segment that is most stretched is at the border between the normal and ischaemic zones.

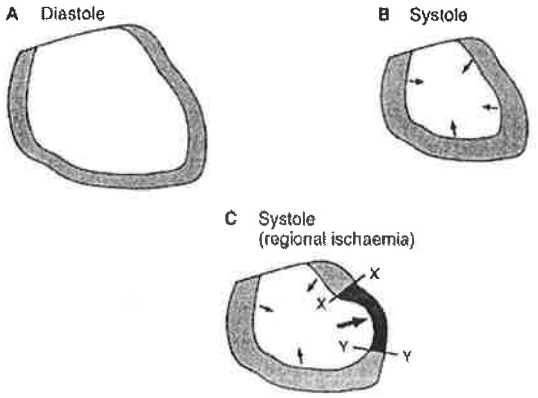


***Figure 7**: Illustration of left ventricular wall during diastole (A) and systole (B). In panel C, a region of acute ischaemia is present in the wall (between-X- and -Y) which moves outwards during systole (large arrow) while the remainder of the ventricular wall contracts normally inwards (small arrows). Diagram obtained from Taggart and Sutton (1999).*

In pathological conditions, where there is altered mechanical loading of the heart, triggered activity (Fig 8) and re-entry (Fig. 9) are the most likely causes for ventricular arrhythmias. If the myocardium is subjected to unphysiological amounts of stretch during the repolarisation phase of the action potential, depolarisations can occur. Triggered activities arise when the magnitude of these depolarisations are sufficient to induce premature action potentials. These activities may result in runs of tachycardia. Re-entry occurs when part of an activating wavefront is confronted with a non-conducting

barrier (eg: a necrotic region of the heart). As activation progresses, part of the progressing wave may pass around through unexcited regions which has been protected by the section of block to re-excite proximally (at region A in Fig. 9). The re-entry circuit may occur repeatedly creating ventricular tachycardia. Re-entry and triggered activity are facilitated by shortened action potential duration. The shorter the action potential duration, the earlier the subsequent action potential can be elicited. As SACs activation can be involved in the shortening of APD, a patient with chronic ventricular overload may be more susceptible to arryhthmias.

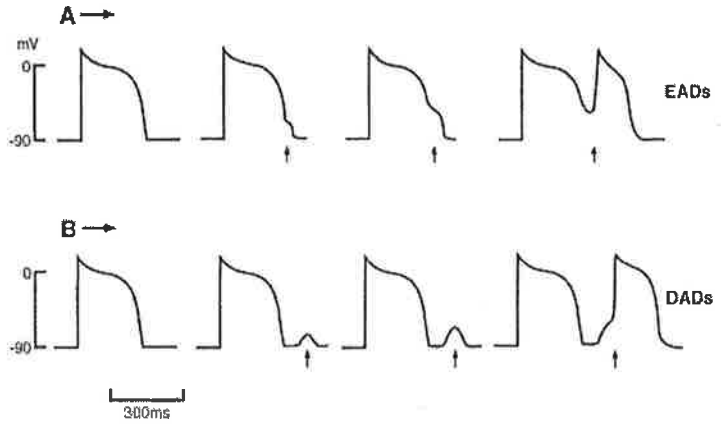


Figure 8: *Depolarisations and ectopic beats triggered by stretch. Diagram of ventricular action potential showing the development of a secondary depolarisation during the terminal part of phase 3 (early afterdepolarisation, EAD) (A) and following repolarisation (delayed afterdepolarisation, DAD) (B). Arrows indicate when stretch was applied. In each case when the amplitude of the EAD or DAD reaches threshold (fourth action potentials in each panel), it triggers a premature action potential. Diagram from Taggart and Sutton (1999).*

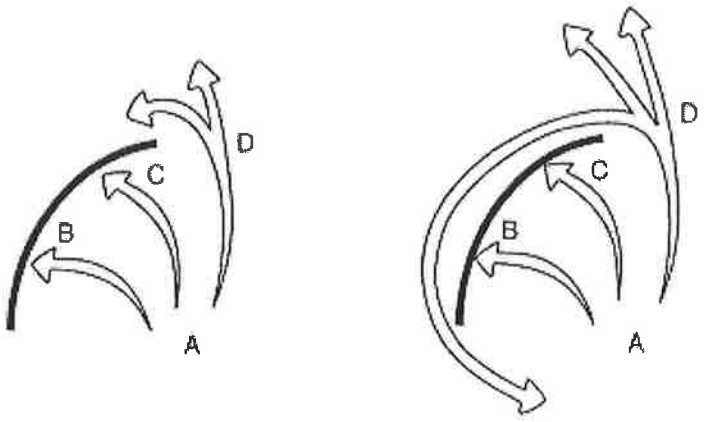


Figure 9: *The basis of a common form of re-entry.*
Left: an activating wavefront starts from point A and is blocked at point B and C when it encounters an area of inexcitability represented by the curved black line. However, at point D, it is able to pass the blocked region. Right: part of the wavefront travels round behind the line of block, which has been protected by the block, and returns to the starting point A, where it may re-excite. Diagram taken from Taggart and Sutton (1999).

1.2.3 Other possible functions of cardiac stretch-activated channels

In conjunction with being involved in the mechano-electric feedback of the heart, there is some evidence that SACs are involved in other regulatory roles. One possible function of these channels is that they might be involved in cardiac cell volume regulation. In ventricular myocytes of rabbits, gadolinium, a SAC blocker, caused a reduction in the amount of cell swelling in hypoosmotic solution (Suleymanian, et. al., 1995). Similarly, in isolated ventricular cells from a rabbit dilated cardiomyopathy model, Grammostola spatulata toxin, a selective cationic SACs blocker, produced a near complete block of the volume-sensitive cation-selective current (Suchyna, et. al., 2001). The effects of SACs blockers on cell swelling and swelling-activated cationic currents suggests that SACs might regulate cell volume by increasing the ability to transport ions. It is also possible that SACs have a protective role in

the preconditioning of the heart. Preconditioning refers to the fact that short periods of cardiac ischemia and reperfusion preceding prolonged ischemia reduces the amount of injury to cells. Preconditioning causes transient swelling of the heart. Ovize and co-workers (1994) found that stretching the heart by rapid volume overload did precondition the heart and that such protection was prevented by gadolinium.

Stretching atrial cells induces the secretion of atrial natriuretic peptide (ANP), a substance that controls blood pressure (Lang, et. al., 1985: Page, et. al., 1991; Ruskoaho, 1992). An increase in cytoplasmic calcium is necessary to evoke the release of ANP. During atrial stretch, activation of stretch-activated channels could be responsible for this increase in calcium influx as this stretch-induced ANP release can be inhibited by gadolinium (Laine, et. al., 1994). Other effects induced by cell stretching include programmed cell death and architectural rearrangement of myocytes (Cheng, et. al., 1995). Although the mechanisms for these phenomena are still unresolved at this stage, it is suggested that stretch-activated channels are involved in these processes.

1.2.4 Stretch-activated potassium channels in the heart

So far, there have only been reports of two types of K^+ selective SACs in mammalian cardiac myocytes. They are the TREK-1 channels (Maingret, et. al.,1999) and the ATP sensitive (K_{ATP}) channels (Van Wagoner and Russo, 1992). There are however some controversies concerning the activation of K_{ATP} channels by stretch. In a study performed by Kim (1992) using adult rat

atrial and ventricular myocytes, K_{ATP} channels were found to be insensitive to stretch. It was reported that in certain patches, suction caused slight activation of K_{ATP} channels but once the channels are activated, their activity was not further increased by negative pressure or reduced when pressure was released. Hence, instead of directly activating the channel, suction could have assisted the wash-out of ATP from the membrane inside the pipette and thus caused the opening of the channel.

1.3 TREK-1 CHANNELS

1.3.1 TREK-1, a member of the two-pore domain K^+ channel family

TREK-1 (TWIK-Related K^+) channels belong to a novel class of K^+ channels which are characterised by the presence of four transmembrane segments and two-pore domains (2P/4TMS) (Patel, et. al., 1998). The first member of this family to be cloned was TWIK-1. Up till now, there are 14 members found in this family of channels. Although members of this family share low sequence identity, they display the same structural motif. Figure 10 shows a simplified model of a 2P/4TMS channel subunit. Each subunit of this family of channels has an extended extracellular loop between the first transmembrane segment and the first pore domain and have both their carboxyl- and amino-termini inside the cell. The P1 domain is located between the hydrophobic M1 and M2 domains and P2 is found between M3 and M4 (Fig 10). This class of channels form dimers via a disulfide bridge (Lesage, et. al., 1996). A Cysteine residue is implicated in the formation of the

interchain disulfide bond. The extracellular M1P1 interdomain which is predicted to form an amphipathic alpha helix, promotes self-dimerization. The M1P1 interdomain might also bind regulatory factors or extracellular ligands that participate in the control of the activity of these channels (Lesage and Lazdunski, 2000). A dimer containing 4P domains is required to form K^+-selective pores. So far, there has not been any evidence of heteromultimerization for this family of channels (Patel and Honore, 2001). An analysis of residues in the pore domains of the 2P/4TMS channels indicates that there is a good conservation with the K^+-selective pore region of other cloned K^+ channels (Patel, et. al., 2001). The 2P domain K^+ channels are associated with unusual functional characteristics. Members of this family can be divided into five main functional classes: the weak inward rectifiers, the acid-sensitive outward rectifiers, the lipid-sensitive, mechano-gated K^+ channels, the halothane-inhibited 2P/4TMS channels and the alkaline-activated background K^+ channels.

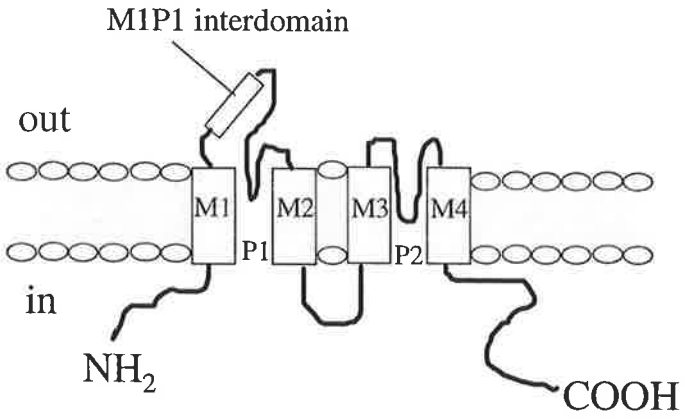


Figure 10: *Schematic representation of a 2P/4TMS subunit. M1-M4 are transmembrane domains while P1 and P2 represents the two pore domains. Both the amino and carboxyl termini of the subunit are present in the cytosol The extracellular M1P1 domain is important for self-dimerization. Modified diagram from Patel, Lazdunski and Honore, 2001.*

1.3.2 The functional characteristics of TREK-1

TREK-1 belongs to the lipid-sensitive mechano-gated category of 2P domain K^+ channels. They are outwardly rectifying channels with conductances of around 94 ± 11 pS at + 60 mV and 64 ± 8 pS at -60 mV (Kim, 1992). These channels are more active at depolarised potentials and they produce rapidly opening and closing currents that do not inactivate (Lesage and Lazdunski, 1998). The channel is activated by shear stress and negative pressure (suction) applied to the cell membrane. Positive pressure has the opposite effect on the channel. In the rat atria, the amount of pressure required to induce half-maximal activation of this channel is -12 mm Hg at 40 mV (Kim, 1992). The channel is more active under stretch when the cytoskeleton is disrupted (eg. by colchicine or excision of membrane patch (Patel, et. al., 2001)). This seems to indicate that the activity of the channel is repressed by

the cytoskeleton. This channel can also be activated by negatively charged polyunsaturated fatty acids such as arachidonic acid (Kim, 1992), eicosapentanoeic acid and docosahexaenoic acid (Patel, et. al., 2001). The observation that the TREK-1 channel can be activated by pressure or by fatty acids raises the possibility that fatty acids may also activate the channel by intercalating into the lipid bilayer and cause a distortion of the membrane which is analogous to that caused by stretch (Kim, 1992). Activation of mouse TREK-1 channels by fatty acids is observed when cyclo-oxygenase and lipoxygenase inhibitors were used, indicating that that this effect is independent of metabolism of the fatty acid (Patel, et. al., 2001). Agents that insert into one of the lipid layers of the membrane and consequently change the shape of the cell would alter the activity of the channel. Lipid bilayer crenators eg. polyunsaturated fatty acids and lysolipids cause activation of the channel while the cationic cup formers inhibit both basal and stimulated activities (Patel, et. al., 2001). However, it is also possible that there is a specific binding site for fatty acids on the channel protein itself. Mouse TREK-1 channels are more active at a low intracellular pH. Decreasing the bath pH for a ripped-off patch shifts the pressure-activation relation of TREK-1 towards positive values and can ultimately cause the channel to be activated even at atmospheric pressures (Maingret, et. al., 1999). TREK-1 channels can also be reversibly activated by heat. An increase in temperature of 10^0 C can raise the channel's activity by ~7 fold (Maingret, et. al., 2000).

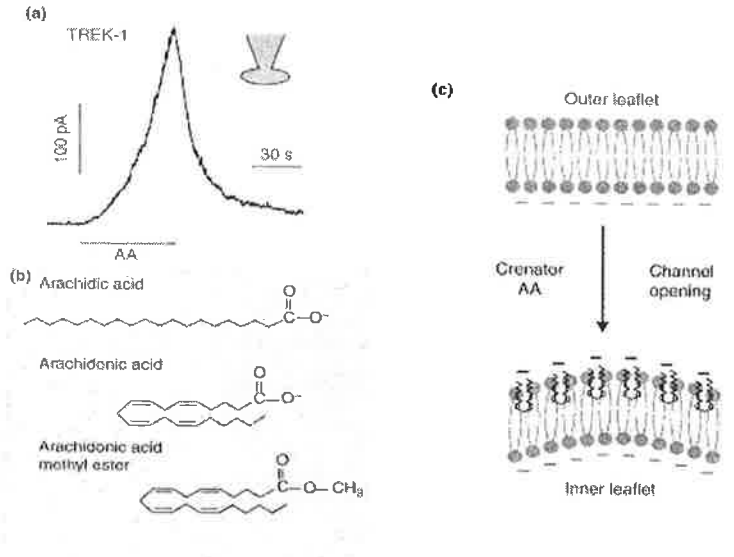


Figure 11: *Polyunsaturated fatty acids are potent openers of TREK-1 channels. Panel A shows TREK-1 whole-cell current recorded from a TREK-1 transfected cell at a holding potential of 0 mV. The current was induced by addition of 10 μM arachidonic acid (AA) to the extracellular medium. Panel B: Negatively charged polyunsaturated fatty acids (e.g. arachidonic acid), unlike neutral (arachidonic acid methyl ester) and saturated fatty acids (e.g. arachidic acid), open TREK channels. Panel C: Anionic amphipaths can enter from both sides of the bilayer. However, there are more negatively charged phosphatidylserines present in the inner leaflet of the plasma membrane, anionic lipids would preferentially insert in the external leaflet of the bilayer, causing a convex curvature of the membrane (crenation). Membrane crenation might be responsible for the opening of the mechano-gated 2P domain TREK channels by anionic PUFA. Positively charged amphipaths including chlorpromazine, on the contrary, will preferentially intercalate into the inner leaflet of the bilayer causing a concave curvature of the membrane and hence inhibit channel opening. Diagram taken from Patel, et. al., 2001.*

1.3.3 The pharmacology of TREK-1 channels

Like the non-selective cationic SACs, TREK-1 is reversibly inhibited by micromolar concentrations of gadolinium (Patel, et. al., 1998; Patel and Honore, 2001a; Lesage and Lazdunski, 2000). It has been recently found that certain local anaesthetics (eg. bupivacaine) inhibit TREK-1 channels (Kindler,

et. al., 1999) but inhalational anaesthetics (eg. chloroform and halothane), activate them (Patel, et. al., 1999). The human TREK-1 channel was markedly activated even at half the maximum concentration of volatile anesthetics used in human for general anesthesia, (chloroform, 0.79 mM, halothane, 0.21 mM, isoflurane, 0.31 mM) (Patel, et. al., 1999). Volatile anesthetics produce depressive side effects on heart function, including reduced heart rate and strength of contractions. These effects are both fully compatible with an increase in the activity of background K^+ channels by volatile anesthetics.

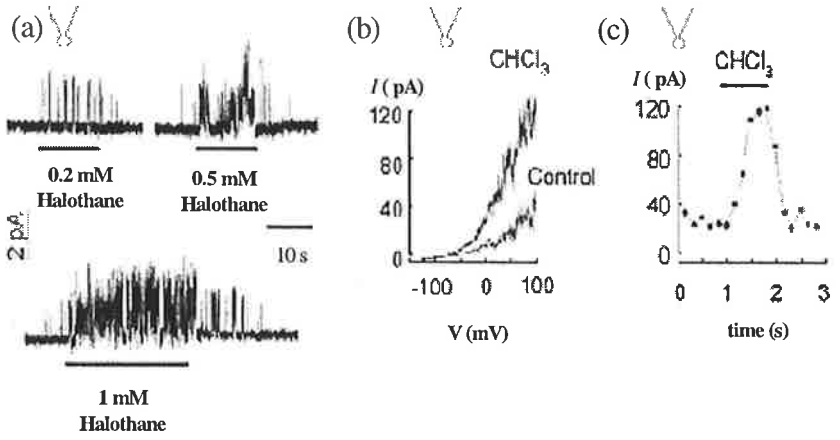


Figure 12: *Activation of TREK-1 channels by anesthetics.*
(a) TREK-1 channel activity in an outside-out patch increases with increasing concentrations of halothane in the bath. The holding potential is 0 mV, and applications of halothane are indicated by horizontal bars. (b) Effect of 0.8 mM chloroform on the I–V curve of TREK-1 in an outside-out patch obtained with a one-second voltage ramp from a holding potential of –80 mV. (c) Kinetics of activation of TREK-1 by 0.8 mM chloroform. Diagram obtained from Patel, et. al.,1999.

TREK-1 is inhibited by activators of PKC and PKA (Fink, et. al., 1996). Riluzole, a neuroprotective agent, inhibits the channel after transiently activating it (Duprat, et. al., 2000). The inhibitory process is attributed to a

rise of the intracellular cAMP concentration by riluzole that causes activation of protein kinase A. At present, an agent that can specifically inhibit this channel is still unavailable.

1.3.4 The carboxyl terminal is of critical importance to TREK-1

Many studies were performed to determine the regions which give TREK-1 its various characteristics. The studies often involved TASK, a member of the 2P/4TMS channel family which has high sequence homology with TREK-1 but is not mechano-sensitive and have very different properties from TREK-1 channels. The cytoplasmic N- and C-termini were focussed upon as they are the least conserved regions between TREK-1 and TASK (Fink, et. al., 1996; Duprat, et. al., 1997). Deletion and chimeric analyses demonstrated that the carboxyl terminal (but not the amino terminal) of the channel is of critical importance for the channel. Deletion in the carboxyl terminal impairs activation by stretch, anesthetics, polyunsaturated fatty acids, temperature and pH (Maingret, et. al., 1999; Patel and Honore, 2001b). The whole segment from Val-298 to Thr-368 in the carboxyl terminal is involved in volatile anesthetics, acidic, arachidonic acid and stretch modulation.

In the carboxyl terminal, the residue E306 is thought to act as an intracellular proton sensor. The replacement of this glutamic residue to either a hydrophobic or positively charged residue mimics intracellular acidification and led to activation of the TREK-1 channel at atmospheric pressures (Honore,

et. al., 2002). Protonation of E306 sensitised TREK-1 channels to stretch. This phenomenon could be caused by a conformational change of TREK-1 which could alter channel mechano-sensitivity (Honore, et. al., 2002; Hamill and Martinac, 2001).

A charge cluster, (**RVISKKTKEE**) was identified in the C-terminal region of TREK-1. It had been found to be critical for activation of the channel and resembles the charge cluster reported in the C-terminus of the mechanosensitive cationic large conductance E.coli MscL channel (**RKKEE**P) (Patel, et. al.,1998; Sukharev, et. al., 1994). It is therefore possible that similar molecular mechanisms involved in mechanosensitivity is conserved through evolution in different species and different types of stretch-sensitive channels. Thr 322 at the carboxy terminus was found to be important for anesthetic activation of TREK-1 as deletion of the residue supressed effects by chloroform and halothane (Patel and Honore, 2001).

1.3.5 Comparisons between human and mouse TREK-1 gene

The human TREK-1 gene is 41 amino acids longer at the carboxyl-terminus compared to its murine counterpart (Meadows, et. al., 2000). Figure 13 shows the gene sequence of hTREK-1. The distribution of TREK-1 in humans is found to be more specific than in the mouse. In the mouse, mRNA expression of TREK-1 was reported in many tissues, while in humans, TREK-

1 is found predominantly in the central nervous system (Meadows, et. al., 2000). Both the human and murine TREK-1 channels have similar biophysical and pharmacological properties. There are however, several differences in TREK-1 between the two species. Glibenclamide (10 μM) which has no effects on TREK-1 channel activity in rat atrial cells (Kim, 1992; Terrenoire, et. al., 2001), is found to block human TREK-1 currents by ~36% at 10 μM (Meadows, et al., 2000). hTREK-1 channels are, however, not as sensitive to glybenclamide as K_{ATP} channels (100 nM glybenclamide is able to antagonise > 90% K_{ATP} channels (Gribble, et. al., 1997)). mTREK-1 is also more sensitive to quinidine compared to hTREK-1. 1mM Quinidine, which completely blocks mTREK-1 did not produce a complete block in human TREK-1 (Meadows, et. al., 2000).

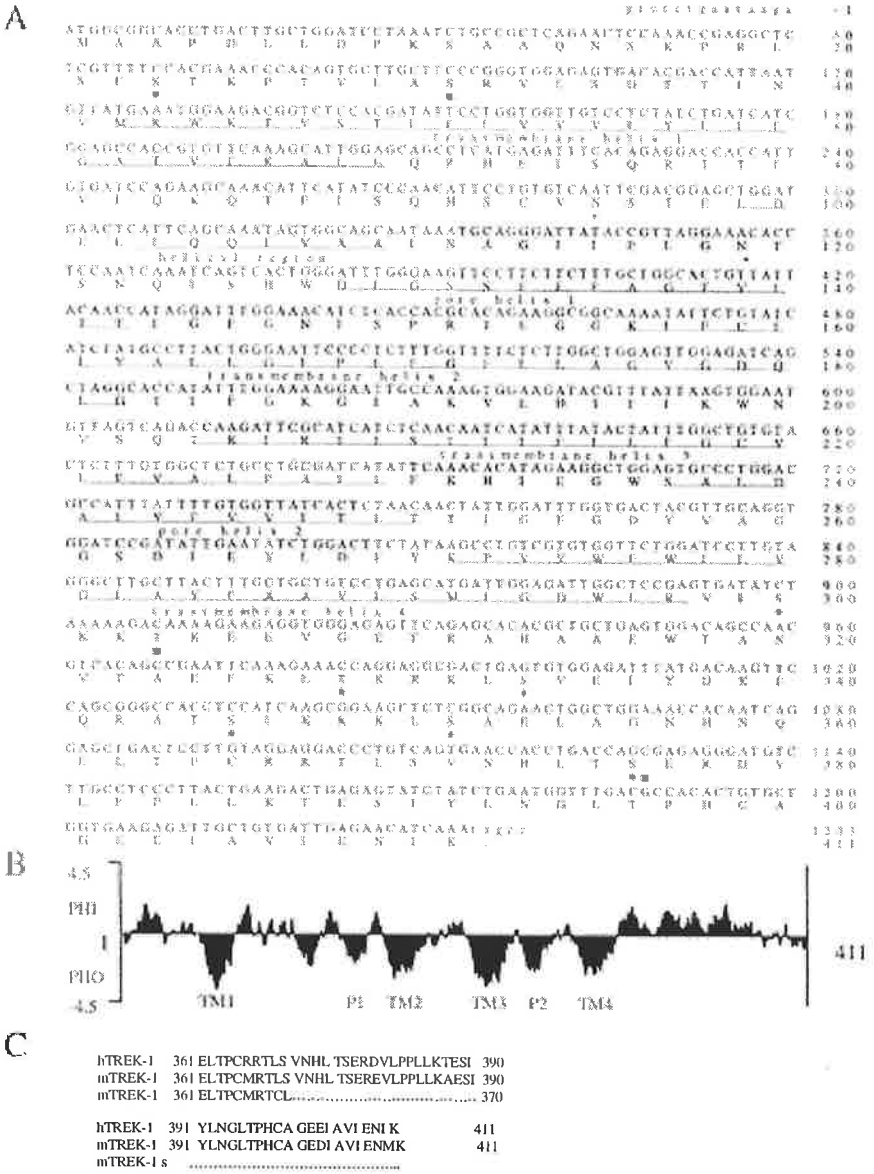


Fig. 13 *Nucleotide and amino acid sequence of hTREK-1.*
The underlined sections show predicted four transmembrane helical regions and two pore helical segments within the gene. An additional, short extracellular, helical region is predicted to exist between amino acids 99 and 109 Consensus sites for phosphorylation by protein kinase C (l), protein kinase A (u) casein kinase II (n) and potential glycosylation sites (V)s are demonstrated. Panel B: Hydropathy analysis for hTREK-1. The plot is based on the Kyte-Doolittle method, using a window size of 11 amino acids, plotted against amino acid position 1–411. Hydrophilic parts of the channel are indicated by positive values and hydrophobic sections by negative values. Panel C: Comparison of C-terminal amino acid data, starting at amino acid 361. hTREK-1, mTREK-1 (in-house data (Meadows, et. al., 2000)) and mTREK-1s (published data). The full-length gene of mouse and human TREK-1 show 96% similarity at the amino acid level. Diagram obtained from Meadows, et. al., 2000.

1.3.6 Possible roles of TREK-1 in the physiology and pathophysiology of the heart.

TREK-1 is a channel which responds to many different stimuli. It is therefore possible that this channel has several regulatory roles in the heart. As TREK-1 channels are stretch-sensitive, they are likely to participate in cardiac mechano-electric feedback. It is possible that these channels may play a role in the beat-to-beat regulation of the heart. TREK-1 could also be involved with the regulation of cellular volume as the TREK-1 current was found to be decreased when the osmolarity of the external solution was increased (Maingret, et. al., 2000; Patel, et. al., 1998). TREK-1 channels are only slightly voltage sensitive and can open at any membrane potential. Thus, they might function as background potassium channels, stabilising the resting membrane potential and shaping the action potential. TREK-1 channels could protect the cell from exaggerated Ca^{2+} entry (Maingret, et. al., 1999). Such an event is hazardous for the cell. Activity of TREK-1 channels induces hyperpolarisation and hence puts the cell's membrane potential further away from the threshold of voltage-sensitive Ca^{2+} channel activation.

During pathological conditions of the heart such as ischemia, where cell swelling, an elevation in free cytosolic arachidonic acid and a decrease in intracellular pH occur (Kim and Duff, 1990; Sparks, et. al., 1984), it would be expected that TREK-1 channels are highly active since mechanical activation of TREK-1 is enhanced by intracellular acidosis and arachidonic acid (Kim,

1992). There is a major K^+ efflux during cardiac ischemia and this efflux is thought to pass through K^+ channels that open under these conditions (Weiss and Venkatesh, 1993; Lazdunski, 1994). This efflux was not eliminated by specific blockers of calcium activated potassium channels and K_{ATP} channels. Thus, the opening of TREK-1 channels may be a major cause of this efflux. Whether such K^+ efflux during ischemia is beneficial or detrimental for the heart cells is still unresolved at this stage.

Non-selective SACs induce a depolarising current when activated during diastole while the TREK-1 current is hyperpolarising. Half-maximal activation of these cationic non-selective channels occur at 1.5mm Hg whereas for TREK-1, it occurs at a higher level of stretch, ie. around 12 mmHg (Terrenoire, et. al., 2001; Kim, 1992), suggesting that TREK-1 activation will occur secondary to the activation of these cationic non-selective SACs (Terrenoire, et. al., 2001). Hence, TREK-1 channels could function as a negative feedback to the stretch-activated cationic non-selective channels. In fact, Nakagawa and colleagues (1988) reported that in isolated ventricular papillary muscles of guinea-pigs, mild to moderate stretch depolarised the resting potential but severe stretch at 130-140% of L_{max} (ie. the length at which a cardiac muscle produces maximum contractile tension), caused hyperpolarisation instead of depolarisation during diastole. This unusual observance could be accounted for by the dominance of potassium selective SACs when ventricular muscle cells experience high levels of stretch. In the atria, stretch causes an increase in intracellular calcium and this effect induces the secretion of natriuretic peptide (ANP) (Lang, et. al., 1985; Lange, et. al.,

1966). It is thought that non-selective cationic SACs are involved in this process (Laine, et. al., 1994). TREK-1 might act as a negative feedback to ANP release by shortening the action potential duration and therefore shortening the period of calcium influx and release from the sarcoplasmic reticulum which occurs during the depolarisation phase of an action potential (Terrenoire, et. al., 2001).

1.4 AIMS OF THESIS:

The aims of this study are to characterise a stretch-activated channel present in rat ventricular myocytes and to determine the distribution of these channels in different regions of the heart. Single-channel patch-clamp techniques were employed to observe the properties of the channel. Firstly, the ionic selectivity and conductance of the channel were determined. Then, experiments were performed to investigate the other physiological and pharmacological properties of the channel. From the channel's characteristics, the type of channel under investigation was determined. Next, the genetic expression of the channel was investigated in the different chambers of the heart. To observe if there was a differential expression of this gene across the left ventricular wall, the mRNA expression in the epicardial as well as in the endocardial cells were quantitated. Finally, whole-cell currents for the channel in epi- and endocardial cells of the left ventricles were compared to determine whether there was any difference in the population of the channels.

2: TREK-1-LIKE CHANNELS IN RAT VENTRICULAR MYOCYTES

ABSTRACT:

A large conductance (c. 111 ± 2.7 pS in symmetrical 150 mM K^+) potassium-selective channel was recorded using patch-clamp techniques in both cell-attached and inside-out membrane patches from acutely isolated adult rat ventricular myocytes. In inside-out patches, the channel was not blocked by 4-aminopyridine (5 mM), TEA (5 mM) or glybenclamide (100 nM) in the bath solution. In cell-attached or ripped-off patches, stretching the membrane by applying hydrostatic pressure to the pipette, increased the open probability (P_o) of the channel (typically, P_o ~ 0.005 with no pressure;~0.328 with 90 cm. H_2O; V_m = 40 mv, pH_i = 7.2). The channel is also sensitive to intracellular pH. Decreasing the pH to 5.5 from 7.2 increased P_o to 0.16 from approximately 0.01 (with no hydrostatic pressure applied and V_m held at 40 mV). The channel was slightly voltage-dependent as depolarisation of the membrane increased the channel's open probability by approximately e-fold for 97 mV (pH_i = 7.2). The properties of this channel are similar to those reported in TREK-1 channels. Results of RT- PCR experiments showed that there is expression of TREK-1 in the rat heart further confirming that the channel under observation is the TREK-1 channel. A property of this channel that has not been reported till now is its activation by intracellular ATP. Channel activation by ATP was more profound at a lower pH eg. using a bath

solution of pH 6, at a membrane potential of 40 mV, 3 mM ATP increased P_o from 0.048 ± 0.023 to 0.348 ± 0.13 while at pH 7, no increase in channel activity was observed. The fact that the channel was activated rapidly (within seconds) and activation of the channel occurred in ripped-off membrane patches suggest that activation by ATP did not involve phosphorylation. Thus, it appears that intracellular ATP might directly activate the channel.

2. 1 INTRODUCTION

Mechanical stress can cause alterations in the electrophysiological properties of the heart. This phenomenon is known as mechanoelectric feedback (MEF). Stretch-activated channels (SACs) are thought to be involved in MEF. All cardiac SACs are cationic (Hu and Sachs, 1997). These SACs can be categorised into two groups: the non-selective cationic SACs which are permeable to K^+, Na^+ and Ca^{2+} ions and the potassium-selective SACs. Activation of cationic non-selective SACs are thought to depolarise the membrane potential while the potassium-selective SACs would induce a hyperpolarising current. There have been many reports of different types of cationic non-selective SACs in the heart but so far there has not been many reports of cardiac potassium-selective SACs. The main ones that have been reported so far are K_{ATP} channels in rat atria (Van Wagoner, et. al., 1992), BK_{ATP} channels in neonatal chick ventricles (Kawakubo, et al., 1999) and TREK-1 channels in rat atria (Terrenoire, et. al, 2001).

This chapter describes the properties of a stretch-activated potassium selective channel that was observed in rat ventricular myocytes. Patch-clamp techniques were employed to determine the characteristics of these channels. It appears that the channel had properties of TREK-1, a member of the newly cloned family of tandem-pore channels (Fink, et. al., 1996). RT-PCR results showed that there was high expression of this channel in both the atria and ventricles of rats. In this study, it was also found that the channel is activated by intracellular ATP, a property that has not been previously ascribed in TREK-1 channels.

2.2 METHODS

Animals used in these studies were cared for according to the National Health and Medical Council *Guidelines for the Care and Use of Animals.* The experimental procedures were subject to the approval of the University of Adelaide Animal Ethics Committee.

2.2.1 Patch-clamp experiments:

(a) Isolation of cardiac myocytes:

Excising and cannulating the heart:

Sprague-Dawley rats weighing 300 - 450 grams were injected intraperitoneally with 2000 units of heparin and 0.5 mls of pentobarbitone. Approximately 20 minutes later, the rats were killed by decapitation. The chest of the rat was opened quickly and the arch of the aorta was freed from its

surrounding tissues. The aorta was tied as far from the heart as possible. The heart was then removed and placed in a container of Calcium-free Tyrode's solution at 0 - 4^0C. Tyrode's solution contained (mM): NaCl, 133.5; KCl, 4; NaH_2PO_4, 1.2; Mg Cl_2, 1.2; HEPES, 10; D-glucose, 11; pH was adjusted to 7.4 using NaOH. A gentle squeeze was given to the heart to expel the blood from the atria, ventricles and blood vessels. For the insertion of the cannula, a cut was made at the junction of the right subclavian artery and the ascending arm of the aorta.

Digestion of the heart

After cannulation, the heart was perfused with Tyrode's solution containing 1 mM calcium for around 4 minutes. The high calcium concentration in the solution caused the heart to beat vigorously, expelling blood from the coronary arteries. The heart was then perfused with Ca-free Tyrode's solution for about 5 minutes to wash out the calcium before introducing the enzymatic perfusate. The glycocalyx of the cells would be damaged if the heart was perfused with Ca-free Tyrode's for more than 5 minutes. The calcium concentration during digestion was reduced to approximately 25 μM, as a high concentration of calcium can cause the isolated cells to be over-digested. The digestive perfusate contained collagenase (83.3 μg/ml Yakult), protease (41.6 μg/ml, Sigma), 2, 3 butane-dione-monoxime (2.5 mg/ml, Sigma) and bovine serum albumin (0.83 mg/ml, Sigma).

Harvesting and storing of cells

After around 30 - 40 minutes of enzymatic digestion, the heart became soft, and enlarged. Sections of the left and right ventricles were cut out into petri dishes containing Tyrode's solution with 25 μM Ca^{2+}, normally at 5 minutes intervals. The pieces of heart tissues were then triturated using a plastic transfer pipette to release the cells. The resultant cell suspension was sieved through a 80 micron nylon mesh into a 50 ml centrifuge tube. Calcium concentration in the solution bathing the cells was increased 10 minutes later to 200 μM by adding the required amount of 1M $CaCl_2$ solution to the side of the tube and gently rotating the capped tube to ensure an even concentration of Ca^{2+} throughout the suspension. 30 minutes later, the concentration of calcium in the tube was raised to 1 mM. The presence of calcium is required for the healing of sarcolemmal tears that occur during digestion. However, if cells are exposed to high concentrations of calcium directly after being harvested, they can hypercontract and shrivel up as calcium enters the cells before healing is complete. Thus, the concentration of calcium was increased progressively to allow time for the healing of damaged membranes.

(b) Single-channel recording

Single-channel recordings were performed at room temperature of 21 ± 2 ^{0}C. In these experiments, the cell-attached and inside-out configurations were used. A cell-attached patch was obtained once the recording pipette had sealed to the membrane of the cell. Inside-out patches were formed by withdrawing the pipette from the cell after a seal had formed. The membrane patch was ripped from the cell but the seal remained intact. Using this

method, the intracellular surface of the membrane was exposed to the bath solution. Therefore, the effects of changing the intracellular environment of the cell (ie. by changing the solutions in the bath) on any particular channel could be easily observed. Unlike whole-cell and cell-attached configurations, the inside-out configuration requires cells that are attached to the coverslips, for if not, it would be difficult to rip the membrane patch from the cell. Polyl-L-lysine hydrobromide (Sigma) or laminin (Sigma) coated on coverslips improved the attachment of cells.

The fabrication of pipettes

For single channel recording, patch pipettes were made from thick walled borosilicate glass capillaries (Clark Electromedical Instruments GC150F-15). Electrodes were prepared from borosilicate glass using a two-stage puller (Narishige Scientific Instruments, Tokyo, Japan). The resistances of the pipettes were between 1-5 MOhms when containing the pipette solution. The tip of the pipette was then polished by bringing it to within 10-20 μm of a heated filament for around 8 seconds. To reduce the pipette-bath capacitance, pipette shanks were coated to within 50 μm from the tip using Sylgard (184 silicone elastomer).

Formation of tight seals

The pipette was filled with pipette solution and all bubbles were removed from the solution by gently tapping the pipette. The pipette was then mounted onto an electrode holder (Narishige, Scientific Instruments, Tokyo, Japan). The solution in the pipette was in contact with a silver chlorided wire

which was connected to an amplifier (Axopatch 200A). Using a stable micromanipulator (Narishige, Scientific Instruments, Tokyo, Japan), the microelectrode was moved towards the cell. The electrode was then lowered towards the cell until it touched the membrane of the cell gently. Suction was then applied onto the patch of membrane to obtain a gigaseal. Gigaseals were easier to obtain if the osmolarity of the solution within the pipette and that of the bath were similar to each other. Flowing bath solution also sometimes aided in the formation of gigaseals.

Recording and analysing of currents

Current recordings were performed using an Axopatch 200A amplifier (Axon Instruments). Channel currents were filtered at 2 KHz and recorded (Axotape - Axon Instruments) via a 12 bit analogue to digital converter operating at 5 KHz. Leakage currents which were produced by incomplete sealing between the micropipette and the cell membrane were compensated using controls on the amplifier. A program called Axotape (Axon Instruments) recorded single channel currents. Single channel open probability, amplitude and burst length were analysed using a software program, Channel 2 (written by M. Smith, John Curtin School of Medical Research).

Suction was applied to the patch pipette using a glass syringe to observe the response of the channel to stretch. The pressure difference was recorded with an electronic pressure gauge (WPI model PM015D, Sarasota, FL), calibrated against a water manometer.

Where rapid switching of the bath solution was required (for example when investigating the time course of the effect of ATP), a rapid-switching perfusion apparatus was employed. In this design, the solutions converged in a manifold machined into a plexiglass rod, with the outflow being through a stainless steel needle placed within a few millimeters of the patch. The dead space of the apparatus was of the order of 100 µl, and other experiments have shown solution-switching times at the membrane of 100-200 milliseconds. Open probability was calculated from the channel records using a threshold-crossing algorithm (threshold generally at 50% of channel amplitude) and summing open (above threshold) and closed (below threshold) times.

(c) Solutions and drugs

For most of the single channel recordings, the cells were superperfused with a bath solution containing (mM): K-aspartate, 140; EGTA, 10; $MgCl_2$, 2; TES, 10; pH adjusted to 7.4 ± 0.5 with KOH. This solution was designed so that the membrane potential of the cells is effectively zero, and hence the potential across the patch in cell attached configuration is known. To test the pH sensitivity of the channels, the pH of the bath solution was lowered to 6.5, 5.5, and 4.5. In some experiments, 2 mM $MgCl_2$ was added to the pipette solution, bath solution or both to observe the effects of Mg^{2+} on the current-voltage curve. The activation of the channel by Ca^{2+} was also tested. To test the pharmacology of the channel, the bath solution was also supplemented with either penitrem A (100 or 300 µM), tetraethylammonium chloride (TEA)

(5 mM), 4-aminopyridine (5 mM), ATP (1 or 3 mM), or glybenclamide (100 nM). The pipette solution contained either KCl or NaCl at concentrations between 150 and 280 mM; TES 10 mM; pH adjusted to 7.4 ± 0.05 with KOH or NaOH as appropriate.

2.2.2 Molecular Biology Techniques:

(these experiments were performed in conjunction with Weihong Liu)

(a)Total RNA extraction and reverse transcription reaction

Six rats were killed by exsanguination under CO_2 anaesthesia. Enzymatic dissociation of cardiac myocytes was performed using the Langendorff perfusion technique as described in section 2.2.1a of this thesis. The left and right ventricles as well as the left and right atria were then excised and collected in separate plastic tubes. Trituration was performed on the tissues to release the myocytes. The tubes were then centrifuged for one minute. The solution in the tubes were discarded and the cells were snap frozen in liquid nitrogen and stored at -80°C until RNA extraction. As RNase activity is hard to inhibit, precautions were taken to prevent RNase contamination throughout the whole sample handling procedure. RNase could come from molds and bacteria from the skin or from contaminated laboratory ware. Disposable gloves and sterile, disposable plasticware and automatic pipettes were used for RNA work. For RNA extraction, TRIzol Reagent (Life Technologies, Inc, Frederick, Maryland, USA), was first added to the sample to protect the RNA as well as to disrupt cells and dissolve cellular components. The tissues were then homogenised, placed into labelled

Eppendorf tubes and centrifuged for 10 minutes. The supernatant was extracted and incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Chloroform was then added and the mixture was incubated for 2 - 3 minutes before being centrifuged for 15 minutes. The colourless aqueous phase containing exclusively the RNA was transferred into fresh Eppendorf tubes. RNA from the aqueous phase was precipitated by incubating the mixture with isopropyl alcohol for 20 minutes at room temperature. The RNA precipitate after centrifugation formed a gel-like pellet on the side and bottom of the Eppendorf tube. The solution was then removed and the pellet was washed with 1 ml 75% ethanol. The pellet was left to dry for ~5 minutes after removal of ethanol. The pellet was dissolved completely using ~60 µl DEPC-treated water (ie. water added with 0.01% diethylpyrocarbonate to ensure that it is RNase-free). The solution containing the RNA was further diluted. Using a photometer, the concentration of RNA was determined. The RNA was then treated with DNase (Life Technologies, Inc, Frederick, Maryland, USA) and reverse-transcribed. The substrates for this reaction were: 5 µl 10x reverse transcriptase, 11 µl 25 $MgCl_2$, 10µl deoxyribonuclease triphosphates, 2.5 µl random hexamer primers, 1 µl RNase inhibitors, 1.25 µl reverse transcriptase enzymes (PE Applied Biosystems, Foster City, CA) and 4 µg RNA.

(b) TREK-1 genes and PCR

PCR was performed for *TREK-1* (genebank accession number U73488) and *GAPDH* (genebank accession number M32599) using cDNAs corresponding to 200 ng total RNA synthesised from left and right atria as well as the left and right ventricles. The gene specific primers were: *TREK-1*, forward 5'TTTGGCTTTCTACTGGCTGGGG3', reverse 5'TCGTCTTCTTA GAGATCACCG3';*GAPDH*, forward 5' ATGTTCCAGTATGACTCCAC TCA CG 3', reverse 5' GAAGACACCAGTAGACTCCACGACA 3'. These primers had been used by other groups to amplify these genes (Aimond, et. al., 2000; Schoenfeld, et. al., 1998). All primers were purchased from GeneSet (GeneSet Pacific Pty. Ltd, Australia). A touch-down PCR protocol was applied, 94 °C, 45 s, 20 cycles, 65 to 55 °C (-0.5 °C per cycle) for 45 s and 72 °C for 90 s followed by 20 cycles at annealing temperature of 55 °C, and a final extension step for 8 minutes at 72 °C.

2.3 RESULTS:

2.3.1 Basic observations:

Figure 1 shows the basic observations that were made of these channels. In panel A, using cell-attached patches, recordings of the channel could be made at positive membrane potentials but not at negative potentials when NaCl was used in the pipette. When KCl pipette solution was used, channel openings could be observed at both positive and negative membrane

potentials. The channels were sensitive to stretch. Panel C shows recordings of the channel in a cell-attached patch at a membrane potential of 60 mV. The channel was activated by 90 cm H_2O stretch applied to the membrane by suction applied to the back of the pipette. The open probability of the channel in this patch was 0.01 when no stretch was applied and 0.3 when 90 cm H_2O stretch was applied. The current-voltage relationship of this channel in a cell attached patch is shown in panel D. The pipette solution used was 140 mM KCl and the bath solution was 140 mM K.Aspartate. The channel could be observed quite frequently, in around 20% of patches where a giga-Ohm seal was obtained.

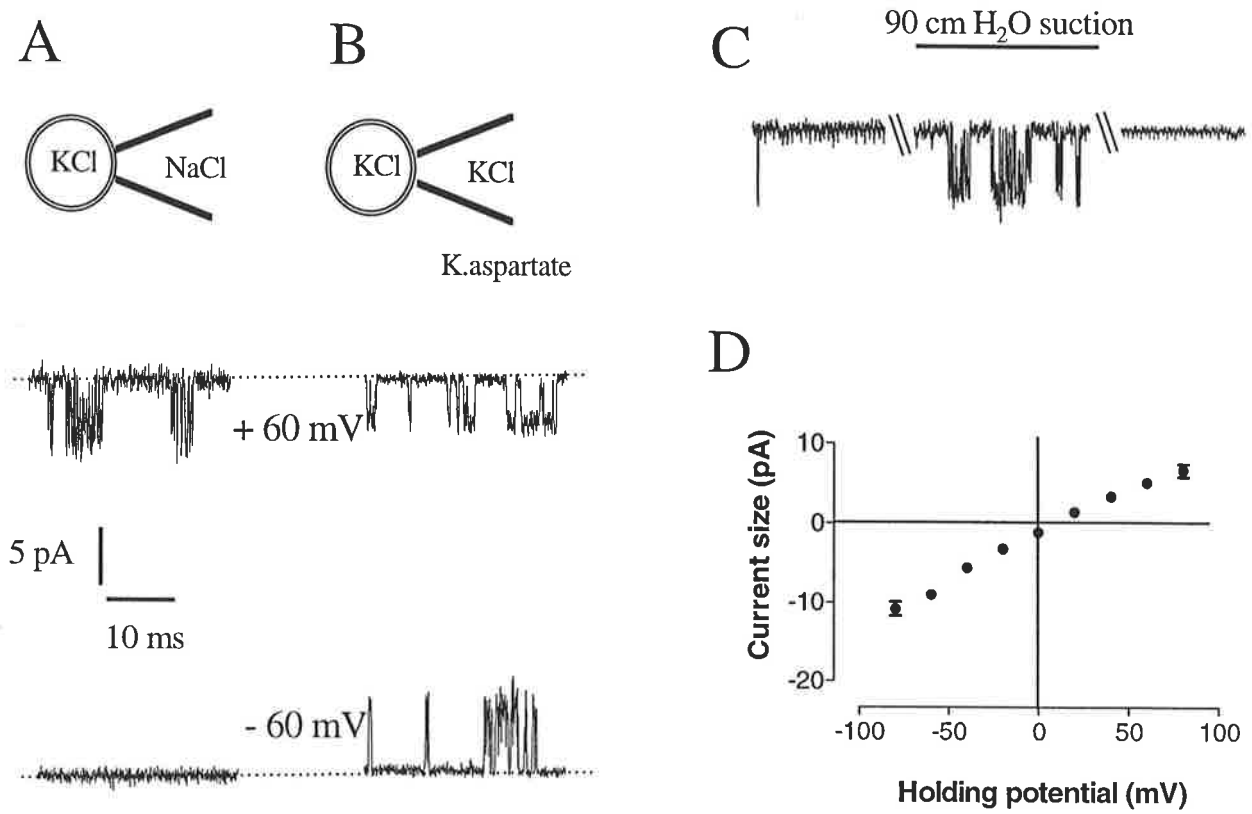


Figure 1: *The basic observations.*
Panel A: examples of channel currents recorded in a cell attached patch with 150 mM NaCl in the recording pipette. At a membrane potential of +60 mV (pipette -60 mV), when slight suction was applied to the membrane, large flickery channel openings could be seen. At a membrane potential of -60 mV, no channel openings could be recorded. When the recording pipette contained 150 mM KCl, channel openings activated by application of suction could be recorded at both positive and negative membrane potentials (panel B). The bath solution contained 150 mM K.aspartate, with other components as detailed in the methods. Panel C: an example of the response of the channel to applied suction (90 cm.H_2O), recorded in a cell attached patch at a constant membrane potential of 60 mV. Panel D: current voltage relation for the stretch activated channel recorded in cell attached patches, with 150 mM KCl in the pipette and the bath solution as given in the methods (membrane potential of the cells should be close to zero). Data points show mean ± SEM (n=7).

2.3.2 Stretch activation

Figure 2 again shows the activation of the channel by stretch, this time, in a ripped-off patch. The channel was subjected to 30, 60 , 90 and 120 cm. H_2O (ie. 2.2, 4.4, 6.7 and 8.9 mm Hg) negative pressure to observe the response of the channel to different amounts of stretch. Panel 2A shows open probability of the channel increasing in a "stretch-dependent" manner. In Panel 2B no change in mean open time was observed with the different amounts of applied suction (definition of mean open time is given in section 2.3.5). There was no change in the size of channels with suction as shown by the amplitude histograms in Panel 2C. The channel was activated in less than a second when stretch was applied.

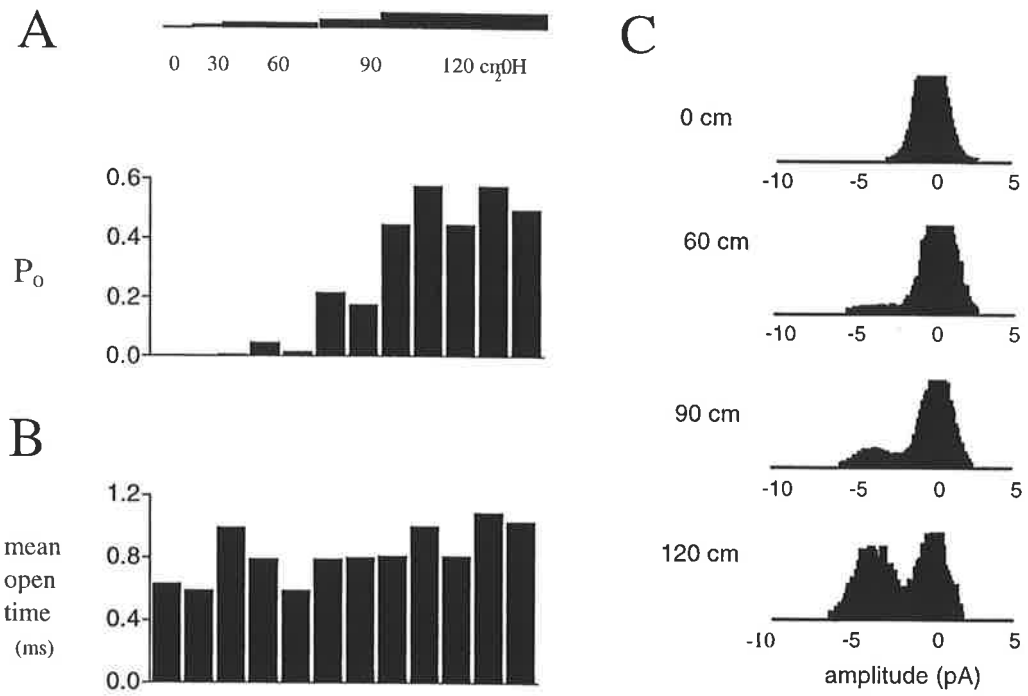
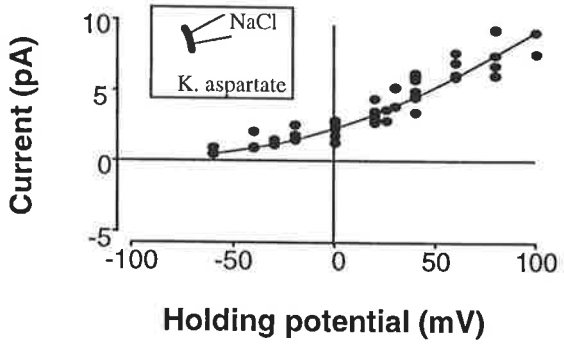


Figure 2: *Response of the channel to hydrostatic pressure.*
Channels were recorded at a constant membrane potential of 60 mV in an isolated membrane patch with 150 mM KCl in the pipette and 150 mM K.aspartate in the bath (pH 7.2). Suction was applied to the pipette side of the membrane and the open probability was measured. As the suction was increased from 0 to 120 cm.H_2O, as depicted in the top bar, open probability increased from near zero to almost 0.6. The time course over which the suction was increased was about 2 minutes, so that each pause at the different suction levels lasted 20 to 30 seconds. The suction at 120 cm.H_2O was held longer, to see if channel activity would "accommodate" to the suction, (ie fall with time). Such accommodation, or inactivation, was not seen in any patch, even for quite lengthy applications of suction. The increase in open probability was primarily due to an increase in frequency of opening, since mean channel open time did not change appreciably (panel B). The amplitude of the channel current did not change appreciably as the suction was increased, as shown by the amplitude histograms constructed from the recordings (panel C).

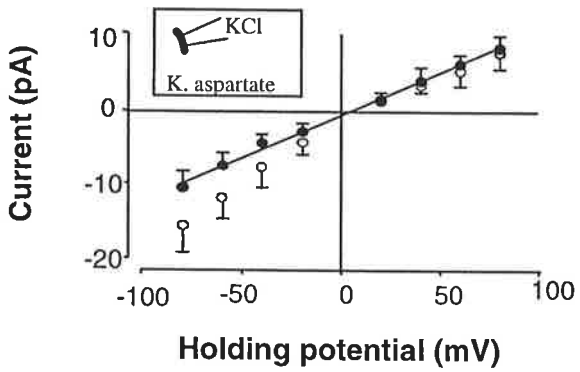
2.3.3 Ionic selectivity and conductance

The current-voltage relation derived from recordings of the channel in ripped-off patches showed extreme rectification when 150 mM NaCl was used in the pipette and 150 mM K.aspartate was used in the solution superfusing the cells (Figure 3A). Recordings made using the same bath solution and 150 mM KCl in the pipette (instead of NaCl), showed a linear current-voltage relation with a slope of conductance of 115 ± 2.7 pS (Figure 3B). A non-linear current voltage relation resulted when 175 mM KCl was used in the pipette. The asymmetric anion concentrations across these patches (chloride in the pipette and aspartate in the bath), together with the observation that substitution of sodium for potassium on one side of the membrane caused extreme rectification, are consistent with the channel being highly selective for potassium. From the data with NaCl in the pipette, the selectivity was calculated as pK/pNa ≈100. When Mg^{2+} was added to the pipette solution, an outward rectification of the channel could be observed (Figure 3C). However, no rectification occured when Mg^{2+} was added in the bath solution (data not shown).

A



B



C

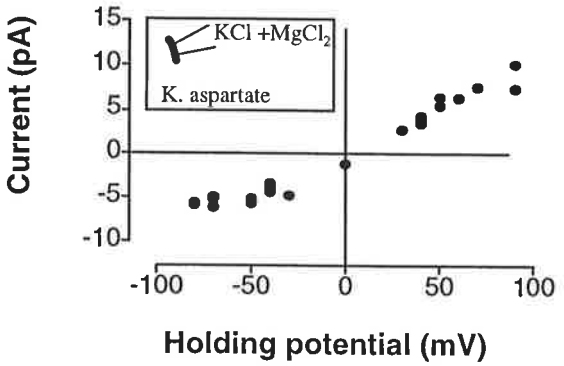


Figure 3: *Current-voltage relations of the channel.*
Panel A shows the current-voltage relation of recordings made from six ripped-off patches with 150 NaCl in the pipette and 150 mM KCl in the bath. Extreme rectification of the channels could be observed. In Figure 2B, recordings made under the same conditions but with 150 mM KCl in the pipette and 150 mM K.aspartate in the bath (filled circles) showed a linear current voltage relation, with a slope conductance of 115 ± 2.7 pS. Using 175 mM KCl in the pipette (unfilled circles) resulted in a non-linear current-voltage relation. Data points show mean ± SEM (n=6). From the data with NaCl in the pipette, the selectivity was calculated as pK/pNa ≈100. In panel C, rectification in the current-voltage relation can be observed when magnesium (2 mM) was included in the patch pipette (n=5).

2.3.4 pH sensitivity

Besides its dramatic response to stretch, this channel was also sensitive to changes in the intracellular pH. Without application of stretch, channel activity was low at pH 7.2. However, as shown in Figure 4A, there was a dramatic increase in the activity of the channel in a ripped-off patch when the pH at the intracellular membrane face was 4.5. Panel B shows the open probability of the channel (held at a membrane potential of 40 mV), plotted against pH of the bath solution. At a more physiological pH, activity of the channel was low, but open probability rose linearly with decreasing pH (ie. exponentially with increasing H^+ concentrations). At a membrane potential of -40 mV, the response of the channel to intracellular pH was similar except that P_o was smaller at all pH values. The interaction between stretch and pH is shown in Panels C (membrane potential is held at 40 mV) and D (membrane potential held at -40mV). The response to stretch was enhanced at a lower pH.

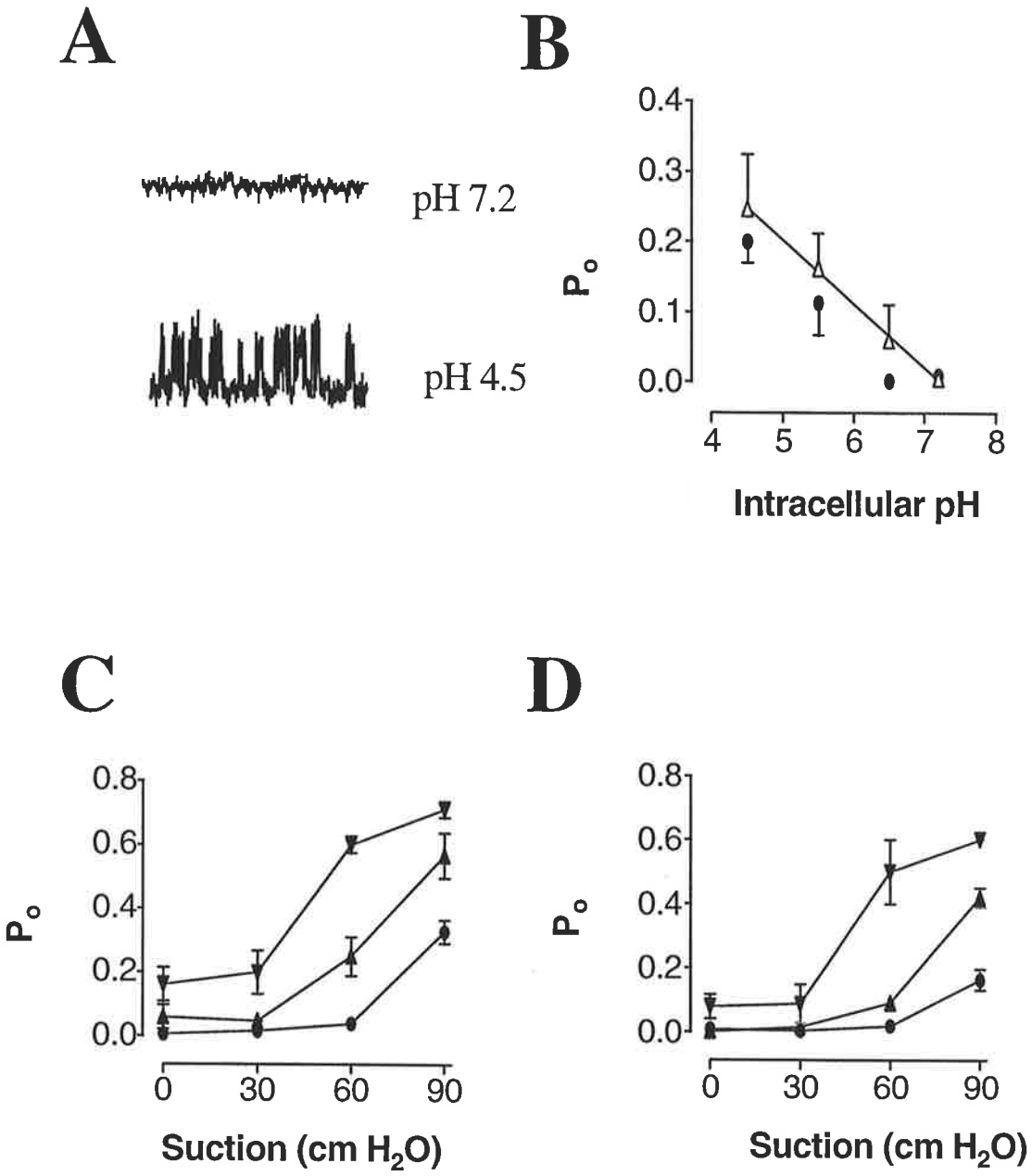


Figure 4: *Sensitivity of the channel to intracellular pH.*
Panel A: Sections of a current record from an isolated membrane patch at a pH of 7.2 and at a pH of 4.5. In 7 isolated membrane patches, the pH in the bath was varied between 7.2 and 5.5 and the channel open probability measured. The pipette contained 150 mM KCl and bath solution 150 mM K.aspartate. The mean open probability is shown plotted against pH in panel B (mean ± SEM). The data was recorded at constant membrane potential of either 40 mV (triangles) or -40 mV (filled circles). Panels C and D shows the interaction of applied suction with pH. In 5 patches, suction of 0, 30, 60 and 90 cm.H_2O was applied to the membrane at a bath pH of 7.2 (filled circles), 6.5 (triangles) and 5.5 (inverted triangles). The data points show P_o (mean ± SEM) plotted against suction. The membrane potential was held constant at 40 mV (panel C) or -40 mV (panel D).

2.3.5 Channel open and close states

Analyses of burst length, mean open time and frequency of bursts were made to determine if the increase in the open probability of the channel at a lower intracellular pH and with membrane stretch, is attributed to changes in these variables. The upper trace in Figure 5, panel A, shows a recording of the channel held at a membrane potential of 40 mV (no suction applied, bath pH = 7.2) . The long and the short closed states of the channel are also demonstrated above the channel record. The lower trace is a simplified diagram showing the open time and burst lengths of the channel in the upper trace. The mean open time of the channel was 0.58± 0.06 ms (n=6) while the mean short close time was 0.18 ± 0.07 ms (n=6). The effect of stretch and pH on the burst length and frequency of the channel is shown in Figure 5, panels B, C and D. Frequency of bursts rose when intracellular pH was decreased (panel B) and when increasing amounts of stretch was applied to the membrane patches (panel C). Panel D shows the combined effects of stretch and pH on the burst lengths of the channel. The graph shows that there was an increase in burst length for all the different bath pH when suction was increased. The length of the bursts was also increased when the intracellular environment was more acidic. However, neither lowering the intracellular pH nor the application of suction had an effect on the mean open time of the channel (Table 1). It therefore appears that suction and lower intracellular pH increased the P_o primarily by increasing the burst length and frequency of openings but not by increasing the mean open time of the channel.

Bath pH	mean open time (ms) no suction	mean open time (ms) 60cm H_2O suction
7.2	0.58 ± 0.065	0.58 ± 0.098
6.5	0.62 ± 0.047	0.97 ± 0.3
5.5	0.75 ± 0.21	0.95 ± 0.18

Table 1: *Mean open time of the channel at an intracellular pH of 5.5, 6.5 and 7.2 when 0 and 60 cm H_2O suction is applied to the patch membrane. Data shows mean± SEM (n=6). No significant difference in mean open time was found between any of the data groups.*

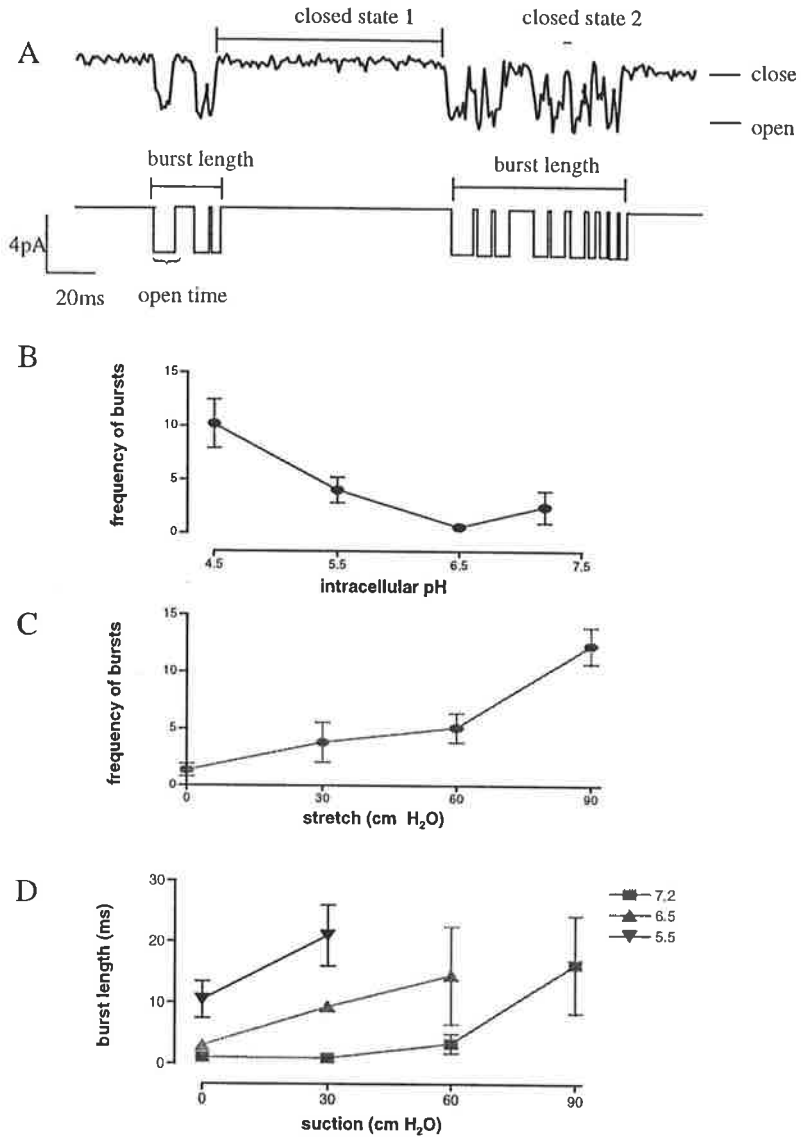


Figure 5: *The effect of stretch and pH on the burst length and burst frequency of the channel.*

The upper trace in panel A shows a recording of the channel held at a membrane potential of 40 mV in a ripped-off patch. When the channel is opened, a downward deflection appears on the record. The lower trace is a simplified diagram showing the open times and burst lengths of the channel in the upper trace. The open time of the channel is the length of time a channel is open. Often the channel can open and close successively for several milliseconds before it remains closed for a longer period of time. The period of flickery channel activity is called a "burst". Channel activities that are separated by an interval where the channel is closed for less that 10 ms is considered in our analysis as belonging to the same "burst" of channel activity. In panel B, frequency of bursts (mean ± SEM) is plotted against intracellular pH for 5 patches. The membrane potential was held at 40 mV. Panel C shows the frequency of bursts (mean ± SEM) when the membrane patches (n=6) were subjected to 0, 30, 60 and 90 cm.H_2O stretch (membrane potential =40 mV, bath pH= 7.2). In Panel D, burst lengths (mean ± SEM)

were plotted against the different amounts of membrane stretch. In 5 patches, suction of 0 to 90 cm.H_2O was applied to the membrane at a bath solution pH of 7.2 (squares), 6.5 (triangles) and 5.5 (inverted triangles). The membrane potential was held constant at -40 mV. Data in panel B, C and D were all obtained from ripped-off patches.

2.3.6 Voltage dependence:

The channel was also slightly voltage dependent. Depolarising the membrane potential in either cell-attached or ripped-off patches caused a noticeable increase in the P_o. An example of this change in P_o is demonstrated in Figure 6A where membrane potential is changed from -90 mV to +90 mV. Figure 6B shows data from a total of 8 patches, plotted as the logarithm of open probability against membrane potential. At a pH of 7.2, a linear regression line fitted to the data set had a slope of 0.01 ± 0.003 (slope ± 95% confidence limit), i.e. an e-fold change in open probability produced by a change in membrane potential of 97 mV. When the pH was 5.0 (open circles), the regression line fitted to the data set had a slope of 0.005 ± 0.002, or an e-fold change for 202 mV. The increase in open probability at more depolarised potentials appears to be again due to an increase in burst-length and frequency of bursts as shown in panels C and D.

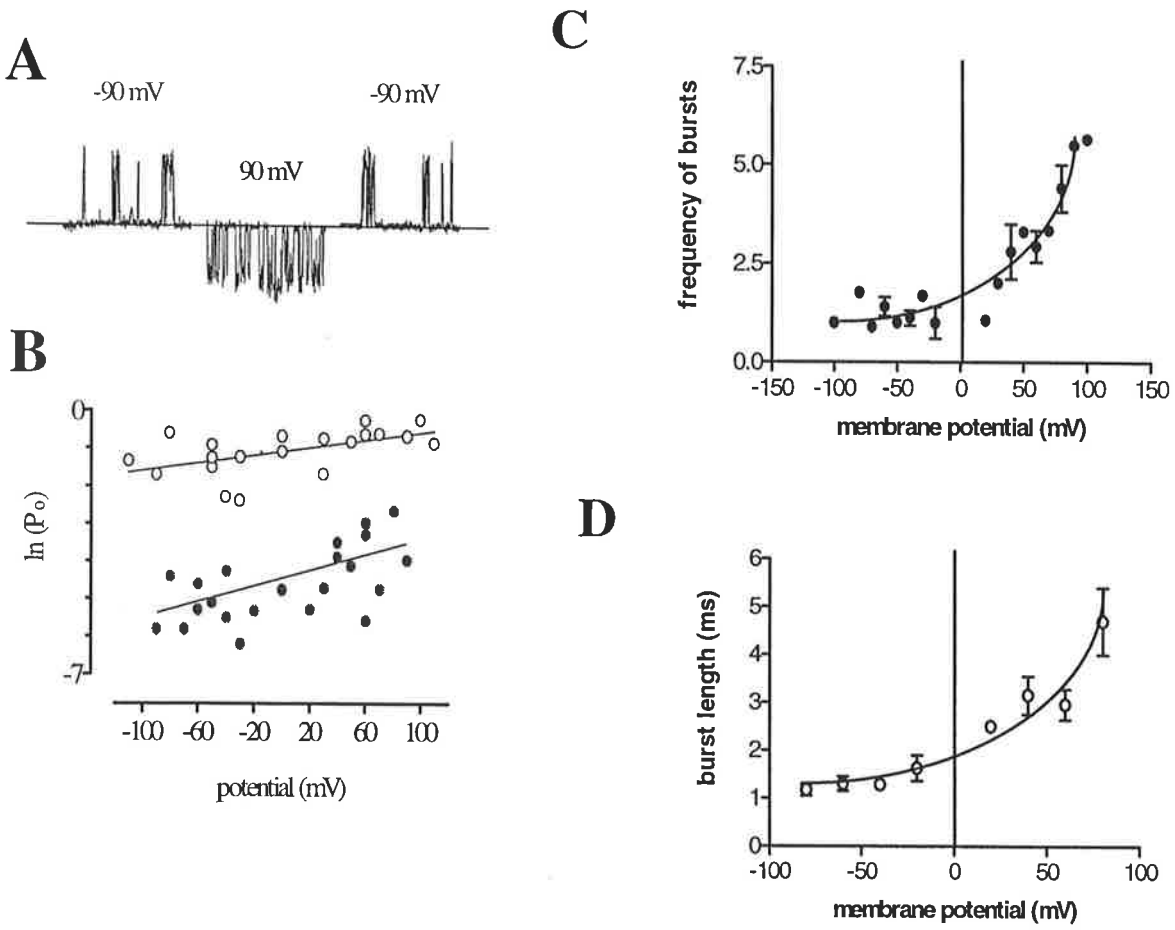


Figure 6: *Voltage dependence of the channel.*
Panel A shows the difference in channel activity in a ripped-off patch (bath solution of pH 6.5) when the membrane potential was held at -90 mV and +90 mV. Data from 8 patches is shown in panel B, plotted as the logarithm of open probability against membrane potential. At a pH of 7.2, a linear regression line fitted to the data set had a slope of 0.01 ± 0.003 (slope ± 95% confidence limit), i.e. an e-fold change in open probability produced by a change in membrane potential of 97 mV. When the pH was 5.0 (open circles), the regression line fitted to the data set had a slope of 0.005 ± 0.002, or an e-fold change for 202 mV. The channel's frequency of bursts (panel C) and burst lengths (panel D) were plotted against membrane potential (n=7, bath pH=7.2) . Error bars show ± SEM.

2.3.7 Pharmacology of the channel:

In Figure 7, panel A shows an example of channel recording in a ripped-off patch held at -40 mV. The channel record and the amplitude histogram below it both show that there were two channels of different conductances present (channels with conductances of ~105 pS and ~45 pS). When glybenclamide (5 μM), a K_{ATP} channel blocker, was introduced into the bath, the channel of lower conductance but not the higher conductance channel was inhibited (disappearance of the lower conductance channel in the recording and the 3-4 pA peak in the histogram (panel B)). The channel was also found not to be affected by potassium channel inhibitors (eg. 4-aminopyridine and TEA), K_{ATP} blockers (eg. 4-hydroxy decanoic acid), and calcium-activated potassium channel blockers (penitrem A, paxilline and charybdotoxin) (n=6) (data not shown). Docosahexaenoic acid (DHA), a polyunsaturated fatty acid, was found to activate the channel. Panel C shows recordings of the channel in a ripped-off patch held at a membrane potential of 40 mV. The open probability of the channel rose from 0.02 to 0.72 after application of 10 μM DHA (bath pH=7.2).

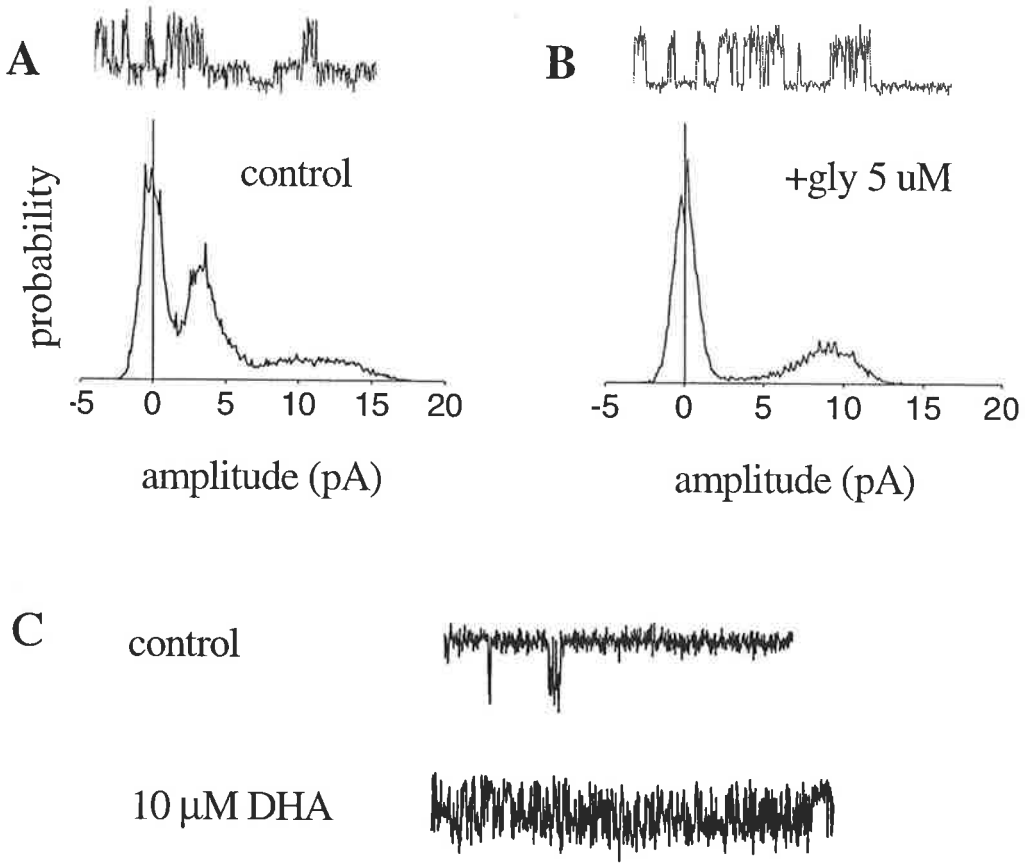


Figure 7: *Pharmacology of the channel.*
Panel A shows a recording of the channel in a ripped-off patch held at a membrane potential of -40 mV. There was also a channel of smaller conductance (~45 pS) present in the record. The amplitude histogram below shows three peaks, ie. a peak at zero pA (baseline), 3-4 pA (small conductance channel) and a broad peak at around 12 pA (larger conductance channel). In panel B, application of glybenclamide (5 μM), a K_{ATP} channel blocker, inhibited the smaller conductance channel but not the channel of larger conductance (disappearance of the lower conductance channel in the recording and the 3-4 pA peak in the histogram). Panel C shows recordings of the channel in a ripped-off patch held at a membrane potential of 40 mV before and after addition of docosahexaenoic acid (DHA) to the bath solution. The open probability of the channel before and after application of 10 μM DHA were 0.02 and 0.72 respectively (bath pH = 7.2).

2.3.8 Expression of TREK-1 gene in the heart.

It appeared that the channel under investigation had all the properties of TREK-1 channels. Using RT-PCR techniques, TREK-1 was found to be expressed in all chambers of the rat heart, further suggesting that the channel being observed was the TREK-1 channel.

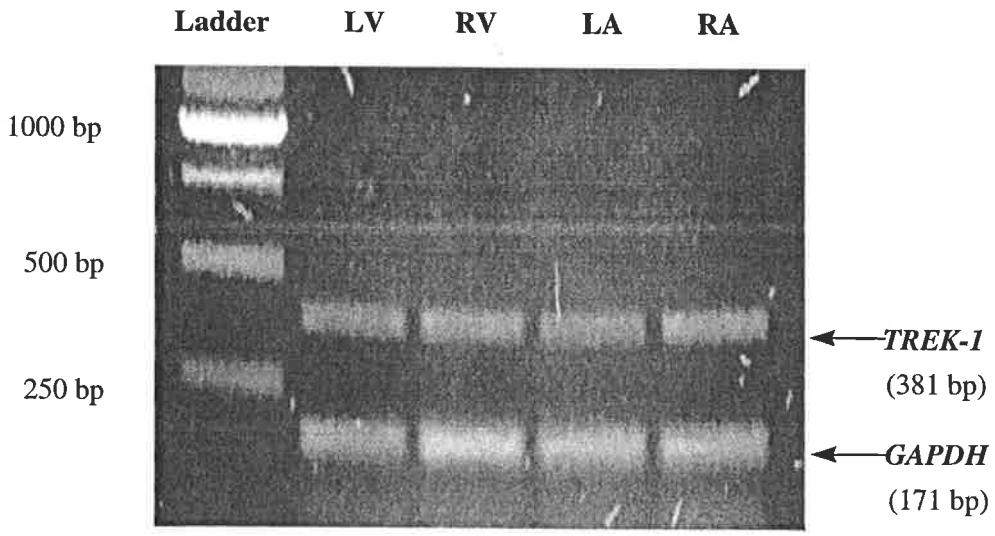


Figure 8: *Genetic expression of TREK-1 in the heart.*
RT-PCR products of both TREK-1 and GAPDH were run on a 1.2% agarose gel and visualised with ethidium bromide. For each lane, 5 μM of each PCR product was added. The expected fragment size for TREK-1 was 381 bp and 171 bp for GAPDH. TREK-1 mRNA was found in the left and right ventricles as well as the left and right atria of the rat (n=6).

2.3.9 Activation by intracellular ATP:

Figure 9A shows examples of channel recordings in a patch with either zero, 1 mM or 3 mM ATP bathing the intracellular membrane face, with the membrane potential held constant at 40 mV, and no suction applied to the patch. Panel B illustrates the interaction of changing ATP concentration and pH in 5 patches while panel C illustrates the interaction of changing ATP concentration and membrane potential. ATP was almost ineffective at increasing P_o at a pH of 7, but was far more effective when pH was lowered slightly. Changing membrane potential had little effect on the response to ATP. The increase in the open probability of the channel with intracellular ATP could be attributed to an increase in the burst-length of the channel and the frequency of bursts. There appeared to be no change in the channel's mean open time (mean open time of the channel at a bath pH of 6.5, with 3mM ATP and 0 mM ATP were 0.66±0.35 ms and 0.63 ± 0.05 ms respectively (n = 5)).

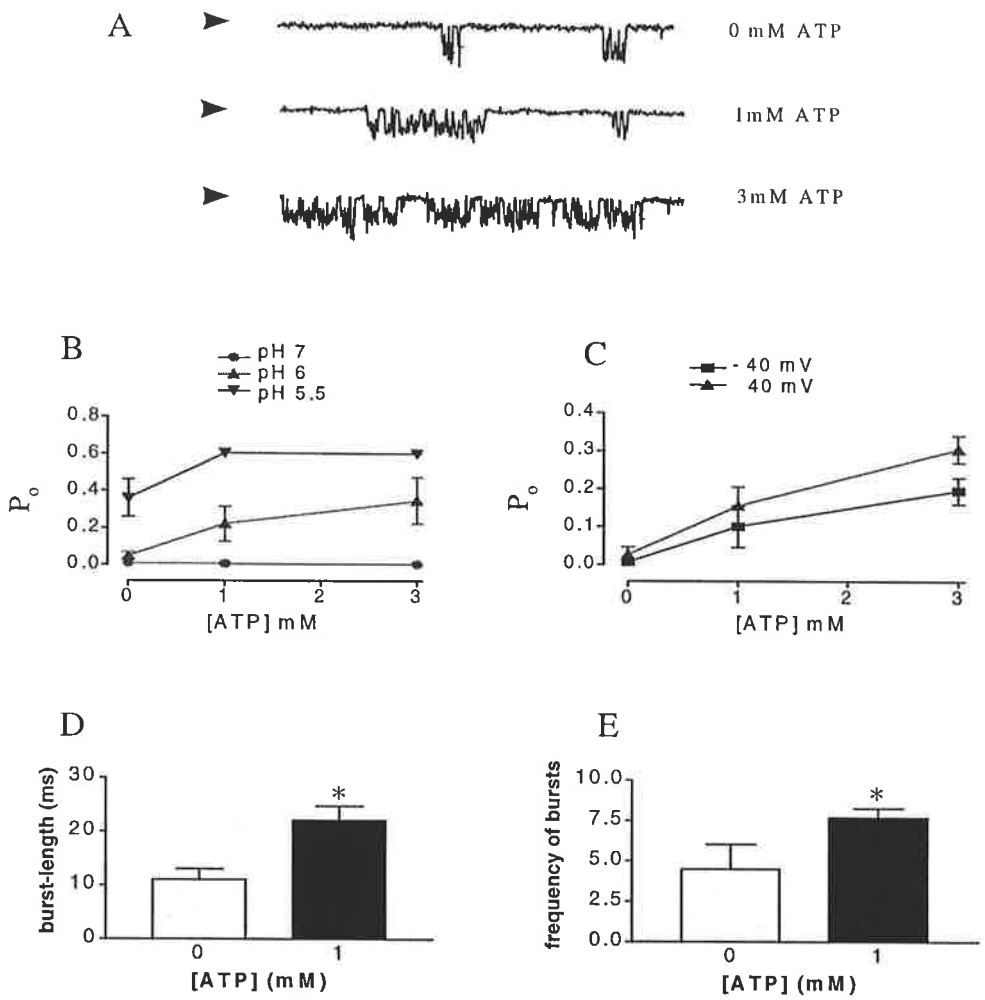


Figure 9: *Effect of intracellular ATP: interaction with pH and membrane potential.*

Panel A shows selected segments of current recordings from a ripped-off patch held at a membrane potential of 40 mV. No hydrostatic pressure was applied and the pH of the bath solution was 6.5. The traces show channel activity when bath solutions with 0 mM ATP (topmost trace), 1 mM ATP (middle trace) and 3 mM ATP (bottom trace) were used. Channel activities increased with increasing concentrations of ATP in the bath solution. Panel B shows the interaction between intracellular ATP and pH. Data was obtained from 5 ripped-off patches held at a membrane potential of 40 mV. The points on the graph show the increase in P_o by ATP at pH 7 (circles), 6 (triangles) and 5.5 (inverted triangles). The error bars show ± SEM. Panel C shows interaction between ATP and membrane potential. The increase in P_o was a result of increasing bath solution ATP from 0 to 3 mM with the membrane potential held at either -40 mV(squares) or + 40 mV (triangles) is shown for 5 different patches. The bar graphs at the bottom of Figure 9 show a significant increase ($p<0.05$) in the channel's burst-length (Panel D) and frequency of bursts (Panel E) in ripped-off patches ($n=5$) when 1 mM ATP was present in the bath solution (bath pH = 6; holding potential = 40 mV).

2.3.10 Time course of ATP activation

There are only a few types of channels that are activated by ATP. A channel which is widely recognised for its activation by ATP is the muscarinic (K_{ACh}) channel. Its activation involves phosphorylation (Heidbuchel, et. al., 1990), a slow process, requiring several minutes to occur (Shui and Boyett, 2000). In our experiment, in order to investigate whether the activation of the channel that we observed involved phosphorylation, we examined the time-course of the channel's activation. Bath ATP concentrations were raised within an order of 100 ms using a rapidly switching perfusion apparatus. The open probability of the channel was seen to rise within 5 seconds (figure 10 B). This rapid increase in channel activity seems to imply that activation of the channel by ATP did not involve a phosphorylation step (see discussion).

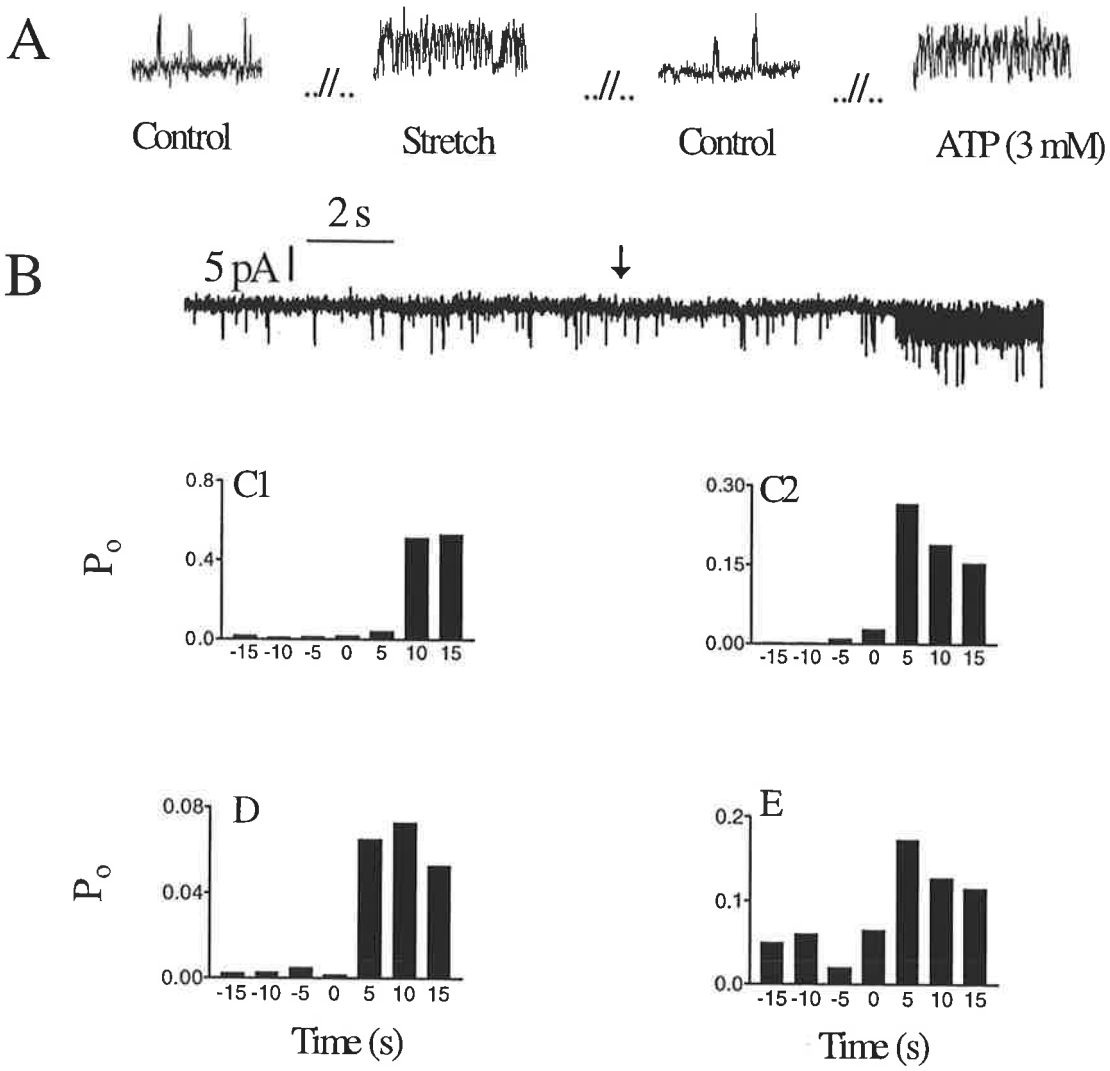


Figure 10: *Time course of the response to ATP.*
Panel A: upper traces show current records from an inside-out membrane patch with a stretch-activated channel in the patch. The potential across the membrane was held constant at -40 mV and bath pH was 6.5. Applying 60 cm.H_2O suction to the patch increased open probability dramatically. With no suction across the same patch, applying 3 mM ATP to the intracellular face of the patch also increased channel activity. B: A section of a recording made from a membrane patch, kept at a membrane potential of + 40 mV. 3 mM ATP was applied to the intracellular face of the patch at the arrow. Lower panels: the time course of the response to ATP is shown as histograms in the panels below. Channel open probability was measured in 5 second bins. ATP was applied at time zero (beginning of bin 0). Graphs C1 and C2 show data obtained from repeats of the experiment in the same patch while panels D and E show data from different patches.

2.4 DISCUSSION

The stretch-activated channel under investigation was found to be a potassium selective channel with a conductance of around 100 pS. Its activity was increased by depolarisation of the membrane potential, intracellular ATP, intracellular acidification as well as polyunsaturated fatty acids. The channel was not blocked by potassium channel blockers such as 4-AP and TEA (broad spectrum K^+ channel blockers), penitrem (large calcium activated (BK) channel blocker) or glybenclamide (K_{ATP} channel blocker). There have not been many reports of potasssium stretch activated channels in the heart. The main ones that have been reported hitherto are BK, K_{ATP} and TREK-1 channels (Kawakubo, et. al., 1999;Van Wagoner and Russo, 1992; Van Wagoner, 1993; Maingret et. al., 1999).

To date, there has not been any observations of channels with this suite of properties. Due to its large conductance, it was initially thought that the channel in question was the BK channel. A property that is shared by all BK channels is activation by intracellular calcium. Thus, attempts were made to determine whether the channel was sensitive to intracellular calcium (10^{-4} M). The amount of calcium required to be added to the bath solution was calculated using a program, EQCAL. Unknown to us at the time, addition of calcium to the bath solution caused a displacement of H^+ ions bound to EGTA. Consequently, there was a dramatic reduction in the pH of the bath solution. Activity of the channel was increased when $CaCl_2$ was added to the bath solution. Erroneously, the augmented channel activity was attributed to an

increase in intracellular calcium and hence it was thought that the channel under observation was a BK channel. However, when the pH of the bath solution containing calcium was corrected to 7.4, there was no change in channel activity using the solution. The channel was also not blocked by penitrem, a known BK channel blocker. Hence, the channel observed was not a BK channel.

The stretch-sensitive channel is also not a K_{ATP} channel. In recordings with two channels of different sizes in the same patch, stretching the membrane increased the activity of the larger conductance channel only. When K_{ATP} channel inhibitors, (glybenclamide or ATP) were added to the bath solution, the channel of lower conductance disappeared, suggesting that the inhibited channel is the K_{ATP} channel. The channel of higher conductance was not affected. From these experiments, it is observed that K_{ATP} channels are not sensitive to stretch. This observation supports the observation of Kim (1992), but is contrary to that of Van Wagoner and Russo, (1992) who reported activation of K_{ATP} channels by stretch in rat atrial myocytes.

It appears that the channel under investigation has many of the characteristics of TREK-1, a stretch activated potassium selective channel belonging to the 2P4T channel family that has recently been cloned. TREK-1 mRNA expression was also found in the atrial and ventricular myocytes, further reinforcing the suggestion that these channels are TREK-1. The first observation of cardiac ionic channels with properties of TREK-1 was by Kim (1992), in rat atrial myocytes. The outward rectification that he noted for the

channels was not apparent in most of the experiments reported here, and it appears likely to be due to Mg^{2+} in the pipette solution (figure 3C). It is possible that this phenomenon is due to partial blockage of the outer channel pore by Mg^{2+}. Surprisingly, Mg^{2+} in the bath solution did not cause any rectification in ripped-off patches. Since the channel can be activated in ripped-off patches, it appears that the mechano-gating of the channel does not require the integrity of the cytoskeleton and the activating force comes directly from the bilayer membrane. It was found in this study, that the response of the channel to suction applied to the patch pipette was similar in cell-attached and ripped-off patches, implying that the cytoskeleton plays little role in the stretch response.

Often, activity of this channel occurs in bursts (rapid opening and closing of channel) followed by periods of channel inactivity. From this observation, it appears that the channel has at least one open state and two closed states. There is a closed state where the channel only closes briefly and another where the channel closes for a longer period of time before another burst of activity ensues. At present, the gating mechanism of TREK-1 is still largely unknown.

So far, there have not been any reports that intracellular ATP activates TREK-1 although Aimond et. al. (2000) reported that TREK-1 channels were activated by extracellular ATP, by a signalling cascade which appears to involve phospholipase A_2. Kim, (1992) reported that the mechanosensitive potassium channel from atrial cells could be activated in the presence of ATP,

although not that it was also activated by ATP *per se*. The activation of the channel by intracellular ATP occurs very quickly (in a matter of seconds). As the response to ATP was rapid and also activation of these channels occur in ripped-off patches, it is unlikely that activation involves phosphorylation of the channel protein. In contrast, K_{ACh} whole-cell currents activation by ATP, of which involves phosphorylation, took approximately 4.5 minutes to increase to a steady-state (Shui and Boyett, 2000). It is possible that ATP activates TREK-1 by binding as a ligand to a receptor on the channel. Shui and Boyett (2000), also reported a potassium channel in atrial cells that was activated by intracellular ATP. They were uncertain of the type of channel they were observing although they went to some lengths to differentiate it from the K_{ACh} channel. It is possible that the channel observed by Shui and Boyett is TREK-1 as it had a conductance of around 100 pS. However, they did not report activation of the channel by stretch or fatty acids. In this study, the activation of intracellular ATP can only be seen at a more acidic intracellular pH. No channel activation by ATP is observed at a physiological pH of 7.4. ATP activation might only occur in pathological conditions of the heart for example, in heart failure or coronary artery disease. Reduced blood flow to the myocardium induces hypoxia in the tissues. Consequently, the ischemic myocardium switches from aerobic to glycolytic metabolism, increasing lactic acid production which causes a decrease in intracellular pH.

At present, it is still uncertain what role TREK-1 channels play in the heart. The channel was observed quite frequently (in about 20-25% of properly sealed patches). Therefore, the channels might carry quite a large

current in the cell. Nevertheless, it is hard to predict what the open probability of the channel is *in vivo* due to the range of interacting factors that activate the channel. Such an estimate would need accurate information on the membrane potential, intracellular pH, ATP concentration and membrane tension. From its properties, its primary role may be to modulate the electrical (and hence contractile) activity of the heart in response to stretch. It is also possible, given the response to pH and ATP, that the channel couples such activity to the metabolic status of the heart. Weiss and Venkatesh (1993), reported that there is a large outflow of K^+ from cells during cardiac ischemia and this efflux could not be abolished by blockers of BK and K_{ATP} channels. This efflux would result in the shortening of the action potential. The channel reported here may resolve this puzzle, since it would couple action potential shortening to the intracellular acidification and increased myocardial stretch during ischaemia. Results of RT-PCR performed indicated that TREK-1 was found to be expressed in atrial cells as well. Channels with properties of TREK-1 were also reported in the atria by Kim (1992) and Terrenoire and co-workers (2001). It is possible that atrial TREK-1 channels are involved in regulating the secretion of atrial natriuretic peptide, a hormone which production is increased when the atria are stretched (Lang, et. al., 1985).

3: DIFFERENTIAL EXPRESSION OF TREK-1 IN EPI- AND ENDOCARDIAL CELLS OF RAT LEFT VENTRICLES

ABSTRACT:

Mechanosensitive TREK-1 channels might play an important role in cardiac mechanoelectrical coupling. mRNA of these channels were found to be differentially expressed in the epi- and endocardial cells of the rat left ventricles. Using real-time PCR techniques, the expression of TREK-1 using GAPDH as a reference, was found to be 0.34 ± 0.14 in endocardial cells compared to 0.02± 0.02 in epicardial cells ($p \leq 0.05$). To observe if there is a correspondingly higher expression of functional TREK-1 channels in the endocardial cells, patch-clamp techniques were employed to study TREK-1 whole-cell currents. Chloroform, an activator of the channel was used to induce this current. TREK-1 normalised current in epi- and endocardial cells were 0.21 ± 0.06 pA/pF and 0.8 ± 0.27 pA/pF respectively ($p \leq 0.05$). A heterogenous expression of TREK-1 channels in the ventricle may correspond to the different amounts of stretch experienced by muscle cells across the ventricular wall during diastole.

3.1 INTRODUCTION

Potassium channels are essential for the functioning of both excitable and non-excitable cells. They are important for regulating the resting potential and controlling the inotrophy and chronotrophy of the heart. There are two categories of potassium channels in the heart: the voltage-gated potassium channels which partake in repolarising the membrane potential at different phases of the action potential and the background potassium channels which stabilise the resting potential and regulate the action potential duration. Potassium channels can be grouped into three major structural classes: the two transmembrane domain, one pore domain (2TM1P)(inward rectifier channels), 6TM1P (voltage gated channels) and 4TM2P families.

All members of the recently cloned 4TM2P family are background potassium channels (Patel, et. al., 1999). They were found to affect the resting membrane potential of the cell as these channels open also at hyperpolarised potentials. TREK-1, a member of the 4TM2P family of channels has been found to be highly expressed in cardiac tissues of rats. Like many of the other background potassium channels, the TREK-1 channel is endowed with several interesting and unusual characteristics, eg. it is activated by stretch, volatile anesthetics and intracellular ATP (Maingret, et. al., 1999; Patel, et.al., 1999; Tan and Saint, 2002). The fact that TREK-1 is highly sensitive to stretch implies that it might play an important role in cardiac mechanoelectric feedback. Although no specific activator or inhibitor of the 4TM2P family of channels had been found, several members of this family are activated by

volatile anesthetics. This is an unusual property as all other cardiac potassium channels are either not affected or inhibited by these agents, except for K_{ACh} channels which are activated by halothane (Terrenoire et al, 2001). Among the potassium channels present in the heart, TREK-1 alone is activated by chloroform (Terrenoire, et al., 2001). The activation of TREK-1 channels by volatile anesthetics might contribute to the decrease in the strength of cardiac contraction which is observed following general anesthesia.

The distribution of several types of potassium channels has been found to be heterogenous in the heart. For example, voltage-activated potassium channels such as Kv 4.2 and Kv LQT1 isoform 2 have been found to be expressed differentially across the ventricular wall (Dixon, et al, 1996; Dixon and McKinnon, 1994; Pereon, et al, 2000). The expression of Kv 4.2, for example, was more than eight times higher in epicardial muscle cells compared to papillary muscle cells (Dixon and McKinnon, 1994). The heterogeneity in the expression of these genes could account for the heterogenous repolarisation occurring along the different layers of the ventricular wall.. This difference in repolarisation rate might be important for establishing synchrony in the contraction of cardiac muscle cells. During diastole, the amount of stretch experienced by the different layers of muscle cells across the ventricular wall is different (Carey Stevens and Peter Hunter, University of Auckland, NZ, unpublished). Consequently, it is possible that there is also differential expression of stretch-activated channels across the ventricular wall to couple the amount of stretch experienced by muscle cells with the strength of contraction.

In this study, the expression of TREK-1 channels in epi and endocardial cells of rat ventricles was examined. Chloroform-induced whole-cell TREK-1 currents in epi- and endocardial cells were also compared as a high mRNA expression of a channel does not necessarily correspond with a high expression of functioning channel proteins (eg. in rat cardiomyocytes, Kv 1.4 protein is very inefficiently expressed, although there is abundant mRNA of the gene (Barry, et al., 1995)).

3.2 METHODS:

3.2.1 Real Time PCR techniques:

(Experiments were performed in association with Weihong Liu)

(a) Total RNA extraction and reverse transcription reaction

Total RNA was extracted from epi-and endocardial tissues of the left ventricles of six adult Sprague-Dawley rats. Please refer to chapter 2 for experimental details on total RNA extraction and reverse transcription reactions (2.2.2 (a) pgs. 43-44)

(b) TREK-1 genes and SYBR Green real-time PCR

Real-time PCR was performed for *TREK-1* (genebank accession number U73488) and a rat house-keeping gene, *GAPDH* (genebank accession number M32599), using cDNAs corresponding to 200 ng total RNA synthesised from left ventricular epicardial and endocardial cells. The gene specific primers were: *TREK-1*, forward 5' TTTGGCTTTCTACTGG CTGGGG 3', reverse 5'

TCGTCTTCTTAGAGATCACCG 3'; *GAPDH*, forward 5' ATGTTCCA GTATGACTCCACTCACG 3', reverse 5' GAAGACACCAGTAGACTCC ACGACA 3'. All primers were purchased from GeneSet (GeneSet Pacific Pty. Ltd, Australia). SYBR Green real-time PCR assays were performed on the cDNA samples in 96-well optical plates on an ABI Prism 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). *GAPDH* assays were run parallel to each different sample. For each 25 µl SYBR Greeen PCR reaction, 2.5 µl cDNA, 1.5 µl sense primer (5 µM), 1.5 µl antisense primer (5 µM), 12.5 µl SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA) and 7 µl PCR-grade water were mixed together. Using SYBR Green PCR Master Mix in the reaction, the direct detection of polymerase chain reaction product can be monitored by measuring the increase in flourescence caused by the binding of SYBR Green dye to double-stranded DNA. The parameters for a two-step PCR were 95^0C for 10 min, 1 cycle, then 60^0C for 1 min, 95^0C for 15 sec, 40 cycles. Before quantitation of real-time PCR data, the specificity of the amplified products was examined by both running the products in 3% agarose gel and dissociation curve analysis.

(c) Quantitation of gene expression level

For comparison of gene expression levels, all quantitation were normalised to endogenous *GAPDH* expression level to account for variability in the initial concentration and the quality of the total RNA in the conversion efficiency of the reverse transcriptase reaction. The amplification efficiency-based method

was used for quantitation and normalisation as developed previously by Liu and Saint (2002).

3.2.2 Patch-clamp techniques:

Adult rat heart cells were prepared as described in the previous chapter (2.2.1 (a) pgs 37-39). Briefly, ventricular myocytes of male Sprague-Dawley rats were isolated by enzymatic digestion using a Langendorff perfusion apparatus. When digestion of the heart was complete, the heart was removed from the perfusion apparatus. The epicardial and endocardial surfaces of the left ventricle were 'trimmed' to remove surface layers of tissue. The epicardial and endocardial tissues were collected in separate petri dishes filled with Tyrode's solution. Trituration was then performed to release the cells.

All electrophysiological recordings were acquired at room temperature of 22-24^{0}C, using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Union City, CA, USA). Electrodes were fashioned from borosilicate glass capillaries (Harvard apparatus PG150T-7.5). The pipette solution contained (mM): KF, 120; $MgCl_2$, 6.8; EGTA, 10; HEPES, 20. Typically, pipettes had resistances of 1-2 MΩ. During formation of gigaOhm seals and initial break-in to the whole-cell voltage clamp configuration, heart cells were perfused with normal Tyrode's solution containing (mM): NaCl, 133.5; HEPES, 10; D-glucose, 11; $NaH_2PO_4.2H_2O$, 1.2; KCl, 4; $MgCl_2$, 1.2. Once a successful break-in is obtained, the perfusing medium was switched to an external recording solution containing (mM): Choline.Cl, 143.5; HEPES,

10; NaH_2PO_4, 1.2; KCl, 4; $MgCl_2$, 1.2. To measure TREK-1 currents, other ionic currents were blocked by adding: 2 mM $CoCl_2$; 5 mM TEA and 10 μM glybenclamide to the bath solution. Data traces were acquired at a repetition interval of 10s using a whole cell recording software, Collect (written by M. Smith, John Curtin School of Medical Research). The holding potential was kept at -50 mV. A voltage step to -70 mV was applied for 400 ms followed by another voltage step to to -30 mV for another 400 ms. Whole-cell recordings were analysed using an analysis program, Canal (written by M. Smith, John Curtin School of Medical Research). mRNA expression and TREK-1 whole-cell currents for epicardial and endocardial cells of the left ventricle were compared using Student's t-test. $p< 0.05$ was considered significant.

3.3 RESULTS

3.3.1 mRNA expression of TREK-1 in the epi- and endocardial cells

Along with the primer Express Software, SYBR Green real-time PCR assay provided a sensitive method for detection of low abundance genes with high specificity. Figure 1A and 1B shows the SYBR Green real-time PCR kinetic data traces of TREK-1 channel genes and the corresponding dissociation curves that checks for primer-dimer formation in the reaction. Dissociation curve analysis showed that the melting temperature for the genes was more than 75^0C indicating no primer-dimer formation (Fig 1B). The specificity of the amplification was also examined by running the product in 3% agarose gel. The gel electrophoresis revealed the single band consistent with the predicted

size for TREK-1. It appears from the gel that there is higher expression of the channel in the endocardial cells compared to the epicardial cells. Using the amplification efficiency-based method developed by Liu and Saint (2002) for quantitation and normalisation of real-time PCR products, the expression of TREK-1 (using GAPDH as a reference) was found to be significantly higher in the endocardial cells compared to the epicardial cells ie. 0.34 ± 0.14 and 0.02 ± 0.02 respectively (Figure 1D).

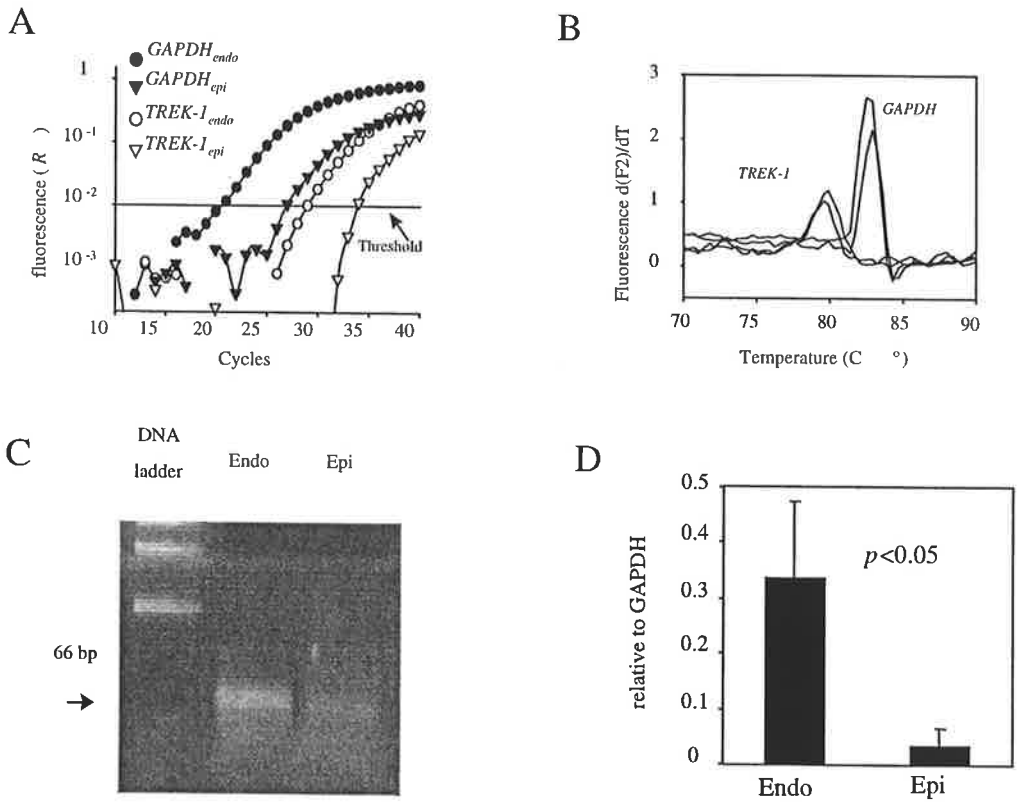


Figure 1: *mRNA expression of TREK-1 in the epi- and endocardial cells of the left ventricles of the rat.*
Figure 1A shows the SYBR Green real-time PCR kinetic data traces for the genes of TREK-1 and GAPDH while Figure 1B shows the corresponding dissociation curves of the real-time PCR products. Dissociation curve analysis shows that the melting temperature for the genes is more than 75^0C indicating no primer-dimer formation. Panel C shows a rough estimate of mRNA expression of TREK-1 in the epi- and endocardial myocytes of the left ventricles of the rat. There appears to be a higher mRNA expression of TREK-1 in the endocardial cells. Panel D: Using real-time PCR techniques, the expression of TREK-1 relative to GAPDH was found to be higher in endocardial cells (0.34 ± 0.14) compared to epicardial cells (0.02 ± 0.02) (n=6).

3.3.2 TREK-1 whole-cell currents in the epi- and endocardial cells

The upper diagram in Figure 2A shows the voltage-step protocol used. Small voltage steps were applied to prevent activation of voltage-gated potassium channels. The resultant whole-cell current for control solution and bath solution containing 0.04% chloroform is shown in the lower diagram. Panel B shows the difference in the whole-cell current of an endocardial cell at -30 mV and at -70 mV plotted before and during addition of chloroform as well as during washout of chloroform. There is a notable increase in the whole-cell current with chloroform in the bath. This current is attributed to the opening of TREK-1 channels. The graph in panel C shows normalised current (whole cell current divided by the cell's capacitance) for the epi- (0.2 ± 0.06 pA/pF) and endocardial cells (0.8 ± 0.27 pA/pF) of the left ventricles of rats. The TREK-1 current in endocardial cells is approximately four-fold larger than in epicardial cells.

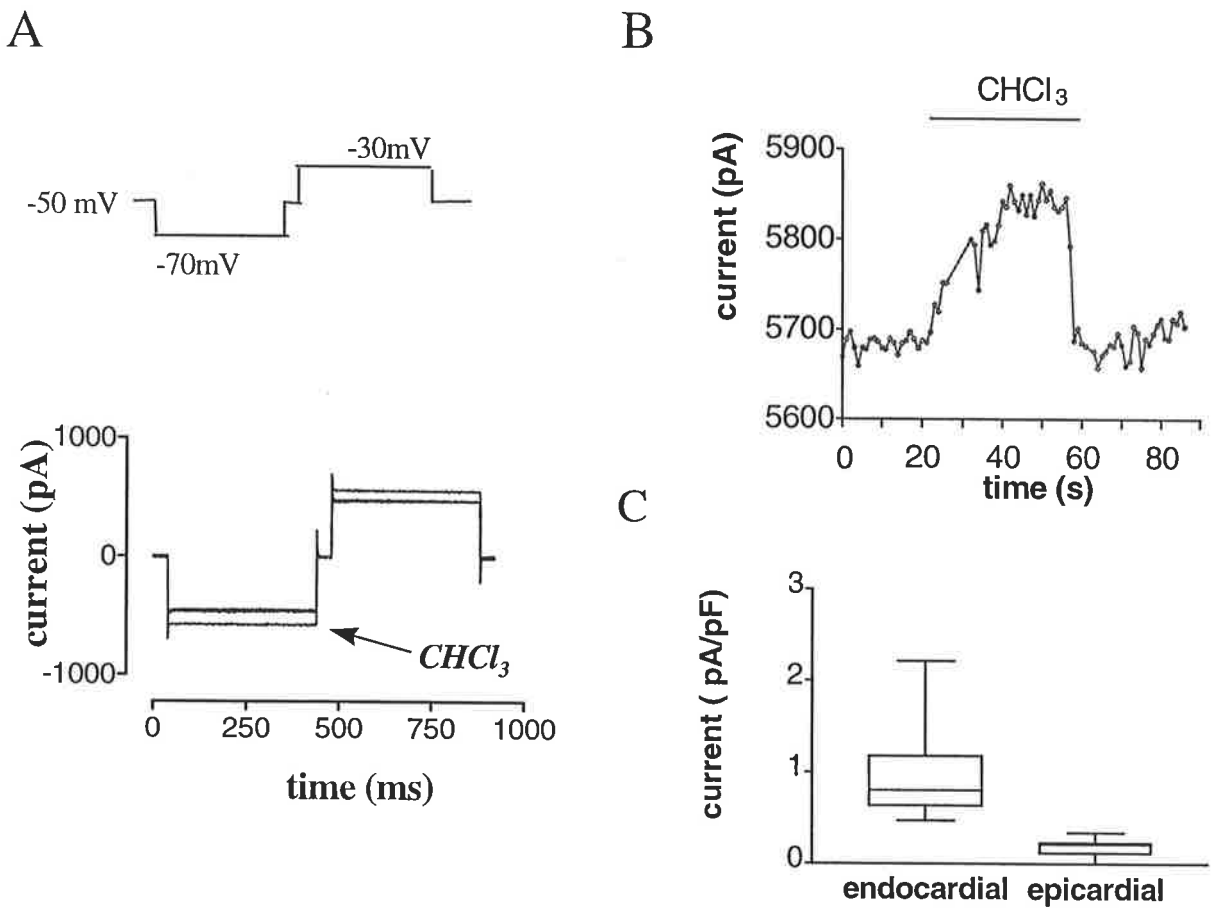


Figure 2: *Whole-cell currents of TREK-1 induced by chloroform.*
The upper diagram in Panel A shows current pulse protocol used to observe whole-cell TREK-1 current. Holding potential was kept at -50 mV. A -70 mV pulse was applied for 400 ms followed by a 400 ms current pulse to -30 mV. This current steps were repeated every 10 seconds. The lower diagram in panel A shows the resultant whole-cell current using this voltage-step protocol for both the control solution and the bath solution containing 0.04% chloroform. Panel B: The difference in the whole-cell current at -30 mV and at -70 mV is plotted before and during addition of chloroform as well as during washout of chloroform. Data shown in panels A and B are obtained from different cells. The box and whiskers graph in panel C shows normalised current (whole cell current divided by the cell's capacitance) for both the epi- and endocardial myocytes of left ventricles for six rats. The mean current for endocardial and epicardial cells are 0.8 ± 0.27 pA/pF and 0.21 ± 0.06 pA/pF respectively. There is a significant difference in the size of TREK-1 whole-cell current between the epi and endocardial cells ($p \leq 0.05$).

3.4 DISCUSSION

In this study, using real-time PCR techniques, it was found that there was a higher expression of TREK-1 channels in the endocardial cells compared to the epicardial cells. There was also a significantly larger TREK-1 current in the endocardial cells compared to the epicardial cells indicating that the mRNA expression of TREK-1 corresponded with the functional protein expression of the channel.

The results of this study demonstrate that there is a heterogenous distribution of these channels between the different regions of the left ventricle. Dutetre and co-workers (1972) reported that mechanically induced changes in action potential duration were dissimilar in different parts of the intact left ventricle supporting the fact that there is a varied distribution of cardiac SACs. So far, there have not been any published reports on the distribution of cardiac potassium SACs. There are, however, reports of heterogeneity in the expression of other potassium channel genes in different regions of the heart (Dixon and McKinnon, 1994; Dixon, et. al., 1996; Pereon, et. al., 2000). For example, Dixon and McKinnon (1994) reported large differences in the expression of rat Kv 4.2 mRNA between atrial and ventricular cells as well as between cells from the epicardium and endocardium of ventricles. This difference in channel expression was thought to cause the variation of electrophysiological phenotype in different parts of the heart.

Using the dimensions of the porcine heart, computer-generated modelling showed that there are differences in the amount of stretch experienced by myocytes in different regions of the porcine left ventricle (shown in Figure 3). The diagram demonstrates that the amount of muscle fibre stretch is not only heterogenous across the ventricular wall but is also different from the apex to the base of the heart. It is likely that cardiac TREK-1 expression is related to the amount of mechanical stretch experienced by the heart cells. In an experiment performed by Takagi and co-workers (1999), epicardial monophasic action potential duration was shortened while endocardial monophasic action potential duration remained unaltered when canine left ventricles were subjected to stretch. From their experimental data, it appears that there is a larger population of SACs in the epicardium while we report a higher density of TREK-1 channels in the endocardium. This discrepancy in results might have been due to the possibility that experimental data was collected from cells along different longitudinal axes of the ventricle or that the pattern of cardiac fibre stretch might vary according to species.

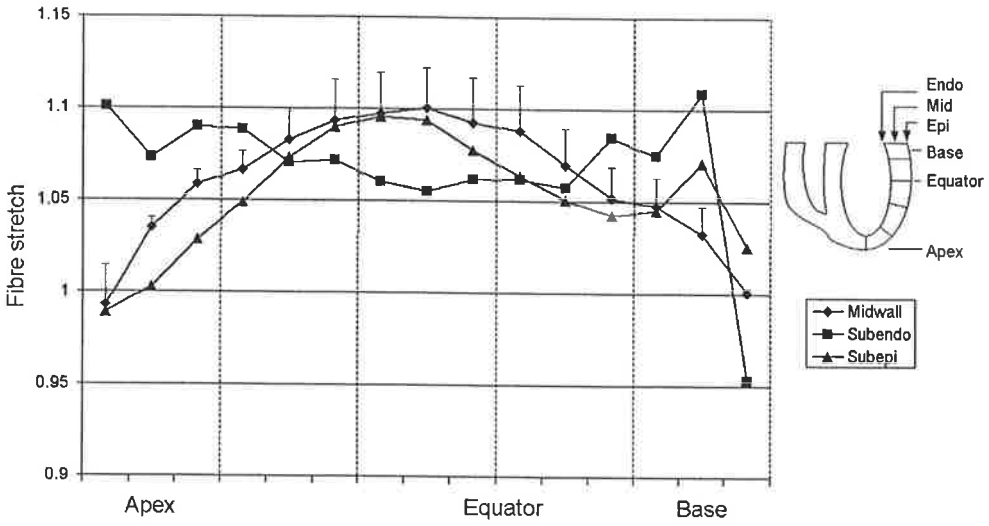


Figure 3: *Fibre stretch variation at end diastole.*
Computer-generated model of stretch experienced by cardiac cells in different regions of porcine left ventricular wall. The diagram shows that the amount of muscle fibre stretch is not only heterogenous across the ventricular wall but is also different from the apex to the base of the heart. Diagram obtained from Carey Stevens and Peter Hunter, University of Auckland, New Zealand (unpublished)

More experiments could be performed to investigate the population of cardiac TREK-1. Expression of channels could differ during development or in aged individuals. In foetal brain, a higher expression of hTREK-1 was found compared to the adult brain (Meadows, et. al., 2000), suggesting that this channel may be important during the early stages of development. The same trend might also be present in the heart. Changes in the genetic expression of certain potassium channels in diseased conditions have been reported (eg. several types of voltage-gated potassium channels have been observed to be down-regulated in the left ventricles of postmyocardial infarcted rats (Huang, et al, 2000) and atrial appendages of patients with atrial fibrillation (Van Wagoner, et. al., 1997)). Hence, it is also possible that there

are alterations in the expression of stretch-activated K^+ channels like TREK-1 in diseased hearts to adapt to changes in levels of myocardial stretch.

The expression of potassium channels was found not only to differ in different parts of the heart but also between species. For example, neither the Kv 1.2 nor the Kv 2.1 gene is expressed at significant levels in the canine heart but both the mRNA and protein products were abundant in the rat heart (Dixon, et al, 1996). These differences may be an adaptation to the different requirements for cardiac function in mammals of different sizes. It is possible that the expression of cardiac TREK-1 in human and in rat might also differ. TREK-1 expression had been found to be high in murine hearts (Fink et.al., 1996) whereas, unexpectedly, a recent report by Meadows and co-workers (2000) stated that the expression of this channel in the adult human heart was very low. The report by Meadows et. al. (2000), however, did not mention the health status of the donors and from which section of the human heart the tissue was extracted. Therefore, it might be too early to conclusively state that there is low expression of TREK-1 in the human heart. Stretch-activated potassium channels are likely to be present in the human heart as stretching of the human myocardia had been found to shorten action potential durations (Taggart, et.al., 1992). It is quite unlikely that an anionic channel is responsible for this effect as there have been no reports of cardiac anionic SACs till now. There is the possibility that a different type of potassium SAC or other members of the 4TM2P channel family which are mechanosensitive are present instead of or in conjunction with TREK-1 channels. It is thought that cardiac K_{ATP} channels are also involved in shortening the action potential

duration during myocardial stretch as this channel is the only other potassium-selective channel besides TREK-1, that has been reported to be activated by stretch in the mammalian heart. The activation of K_{ATP} channels by stretch is however still controversial at this stage as there have also been reports that disagree with the stretch sensitivity of this channel (Kim, 1992; Tan and Saint, 2002).

Further investigation into the population of TREK-1 channels in the human heart should be undertaken as these channels might play an important role in the mechano-electric feedback of healthy and diseased hearts. A difference in the population of K^+-selective SACs in the ventricular wall would result in a more pronounced shortening of action potential in certain areas of the ventricle when stretched, thereby increasing the difference between the action potential duration in different areas of the heart. If the ventricles are under a high level of tension, such as in heart failure or congestive heart diseases, this heterogenous shortening of action potential duration is potentially hazardous as they might disrupt the heart's rhythmic pattern of excitation and contraction by causing premature ventricular contractions (Takagi, et. al., 1999) or re-entry circuits (Kohl, et. al., 1999). However, in healthy hearts, a heterogenous population of SACs in the ventricles may serve to couple the amount of mechanical stretch experienced by cardiac cells from different areas of the heart with the force of contraction. As activation of cation non-selective SACs induces membrane depolarisation, whereas, opening of K^+-selective SACs causes hyperpolarisation, a possible function of cardiac K^+ SACs is to act as a negative feedback to the cation non-

selective SACs to prevent ectopic beats from occurring when the myocardium is stretched. There could probably be a correlation between the population of potassium SACs and cationic non-selective SACs in the different regions of the heart. More studies are required to investigate this possibility.

4: GENERAL CONCLUSION

4.1 FUTURE DIRECTIONS

4.1.1 Experiments to further investigate the biophysical properties of TREK-1 channels

The activation of TREK-1 by intracellular ATP occurred rapidly and also in ripped-off patches, suggesting that phosphorylation was not involved in this process. To further investigate the involvement of phosphorylation in TREK-1 activation, a non-hydrolysable analogue of ATP eg. adenylyl-β, γ - imidodiphosphate, can be used. If the channel is activated by this agent, it is possible that the activation of the channel by ATP is a receptor-mediated process. It would also be interesting then to locate this ATP receptor site. To do this, deletion or site-directed mutagenesis studies would need to be performed. A commonly targetted site is the carboxyl terminus as this region of the channel has been found to be important for many of the biophysical properties of the channel.

Preliminary experiments in our laboratory have shown that the channel is sensitive to temperature. Figure 1 shows the open probability of the channel when the bath temperature is raised.

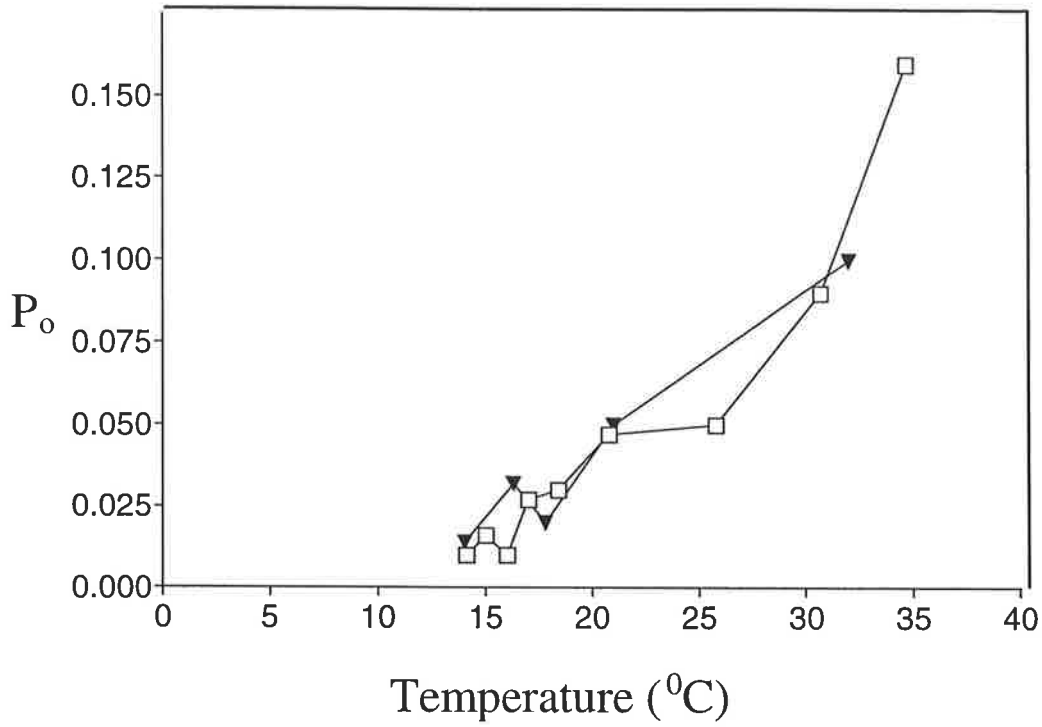


***Figure 1**: Temperature dependence of ventricular TREK-1 channels. Preliminary data shows open probability of the channel increased dramatically with a rise in the temperature of the bath solution. (n=2).*

If the open probability of a channel is very temperature dependent, it is likely that the protein structure of the channel can undergo large conformational alterations. At present, the transduction mechanism of stretch in TREK-1 channels is still unknown. Published observations (Maingret, et. al., 2000) and preliminary data collected from our laboratory demonstrate that TREK-1 activity is highly influenced by temperature. It is thus suggested that the channel is capable of undergoing large conformational changes that could enhance the activity of the channel when stretched or when there is a drop in intracellular pH. To pin-point the residues that are involved in altering the conformation of the channel, site directed mutagenesis of the channel could be performed. Deletion or

mutation of residues responsible for conformational changes in the channel would alter the channel's response to temperature.

Applying suction on the patch membrane would cause an increase in membrane tension. However, the membrane distortion caused by suction might also be analogous to the distortion of the membrane during cell-swelling ie. the lipid bilayer of the patch is crenated when suction is applied. Therefore, negative pressure applied to the cell membrane may not purely be a stretch stimuli. TREK-1 whole-cell or single channel recordings peformed when the whole cell is stretched would be ideal although technically difficult. Methods that have been employed include using a pipette to squeeze the cell against the coverslip (Hu and Sachs, 1996) or using carbon fibres (Hongo, et. al., 1996) or microglasstool tips (Sasaki, et al., 1992) to stretch the cell.

4.1.2 Experiments to investigate the distribution of TREK-1 channels in the human heart

More experiments could be performed to investigate the distribution of stretch-activated channels, especially in the human heart. It is very likely that there is a heterogenous distribution of these channels in the human heart as different regions of the heart might experience different amounts of mechanical stretch. These channels might not only be heterogeneously expressed in the epi - and endocardial cells, but they might also be variably expressed in the different

chambers of the heart. A change in channel population might also occur in diseased states. It is also possible that the expression of the channels might differ throughout the different stages of life. Changes in expression of certain channels during development has been seen in studies of voltage-activated channels, for example, it has been found that at early stages of development, Ca^{2+} channels are dominant in excitation, while at later stages, Na^{+} channels become dominant (McDonald and Sachs,1975; Fujii, et. al., 1988; Davies, et. al., 1996). Another method to determine the population of TREK-1 channel proteins is by using immunohistochemistry assays. These experiments involve using monoclonal antibodies carrying flourescent tags that would bind to the channel protein. The higher the flourescence that can be detected, the higher the population of channel protein. These experiments are possible to perform as there are TREK-1-specific antibodies available.

4.1.3 Experiments involving isolated perfused hearts to investigate the role of cardiac stretch-activated channels

The Frank-Starling law states that the strength of contraction of the muscle increases with increasing amounts of applied stretch. This increase in contractility is thought to be due to an increase in the calcium release from the sarcoplasmic reticulum and a reduction in the spacing between the actin and myosin filaments. There has not been any mention on the contribution of stretch-activated channels. As activation of mechanosensitive channels can influence the shape and duration

of cardiac action potentials, these channels would theoretically influence cardiac contractility when the myocardium is mechanically stretched. To determine the role of SACs on the Frank-Starling curve, experiments involving isolated hearts attached to the Langendorff perfusion system can be performed. Using this method, the ventricle can be given different amounts of stretch via an intraventricular balloon. The contribution of SACs to the Frank-Starling curve can be determined by comparing results obtained from experiments performed with a blocker or activator of these channels to data obtained from control experiments.

Another worthwhile study is to investigate the effects of SACs on the electrical responses of the heart when stretch is applied at different phases of the cardiac cycle. An electronic circuit that can trigger a stretch pulse at different time intervals can be used for this experiment and monophasic action potentials can be recorded from the surface of the heart using suction electrodes (Babuty and Lab, 2001). An activator or blocker of SACs can be used to observe the effects of blocking or activating SACs.

As fibrillation commonly occurs in association with passive stretching of the myocardium, experiments could be performed to investigate whether SACs are involved in the development of the stretch-induced fibrillation. The contribution of SACs to stretch-induced fibrillation could be determined by

comparing the amount of stretch required to trigger fibrillation with and without a SAC blocker or activator.

To observe the effects of cationic non-selective SACs, experiments can be performed using toxin of Grammastola spatulata, a specific blocker of cationic non-selective SACs (Niggel, et. al., 1996). The contribution of TREK-1 channels can be investigated by altering the intracellular pH of cardiac myocytes to change the activity of these channels. To induce intracellular acidosis, solutions with HCO_3^- are commonly used (Maingret, et. al., 1999). Alternatively, chloroform [which in the heart, activates only TREK-1 channels (Terrenoire, et. al., 2001)], could be added into the perfusates to raise the activity of these channels. However, the effects observed when changing the intracellular pH or perfusing the heart with solutions containing chloroform may not be solely due to TREK-1 channel activation as other cardiac channels might also be affected by these treatments (Mongo and Vassort, 1990; Moncada, et. al., 2000; Ito, et. al., 1992). A specific modulator of TREK-1 activity would be useful for these experiments but at present such an agent is still unavailable.

As most life-threatening ventricular arrhythmias occur in patients with myopathic ventricles, it would be relevant to perform whole-heart experiments using animals with induced cardiac disorders. For example, right ventricular congestive failure in rats can be induced by a intravenous administration of monocrotaline (Comini, et. al., 1996; Agnoletti, et. al., 1990; Jones, et. al., 2002).

Monocrotaline causes impairment of blood circulation in the lungs. The right ventricles would be under a high level of stretch as blood is not readily pumped through the pulmonary blood vessels. To induce an *in vivo* situation where the left ventricles are chronically stretched, the aorta can be constricted by ligation

4.2 CONCLUDING REMARKS

Ventricular arrhythmias have been found to be caused by abnormalities in impulse formation and conduction (Hoffman and Cranefield, 1964; Hoffman and Rosen, 1981). However, these electrophysiological mechanisms do not explain why most deaths from arrhythmias happen in patients with regionally or globally dilated ventricles and heart failure (Taggart, 1996). In pathological conditions where the heart experiences unphysiological amounts of stretch, triggered activity and re-entry are likely to occur. These stretch-induced phenomena can cause ventricular arrhythmias.

The drugs currently used to treat heart diseases mainly target voltage-activated channels, beta adrenoceptors or phosphodiesterases. These pharmacological agents, eg. digitalis, dobutamine and milrinone have been found to promote serious ventricular ectopy in cardiac patients (Steiness and Olesen, 1976; David and Zaks, 1986; Ludmer, et. al., 1987). Hence, more efficacious drugs are required to treat and prevent deaths from cardiac disorders. Treatment of arrhythmias by agents that can modulate the activity of stretch-activated

channels should be given serious considerations. At present, there is a specific blocker of cationic non-selective SACs, Grammastola spatulata toxin (Niggel, et. al, 1996), which would enable researchers to look at the effects of blocking cardiac cationic non-selective channels. In fact, there have been observations from previous experiments that seem to suggest that cationic SACs are involved in stretch-induced arrhythmias, as this phenomena can be blocked by inhibitors of these channels (Hansen, et. al., 1991; Stacy, et. al., 1992; Suchyna, et. al. 2000; Hu and Sachs, 1996). However, as there is still no known unequivocal blocker or activator of TREK-1, it is difficult to investigate the roles of these potassium-selective SACs in the heart. Since TREK-1 channels are potassium-selective, the activation of these channels would theoretically induce action potential shortening which can predispose ailing hearts to re-entry arrhythmias and premature excitations. On the other hand, TREK-1 activation might also play a role in preventing arrhythmias by hyperpolarising the membrane potential and thus suppressing the formation of ectopic beats in hearts experiencing pathological amounts of stretch. TREK-1 activation may also be beneficial in diseased hearts as it may prevent or reduce detrimental electrical dispersions in injured regions of the heart, which may have defective electrical conduction.

TREK-1 can be activated by stretch as well as membrane crenators such as unsaturated fatty acids. Positive amphipaths which are membrane cup-formers on the other hand cause inactivation of the channel (Patel, et. al., 2001). It may therefore be possible to develop a drug based on the fatty acid molecule to

modulate the activity of these channels. The binding site of volatile anesthetics could also potentially be a site targetted by agents to control the activity of the channel. During cardiac ischemia, phospholipase A_2 (PLA_2) is activated, causing the release of arachidonic acid which in turn can activate TREK-1. New drugs which target PLA_2 or its activation pathway might also be efficacious in treating arrhythmias. Other target avenues could include the phosphorylation sites or the pathways of PKA and PKC as TREK-1 is inhibited by activators of these protein kinases. However, therapeutic manipulation of the ion channels by cellular signalling pathways is difficult and other channels besides the targetted one might be affected.

The study of SACs pathways in cardiac mechano-transduction is still in its early stages. A better knowledge of SACs and their involvement in the mechanical modulation of the heart is important for a fuller understanding of the physiology and pathophysiology of the cardiac system. Such knowledge should be useful for the development of more efficacious antiarrhythmic treatments in future. A major problem with targetting stretch-activated channels for therapeutic purposes is that these channels are usually found in more than one tissue type. Therefore, one might expect some undesirable side-effects from such a drug. This is however also true for other voltage-dependent channels targetted by anti-arrhythmic drugs and therefore researchers should not be discouraged from efforts to use SAC modulators for anti-arrhythmic therapy.

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