A PATCH CLAMP STUDY OF PHARMACOLOGICAL AND PHYSIOLOGICAL REGULATORS OF LARGE CONDUCTANCE CALCIUM-ACTIVATED POTASSIUM CHANNELS IN ARTERIAL SMOOTH MUSCLE CELLS.

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by

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A patch-clamp study of pharmacological and physiological regulators of large conductance calcium activated potassium (BK_{Ca}) channels in arterial smooth muscle cells.

Electromechanical coupling is an important regulator of vascular smooth muscle contractility, with membrane hyperpolarisation and depolarisation producing vasorelaxation and vasoconstriction respectively. In the 1980s a group of vasodilator drugs were found to possess a common mechanism which involved opening K_{ATP} channels located in the cell membrane of smooth muscle cells, leading to vasorelaxation via membrane hyperpolarisation. A number of additional K^+ channels have been identified in vascular smooth muscle cells and one of these channels, the large conductance calcium-activated K^+ channel (BK_{Ca}), is a promising therapeutic target.

This thesis uses the various configurations of the patch-clamp technique to examine the effects of NS1619, nitric oxide (NO) and compounds resulting from the activation of the cGMP signalling pathway by NO, on the activity of ion channels.

Using single smooth muscle cells enzymatically isolated from the rat basilar artery, NS1619 was confirmed as an effective BK_{Ca} channel opener and hyperpolarising agent. Studies of the mechanism of action of NS1619 concluded that it has a direct effect on the BK_{Ca} channel itself or an associated regulatory site, possibly leading to an increase in the Ca^{2+}-sensitivity of the BK_{Ca} channel. NS1619 also blocked at least two other channels, voltage-activated K^+ channels and DHP-sensitive Ca^{2+} channels, and this latter effect almost certainly explains the functional vasorelaxation produced by NS1619, actions which will certainly limit the use of NS1619 in defining physiological roles for BK_{Ca} channels..

Nitric oxide activated whole cell currents when applied to single smooth muscle cells, which were blocked by specific BK_{Ca} channel blockers. Additional experiments determined that this action of NO could not be explained by a direct effect on BK_{Ca} channels but probably occurred by a mechanism involving a phosphorylation reaction catalysed by cGMP-dependent protein kinase. This stimulatory effect of cGMP-dependent protein kinase on BK_{Ca} channels may be involved in the vasorelaxation produced by nitric oxide and nitrovasodilators.

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CONTENTS

CHAPTER ONE Introduction

This chapter will introduce the concept of what ion channels actually are and will attempt to clarify their role in the regulation of the membrane potential of vascular smooth muscle and how this relates to vascular contractility or tone.

1.1 Historical perspectives: Ringer to Neher

The first demonstration that ions play a pivotal role in the excitability of living cells was provided by a series of pioneering experiments performed by Sidney Ringer in the 1880s. He demonstrated that for an isolated frog heart to continue beating, the perfusing solution must contain Na^+, K^+ and Ca^{2+} in definite proportions. Later, Julius Bernstein, at the beginning of this century, proposed that excitable cells were surrounded by a membrane which was selectively permeable to K^+ ions at rest, but during excitation its permeability to other ions increased, via a process he termed 'membrane breakdown'. His membrane hypothesis explained why the resting membrane potential of excitable cells was negative, as a result of the diffusion potential that developed as positively charged K^+ ions diffused from an area of high concentration, in the cytoplasm, to a low concentration in the extracellular solution. Critically, Bernstein also found that the injury potential of cut fibres was close to the potassium equilibrium potential (E_K) predicted by the Nernst equation for the known K^+ concentrations.

Kenneth Cole and Howard Curtis in the late 1930s demonstrated, using squid giant axons, that during the nerve action potential the resistance of the axon cell membrane was greatly reduced, when compared to its resting value. This confirmed Bernstein's theory that the permeability of the membrane increased during excitation, although his idea of membrane breakdown had to be modified, as the conductance of the membrane during the action potential was far lower (1 millionth) than the equivalent thickness of seawater, implying that actual membrane breakdown did not occur. Alan Hodgkin and Andrew Huxley and Curtis and Cole in the late 1930s and early 1940s finally succeeded in measuring the full action potential of an axon using an intracellular micropipette. This led Hodgkin and Huxley in the 1950s, using voltage-clamp techniques, to propose that the current comprising the nerve action potential was composed of two major components: a sodium and a potassium current, I_{Na} and I_K.

This was quickly followed in 1955 by another study by Hodgkin, on this occasion working with Richard Keynes, into the K^+ permeability of nerve axons, where they proposed that the movement of radioactive K^+ was best explained if the K^+ ions passed through discrete 'channels' located in the axon membrane. Therefore, the idea of distinct ion channels, which were responsible for the movement of ions across the cell membrane, was proposed approximately twenty years before Bert Sakmann and Erwin Neher in the late 1970s and early 1980s, developed and refined the revolutionary patch-clamp technique. This powerful technique, for which Sakmann and Neher received the Nobel prize for Physiology and Medicine in 1991, allowed for the first time the tiny currents (10^{-12} of an amp), which flow through individual ion channels when they open, to be resolved and the properties of the channels and the accompanying currents to be studied (for general reviews of the patch-clamp technique and its applications see Cahalan & Neher, 1992; Gibb, 1995; Aidley & Stanfield, 1996; Standen *et al.*, 1997)

1.2 Ion channels

The ability to record the currents that flow through ion channels inevitably leads to the question: What are ion channels? A general definition of an ion channel is of a protein anchored in the lipid bi-layer of the cell membrane, where it forms an aqueous, membrane spanning pore through which ions can diffuse down their electrochemical gradient when the pore is open. The conducting pathway of the ion channels can be composed of either a single peptide, for example Na^+ and Ca^{2+} channels, or the co-assembly of a number of peptide subunits, as is the case for voltage-activated K^+ (K_V) channels. Additional channel subunits may also be present which may regulate channel function.

1.2.1 General properties

1.2.2 The channel pore. For ions to diffuse across the plasma membrane, a channel pore of some kind must exist. The pore is assumed to be a water filled space which allows ions to diffuse through it. The concept of an actual membrane pore was first discussed in the 1840s but took until the 1970s to be universally accepted. Major evidence that ion channels are pores rather than carriers is that ion channels move millions of ions per second whereas no known carrier systems approach such a speed.

If a K^+ channel for example has a unitary current amplitude of 5 pA, then the number of ions flowing when the channel opens is given by Equation 1:

Current × Avogadro's number / Faraday's constant [1]

which equals $5 \times 10^{-12} \times 6 \times 10^{23} / 96500$

$=3.1 \times 10^{7}$ ions s^{-1}

Thus the rapid movement of ions must represent their movement through a pore or channel rather than via carrier mediated systems which are slower than the rate of ionic flux.

1.2.3 Selective permeability. The majority of ion channels are highly permeable to certain ions but not to others, in other words they exhibit selectivity. K^+ ions, for example, pass through the K^+ channel pore ten thousand times more readily than the smaller Na^+ ion. Therefore, when an ion channel opens only the permeant species passes through the channel down its electrochemical gradient (notable exceptions are non-selective cation channels). How does an ion channel achieve such selectivity?

One mechanism proposed to account for the selective nature of voltage-activated Na^+ channels is that they possess a specialised structure called a 'selectivity filter' This term was first coined by the celebrated American biophysicist Bertil Hille to describe a putative short, narrow area of the channel pore which confers ionic selectivity (Hille, 1971; 1972; for general accounts of the processes involved in producing channel selectivity see Hille, 1992; Aidley & Stanfield, 1996, for reviews see Tsien *et al.*, 1987; McCleskey 1994). For a channel to be highly selective, Hille reasoned, the pore must be narrow enough to force permeating ions into direct contact with part of the channel protein so they can be somehow 'sensed' by an interaction with this selectivity filter (Hille, 1971, 1972). The simplest way of thinking of a selectivity filter is one which purely differentiates between ions on the basis of size and shape, with only ions smaller than the dimensions of the filter passing through the channel. Hille determined that the permeability of cations through voltage-activated Na^+ channels decreased as the atomic radius of the ion increased, therefore indicating that an actual physical cut off point existed and ions larger than this critical size were impermeant.

Hille also found that all the ions which permeated voltage-activated Na^+ channels shared the ability to form hydrogen bonds with oxygen atoms that lined the selectivity filter. Hille predicted, using data from permeation studies, that the selectivity filter in a voltage-activated Na^+ channel would have a radius of ~3Å and a length of ~5Å. A filter of these dimensions would allow a Na^+ ion with one water molecule bonded to the Na^+ ion in its transverse plane to pass through.

A physical 'hole' however cannot fully explain the selectivity filter. Na^+ ions for example are smaller than K^+ ions but they do not readily pass through K^+ channels. Hille suggested that when an ion enters the narrow selectivity filter it is forced to shed most of its hydration shell, that is the water molecules that surround the ion and form ion to dipole bonds with it in solution (see Hille, 1992). The ion-dipole bonds to the oxygen of water molecules are replaced by similar bonds to oxygen atoms that line the selectivity filter and act as 'surrogate' water molecules. If these oxygen atoms are fairly rigid structures that bond a K^+ ion, then it follows that a structure that is smaller than a K^+ ion, such a Na^+ ion (the crystal radius of a K^+ ion is 1.33 Å, the crystal radius of a Na^+ ion is 0.95 Å), will not form the same number of bonds and will not enter the selectivity filter so readily. The K^+ channel is thought to contain three or more binding sites with different affinities for K^+ within the pore (see Hille, 1992). Permeation of K^+ ions through the K^+ channel is achieved by several K^+ ions occupying the various binding sites within the pore and electrostatic repulsion 'shunts' the ion which occupies the lowest affinity sites into the cell.

An alternative attempt to solve the puzzle of selective permeability has focused on voltage activated Ca^{2+} channels. Voltage-activated Ca^{2+} channels are amongst the most selective ion channels known, selecting for Ca^{2+} ions over their main competitor Na^+ ions by a ratio of ~1000:1 (Tsien *et al.*, 1987). This is despite Na^+ and Ca^{2+} ions having similar crystal radii (Ca^{2+} 0.99Å, Na^+ 0.95Å) and the presence of an electrochemical gradient which favours the influx of both of these ions (however it should be noted the chemical gradient for Ca^{2+} ions is far greater ($[Ca^{2+}]_o/[Ca^{2+}]_i$ =15000, $[Na^+]_o/[Na^+]_i$ =12). Also the pore diameter of voltage-activated Ca^{2+} channels is as wide as the nicotinic acetylcholine receptor pore, which shows little selectivity. This indicates that the channel selects for Ca^{2+} by a different mechanism than the molecular 'sieve' model used to explain the selectivity of voltage-activated Na^+ channels by Hille. Interestingly, when the external $[Ca^{2+}]$ is reduced, voltage-activated Ca^{2+} channels become permeable to Na^+, illustrating that the selectivity of the channel is dependent on the presence of extracellular Ca^{2+}.

This observation led to the proposal that each Ca^{2+} channel selects Ca^{2+} through two high affinity binding sites (K_D 0.7 μM) located within the channel pore (Tsien *et al.*, 1987). When submicromolar Ca^{2+} is present extracellularly these sites are unoccupied by Ca^{2+} so Na^+ ions freely pass through the channel. When however a physiological extracellular Ca^{2+} concentration exists the high affinity sites are saturated with Ca^{2+} ions and electrostatic repulsion rejects Na^+ ions, therefore producing selectivity, and also 'shunts' Ca^{2+} ions through the channel into the cell (Hess & Tsien, 1984).

This model was recently modified following the discovery that four glutamate residues, one donated by each structural repeat, reside at an equivalent position in the pore region (H5) of voltage-activated Ca^{2+} channels but not in K^+ or Na^+ channels (Yang *et al.*, 1993). The removal of one of these residues significantly altered the affinity of the pore for Ca^{2+} (Yang *et al.*, 1993). If two identical and independent Ca^{2+}-binding sites existed, this type of result would not have been expected as one high affinity binding site would still be present. Yang and co-workers therefore proposed an alternative model, namely that the four glutamate residues together form a single high-affinity Ca^{2+} 'pocket' which initially binds one Ca^{2+} ion. The high affinity of this site for Ca^{2+} and the positive charge on the Ca^{2+} ion serves to repel Na^+ entry, therefore producing a Ca^{2+} selective channel. When a second Ca^{2+} ion enters the pore the glutamate residues are 'shared' between the two Ca^{2+} ions which basically produces two lower affinity Ca^{2+} binding sites. The diminished affinity of the binding sites ensures that the first Ca^{2+} ion moves into the cell due to electrostatic repulsion between it and the second Ca^{2+} ion. Electrostatic repulsion therefore serves to maintain ionic selectivity and produce permeation of the ionic species. In summary two mechanisms therefore appear to endow individual ion channels with selectivity:

a). A selectivity filter.

b). The availability of multiple high affinity binding sites for the selectively permeant species.

1.2.4 A voltage sensor. Many ion channels are voltage-dependent and possess a structure that allows the channel to detect changes in the membrane potential and respond accordingly. What and where is this voltage sensor?

Following the cloning of voltage-activated channels, the so-called S4 region (see section 1.4 on general molecular structure of ion channels) of the channel α subunit was identified as the most likely voltage sensor (Noda *et al.*, 1984 for review see Catterall, 1986). This idea was based on the extreme conservation of this S4 region amongst voltage-dependent channels and the net positive charge of this region, due to the presence of positively charged lysine or arginine amino acid residues at every third position on the S4 helix. Point mutation studies using cloned voltage-activated Na^+ channels demonstrated that neutralising positively charged residues in the S4 region, with either neutral or negatively charged residues, reduced the gating charge, decreased the voltage-dependence and altered the gating properties of the mutant channels (Stuhmer *et al.*, 1989). William Catterall proposed a working model, the *sliding helix model*, to explain how movement of the S4 region resulted in voltage-dependent activation of Na^+ channels (Catterall, 1986). Caterall suggested that at rest the positive charged residues on S4 are paired with fixed negative charges on other transmembrane segments of the channel. These forces, together with the negative internal membrane potential serve to hold the S4 helix in position. Upon depolarisation, the forces holding S4 are reduced and S4 undergoes a 'screwlike' outward movement of ~5 Å and a rotation of 60°. This creates a gating current resulting from an unpaired positive charge on the outward surface of the membrane and an unpaired negative charge on the inward surface of the membrane. This process moves positively charged residues away from the inner pore of the channel, where they may function as an electrostatic barrier which arrests the passage of cations. Alternatively, movement of the S4 region may uncover charged residues which may initiate a subsequent conformational change in the channel structure, moving a 'gate', possibly the S4-S5 region (Isacoff *et al.*, 1991), away from the mouth of the pore enabling ions to flow through the channel. Support for the sliding helix model was recently provided when the movement of the S4 segment in K_V channels was shown to correlate with the production of a gating current (Mannuzzu *et al.*, 1996). Using fluorescent labels Mannuzzu and her colleagues discovered that a stretch of at least seven amino acids of the S4 region moved from a 'buried' position into the extracellular environment and produced the gating current, so providing actual physical evidence that S4 is the voltage sensor of voltage-activated channels.

1.2.5 Channel gate. Integral with the concept of a voltage sensor is the idea of an area of the channel protein which acts as a voltage-dependent 'gate', controlling transmembrane ionic flux.

These gates are thought to move in response to the movement of dipoles in the S4 region following membrane depolarisation. What therefore are these gates? Many theories have been proposed to explain the nature of channel gates (see Hille, 1992 for an account of possible gating mechanisms). These include ideas such as sliding doors and soluble or tethered gating particles or a 'field effect', where a local electrical potential actually within the pore attracts or repels permeant ions. Clay Armstrong in the early 1970's discovered that perfusing pronase, a mixture of proteolytic enzymes, into squid giant axons abolished the rapid Na^+ channel inactivation without affecting the K_V current, suggesting that Na^+ channel inactivation was dependent upon some mobile component of the channel protein located on the inside of the membrane (Armstrong *et al.*, 1973). Similarly fast inactivation of *Shaker* K^+ channels can also be removed by treatment of the cytoplasmic side of the cell membrane with proteases (Hoshi *et al.*, 1990). This led to the 'ball and chain' model to explain this effect. Briefly this model predicts a mobile positively charged 'ball' probably located at the amino- (N) terminal region, which is tethered to the channel by a 'chain'. When the channel opens the ball is displaced from a series of negatively charged residues site close to the pore and Na^+ or K^+ ions briefly flow through the open channel, before the ball 'swings' back into place blocking further permeation of ions. Na^+ channel inactivation is thought to be voltage-independent as no current associated with channel inactivation has been reported (Catterall, 1986).

1.3 Gating

1.3.1 Classification

Ion channels are usually broadly classified according to their ionic selectivity and the various processes that influence the opening and closing or *gating* of the channel. A channel selective to K^+ and activated by depolarising potentials will by this definition be termed a voltage-activated K^+ channel, which is usually abbreviated to K_V channel. More recently an alternative way of classifying channels is to group them together according to their molecular structure.

If, for example, a K^+ channel is isolated, cloned and its predicted molecular structure is similar to the previously identified *Shaker* Kv channel (K_V denotes a voltage-activated K^+ channel; Kv denotes genetic material which encodes the expression of a voltage-activated K^+ channel subunit see section 1.6.4 for more detailed description) it will be given the prefix Kv and grouped together with similar genes encoding *shaker*-like K_V channels. Gating is the act of the channel 'gate' opening or closing and is influenced by three main processes:

1.3.2 Voltage-activated. Many channels, for example voltage-activated Na^+, Ca^{2+} channels and K_V channels are thought to undergo a structural rearrangement following depolarisation of the cell membrane. Hodgkin and Huxley, for example, in their classic description of the delayed I_K published in 1952, proposed that four charged gating particles needed to become aligned for the K^+ channel to open (Hodgkin & Huxley, 1952). Kinetic models proposed that K_V channels exist in several conformational states which may correspond to the respective movement of the gating particles proposed by Hodgkin and Huxley.
These are: C, closed resting state(s), C^{*n}, activated closed state(s), O, open state(s) and I, inactivated state(s) (for a detailed descriptions see Hille, 1992; Aidley & Stanfield, 1996 for general reviews see Pongs, 1992; Pallotta & Wagoner, 1992; Jan & Jan, 1992).

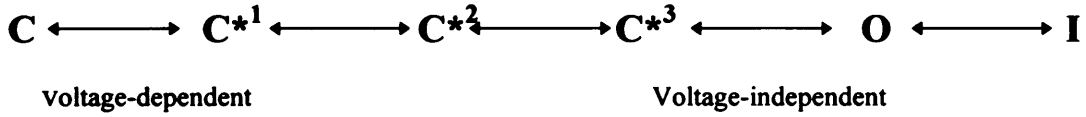


Figure 1.i Simple representation of the transitions between the various hypothetical conformational states in a voltage-activated K^+ channel

Using a K_V channel as an example, initially in the C state at the negative resting membrane potential. Following membrane depolarisation this C-K_V channel undergoes three voltage-dependent conformational changes into the C^{*3} state, possibly due to the movement of the S4 region in the channel subunits which comprise the K_V channel. This is followed by a voltage-independent conformational change into an O state where the 'gate' is moved, possibly by removing an unfavourable charge or a physical barrier from the pore. This opens the channel and allows the permeant ionic species, in this case K^+, to flow through the channel down its electrochemical gradient.

The movement of the charged S4 region in response to changes in the membrane potential produces small currents (0.3 % of the total inward Na^+ current in a squid giant axon) termed 'gating currents', which were first identified in the early 1970s (Armstrong & Bezanilla, 1973). Although first quantified in the 1970s, the existence of gating currents was predicted far earlier by Hodgkin and Huxley, who had envisaged the existence of currents associated with the movement of regulatory charged regions within the membrane immediately prior to the onset of the flow of ionic currents. The S4 region is therefore the most likely voltage sensor and architect of these gating currents in voltage-dependent channels. Following the movement of S4 the actual gate is opened by mechanisms which are not as yet understood.

1.3.3 Ligand-gated. A second group of channels shares one feature, that their gating can be influenced following the binding of a ligand. The ligand can be extremely diverse and includes synaptic neurotransmitters such as acetylcholine, adenosine triphosphate (ATP) the 'fuel' which drives most physiological processes, intracellular Ca^{2+} and intracellular second messengers such as cyclic guanosine monophosphate (cGMP). While these ligands are very different, they all share one common feature, that when they bind to either an external receptor or intracellular binding sites they directly influence channel gating. Two widely studied ligand gated ion channels are found in vascular smooth muscle. These are the large-conductance Ca^{2+} dependent potassium (BK_{Ca}) channel and the ATP-dependent potassium (K_{ATP}) channel which are discussed in more detail later.

1.3.4 Stretch-activated. More recently a third group of channels have been described whose gating is influenced by physically distending the cell membrane, a property which has led these channels to be dubbed stretch-activated or mechanosensitive ion channels. Mechanosensitive ion channels were first identified in the membranes of skeletal muscle cells (Guharay & Sachs, 1984) and have subsequently been identified in almost every cell type studied, including vascular smooth muscle (for reviews see Nakayama & Tanaka, 1993; Sackin, 1995; Bett & Sachs, 1997). Even though the identification of mechanosensitive ion channels occurred fairly recently, the initial observation that blood vessels could respond to physical stimuli was reported at the turn of the century by Bayliss, and the phenomenon of stretch producing depolarisation and contraction of vascular smooth muscle was described as early as the 1950s.

In vascular smooth muscle, for example, activation of mechanosensitive Ca^{2+} channels may be partly responsible for stretch-induced arterial contraction, as demonstrated by the ability of Ca^{2+} antagonists to attenuate a stretch-induced contraction (Brayden & Nelson, 1992; Nakayama & Tanaka, 1993). Physiologically, therefore, the activation of mechanosensitive Ca^{2+} and cationic channels in vascular smooth muscle may be important during autoregulatory vasoconstriction in response to increasing intravascular pressure.

1.4 General molecular structure of ion channels

To avoid unnecessary repetition with later sections where the molecular structures of the various K^+ channels are described in greater detail, this section will deal with the basic molecular structures of ion channels, using Na^+ and K^+ channels as examples.

Attempts to determine the molecular structure of ion channels began with the actual isolation of the voltage-activated Na^+ channel protein (Noda *et al.*, 1984), and the determination of its molecular structure, a process made possible by two discoveries.

1. The isolation of tetrodotoxin (TTX), a neurotoxin, from the *fugu* puffer fish which binds with high affinity and selectivity to Na^+ channels.

2. The finding that the electric eel, *Electrophorus electricus*, possesses a highly specialised electric organ, the *electroplax*, which it uses to stun prey. The electroplax was subsequently found to contain enormous numbers of Na^+ channels.

TTX was used to label and isolate the Na^+ channel protein and hydrophobicity studies revealed that it consisted of an α subunit composed of four homologous repeats or domains with approximately 24 putative transmembrane α helices connected by extra-and intracellular loops (Noda *et al.*, 1984; for comprehensive account see Aidley & Stanfield, 1996). Each of these individual domains possess six putative membrane spanning α helices, labelled S1-S6 (Noda *et al.*, 1984) and the four domains form the so-called α subunit which are assumed to cluster together to form the functional ion channel in the manner illustrated in figure 1.ii. The protein comprising voltage-gated Ca^{2+} channels was later purified in a similar manner using dihydropyridine Ca^{2+} channel blocking drugs as a high affinity ligand and was also found to be composed of a single protein chain with four homologous domains.

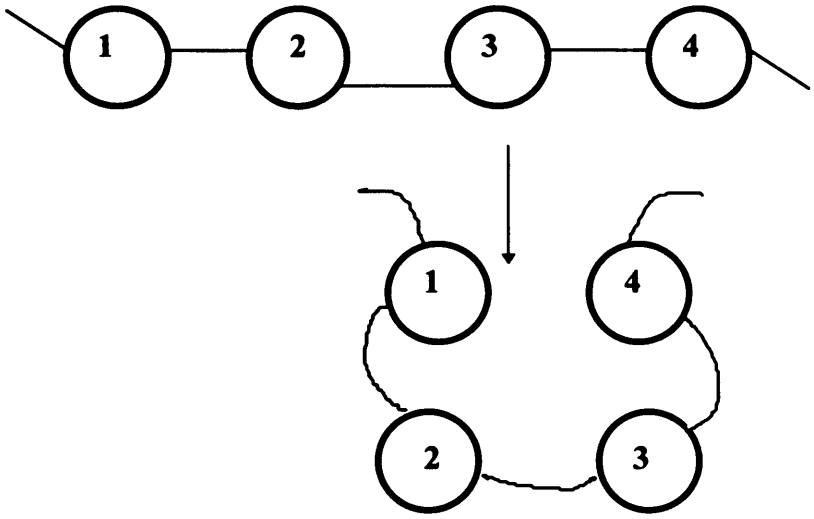


Figure 1.ii Composition of the α subunit of voltage-gated Na^+ and Ca^{2+} channels. 1-4 represent the four domains each of which is composed of 6 putative membrane spanning α helices, S1-S6.

Following the isolation and expression of K_V channels, using alternative methods to those used to isolate Na^+ and Ca^{2+} channels (described in greater detail later in this chapter), hydrophobicity studies predicted that the gene product, which is preceded by the prefix Kv, encoded a channel α subunit composed of 6 not 24 membrane spanning α helices (S1 to S6). This α subunit is equivalent to one of the four homologous repeats identified in voltage-activated Ca^{2+} and Na^+ channels. By analogy, therefore, with Na^+ and Ca^{2+} channels it is likely that functional Kv channels are formed by the assembly of four of these α subunits (see figure 1.iii) (see Pongs 1992; Jan & Jan, 1992).

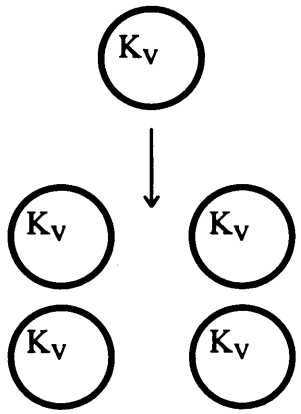


Figure 1.iii Co-assembly of 4 Kv α subunits to form a functional K_V channel

Two structures within each channel subunit are extremely important; these are the S4 α helix, which as previously described acts as a voltage sensor in all voltage-activated ion channels, and a region of ~20 amino acids between helices S5 and S6, the S5-S6 loop, which is also referred to as the H5 or P region, which may contribute to part of the channel pore and define some of the channel pharmacology (see figure 1.iv). The structure of S4 is a highly conserved feature of all voltage-activated channels, while the H5 region is highly conserved between Na^+, Ca^{2+} and K^+ channels. The first evidence that the location of the pore was close to the S5-S6 loop of K_V channels was provided when a negatively charged glutamate residue located at position 422 of the Kv subunit, which is located on the S5-S6 loop, was neutralised or replaced with a positively charged residue. This mutation resulted in reduced block of the channel by the positively charged toxin, charybdotoxin (CTX). The half inhibition constant for CTX block was increased from 4.2 nM to 14.5nM if the glutamate residue was replaced by the neutral residue glutamine and to 48 nM if it was replaced by the positively charged amino acid lysine, 3-12 fold reductions in the affinity of CTX for the channel respectively (Mackinnon & Miller, 1989). Another study reported that a stretch of 21 amino acids comprising the S5-S6 loop defined essential biophysical properties of the channel such as conductance and block of the channel by the tetraethylammonium ion (TEA^+) (Hartmann *et al.*, 1991). This elegant study used two related Kv channels with differing pore phenotypes; Kv2.1 which is blocked by internal TEA and Kv3.1 which is blocked by external TEA and whose K^+ conductance is 3 times larger than Kv2.1. When a region of 21 amino acids from the S5-S6 region of Kv2.1 was removed and replaced with the corresponding region from Kv3.1, the resulting chimeric channel had the properties expected of Kv3.1. This study established that this stretch of 21 amino acids comprised part, if not all of the pore region, including the sites that determine TEA^+ block and channel conductance.

Following the identification that the shaker gene encoded Kv channel α subunits an enormous number of related gene products were subsequently identified in vertebrates which also encoded Kv channel α subunits. This led to different nomenclature being employed to describe essentially the same channels until the common prefix, Kv, was proposed (Chandy, 1991). The Kv channel family is a genetic super-family and encodes numerous related but distinct gene products. This large number of related gene products (see Chandy, 1991) may co-assemble with different subunits and may explain the relative diversity of K^+ conductances when compared to Na^+ and Ca^{2+} conductances.

In addition to the α subunit, auxiliary subunits may also be present which can influence channel function. An excellent example of the effects of additional subunits was provided by studies of the large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel. The cloned α subunit (*slo*) alone of this channel, when expressed in purified cell systems, did not fully account for all the properties of native BK_{Ca} channels.

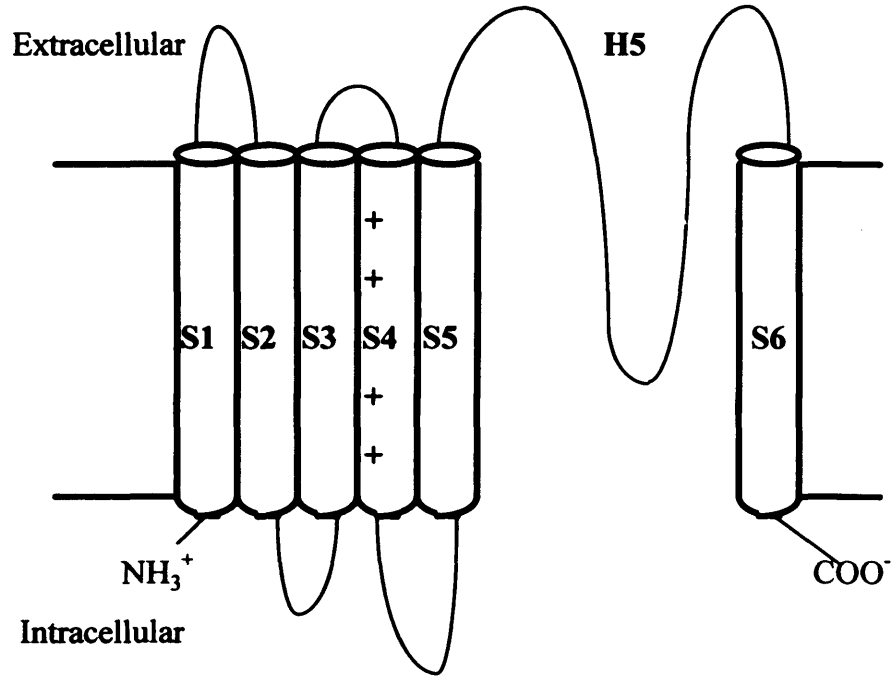


Figure 1.iv. Putative topological organisation of a region corresponding to a Kv channel α subunit or a single voltage-activated Na^+ channel domain

The lack of expression of a channel β subunit was later found to explain these differences (Kaczorowski *et al.*, 1996), and highlighted a problem incumbent when using molecular biological techniques to isolate ion channels; namely that the identification of important secondary structures which do not resemble the typical molecular structure of ion channels, but which are vital to channel function, may be neglected.

1.5 Vascular smooth muscle

1.5.1 Morphology and classification

Smooth muscle is anatomically and functionally distinct from skeletal muscle (see Guyton, 1991). Skeletal muscle cells for example, possess bundles of visible filaments composed of actin and myosin, termed myofibrils.

These myofibrils are aligned transversely and give skeletal muscle cells their characteristic banded or *striated* appearance. Actin and myosin are both present in smooth muscle but they are not arranged in regular arrays, consequently the striations are absent giving an apparent 'smooth' appearance. Skeletal muscle cells are also enormous multinucleated cells up to 80 μM in diameter and sometimes many centimetres long, whereas smooth muscle cells are small mononucleated, spindle shaped structures up to 5-10 μM in diameter and 20-500 μM long. There is a considerable degree of variation in the characteristics of smooth muscle in different parts of the body. However, for the sake of simplicity smooth muscle can be broadly divided into two major types:

1. Multiunit smooth muscle. This type of smooth muscle is composed of discrete smooth muscle fibres which operate independently of each other and are often innervated by a single nerve endings, with each nerve fibre controlling an individual smooth muscle cell. This characteristic contrasts with many vascular and visceral smooth muscles which lack innervations and are mainly controlled by other stimuli. Multiunit smooth muscle fibres are often covered with a mixture of collagen and glycoprotein which forms a thin basement membrane and acts to insulate the individual fibres. Examples of this type of smooth muscle are found in the iris and ciliary muscle of the eye, where fine graded involuntary contractions are required to maintain visual acuity, and also in the erector pili muscles in the skin, which respond to sympathetic stimuli by contracting and raising the hair to which the muscle is attached, a process termed *piloerection.*

2. Single-unit or visceral and vascular smooth muscle. Single-unit smooth muscle is the 'classic' type of smooth muscle. It consists of many individual smooth muscle cells arranged in sheets or bundles joined to adjacent cells via the cell membrane, allowing the contractile force generated in one fibre to be transmitted to adjacent. They are also electrically and chemically linked by numerous gap junctions through which ions can flow freely from cell to cell. This allows an electrical signal such as a depolarisation to flow from one cell to another, enabling all the smooth muscle cells to contract together as a single unit which is termed a functional syncytium. Single-unit smooth muscle can also be sub-divided into tonic and phasic smooth muscle:

1. Tonic smooth muscle. This type of smooth muscle is found throughout the vasculature and is the type of smooth muscle that has been the focus of the experiments described in this thesis. Tonic smooth muscle has a stable membrane potential, is electrically quiescent and responds to stimuli with slow, graded changes in membrane potential.

2. Phasic smooth muscle. This type of smooth muscle is found primarily in the walls of hollow viscera, for example the intestines and uterus. It is also present in some parts of the vasculature, the portal vein being a good example. Visceral smooth muscle has a characteristic unstable membrane potential and produces action potentials and continuous irregular contractions that occur independently of innervation.

1.5.2 Vascular smooth muscle and blood flow

Blood flows through blood vessels primarily due to the pressure caused by the pumping of the heart. The actual resistance to flow depends to a minor degree on the viscosity of the blood but mainly upon the diameter of the blood vessels, principally small arteries and arterioles.

1.5.3 Relationship between flow, pressure and resistance

If we discount the viscosity of blood, which only becomes a significant factor when the body suffers from extreme dehydration or if an individual suffers from polycythemia, a condition where the haematocrit increases dramatically raising blood viscosity, or severe anaemia where the haematocrit and viscosity fall, then the flow of blood through a vessel is determined by two factors:

1. The pressure difference between the two ends of a vessel which constitutes the force which pushes the blood through the vessel.

2. The resistance of the vasculature to the flow of blood, so-called vascular resistance.

The relationship between blood flow, blood pressure and vascular resistance is analogous to the relationship of current flow, potential difference and the electrical resistance in an electric circuit as defined by Ohms law which states:

Current = potential difference or voltage / resistance [2]

Blood flow can therefore be defined by the equation:

Flow = pressure difference (P_1-P_2) / resistance [3]

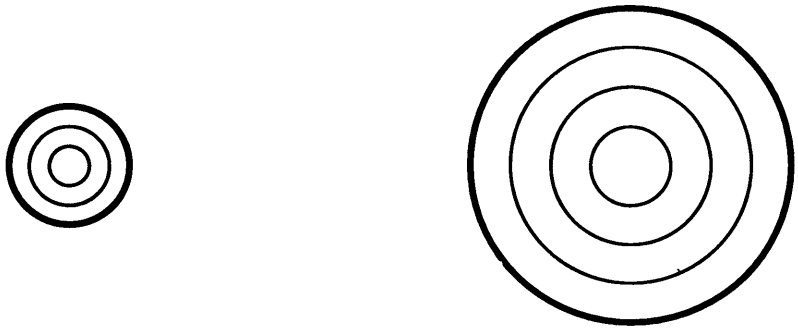
where the pressure difference is defined as the mean intraluminal pressure at the arterial end minus the mean pressure at the venous end. This formula states that blood flow is directly proportional to the pressure difference but inversely proportional to the resistance of the blood vessel. Vascular conductance, which is a measure of blood flow through a blood vessel at a given pressure difference, is also related to resistance. Vascular conductance, like electrical conductance, is defined as the reciprocal of resistance and is expressed by the equation:

Conductance = 1 / resistance [4]

Vascular diameter greatly influences conductance in a blood vessel and the application of physical principles can help explain why.

Conductance of perfect fluid in a rigid vessel is proportional to the fourth power of the diameter of the vessel. Although blood vessels are far from rigid tubes and blood is not a perfect solution but a two-phase system of liquid and cells, these principles do help to explain the relationship between blood flow, blood vessel diameter and resistance to blood flow. Because conductance is related to the fourth power of the diameter only slight changes in the diameter of a vessel significantly affect the conductance of the vessel. To better illustrate this point, consider fluid flowing through three separate vessels with relative diameters of 1, 2 and 4, with an identical pressure difference across the ends of the vessels. The conductance in each vessel will be given by the product of the fourth power of the diameter or 1^4, 2^4 and 4^4. Thus giving relative values for conductance of 1, 16 and 256 respectively. While the relative diameter of vessel 3 increased only 4-fold with respect to vessel 1 flow increased 256-fold.

To explain why these enormous increases in conductance occur following small increases in diameter, consider that figure 1.v represents transverse cross sections of two vessels with different diameters. The sets of concentric circles illustrate the different velocities of streamline or laminar flow of fluid in the vessel.



Small Vessel Large Vessel

Figure 1.v Concentric rings of flow of fluid in two vessel illustrating the increasing velocities of laminar flow; the farther away from the vessel wall the faster the flow.

If figure 1.v represents blood flow in two blood vessels then the velocity of the first ring of blood adjacent to the vessel wall is slow or even stationary. This is due to frictional drag of the large numbers of red cells in the blood with the vascular endothelium. The second ring avoids the endothelium and the first ring of blood and therefore slips past at a higher velocity. Likewise the second and third rings flow past at progressively increasing velocities. Obviously in larger vessels successive rings are further away from the wall of the vessel and are faster flowing, conversely in a smaller vessel essentially all the blood is close to the wall and the middle regions of faster flowing blood simply do not exist. By integrating the velocities of all the rings of fluid and multiplying these by the areas of the vessel, Equation 5 was derived by Poiseuille to explain the flow of fluid in tubes.

$$\mathbf{Q = \pi \Delta P r^4 / 8 \eta l} \qquad [5]$$

Where Q equals the rate of flow, ΔP is the pressure difference between the ends of the tube, r and, l are the radius and length of the tube respectively and η is the viscosity of the fluid. The rate of flow is directly proportional to the fourth power of the radius of the tube indicating that the diameter of the tube plays a pivotal role in determining the rate of flow.

Most of the resistance to blood flow in the systemic circulation is provided by small arteries and arterioles which are sometimes referred to as the resistance vessels. These vessels exist *in vivo* in a state of partial contraction termed 'tone' from which they can constrict further or dilate according to local metabolic demands and the demands of the circulation as a whole. Theoretically according to the fourth power law it is possible for a vessel to increase or decrease blood flow enormously. The mechanisms involved in controlling vessel diameter, if they can be manipulated pharmacologically, constitute potentially powerful vasodilator and vasoconstrictor mechanisms. Therapeutic agents acting via these mechanisms could be employed to raise or lower vascular resistance and increase or decrease blood flow and blood pressure accordingly. Pathophysiological conditions where it may be advantageous to increase the diameter of these so-called resistance vessels are essential hypertension and stroke.

1.5.4 Essential hypertension

Essential hypertension is a sustained elevation of the systemic arterial pressure of unknown origin. It is by far the commonest form of hypertensive disease and approximately 90 % of all individuals diagnosed as suffering from hypertension are defined as having essential hypertension. An individual is said to have hypertension when his or her mean arterial pressure is greater than 110 mm of Hg. This occurs when the diastolic pressure, where the heart is filling with blood, is >90 mmHg and the systolic pressure, when the heart is contracting is >140 mm Hg. Severe essential hypertension is characterised by the following vascular modifications:

1. A diffuse constriction of the arterioles which increases total peripheral resistance by 40-60 %.

2. An increase in the mean arterial pressure by a corresponding amount, 40-60 %.

3. A startling 2- to 4-fold increase in the resistance to kidney blood flow which decreases renal blood flow to approximately 50 % of its normal level. The maintained high arterial pressure may be an adaptive measure to overcome the increased resistance and decreased perfusion of the renal system to enable normal excretion of water and salt to occur.

Although patients with essential hypertension have a 'normal' cardiac output, the heart is obviously working harder to maintain this output, as demonstrated by the increased systolic pressure, when faced with the increased arterial resistance or 'pre-load' it is working against. One logical approach to the treatment of essential hypertension is therefore to reduce the preload. This can be achieved by increasing the diameter of the diffusely constricted arteries and arterioles which will clearly reduce the arterial resistance and, while blood flow may not increase to the fourth power of the radius, as proposed by Poiseuille's equation, it will certainly increase considerably. The work rate of the heart will be reduced accordingly as the pressure it is pumping against is decreased and the accompanying risk of, for example, myocardial infarction will be reduced. From a clinical point of view it is imperative that the mean arterial pressure is reduced as even moderate elevations of the mean arterial pressure can shorten life expectancy. The effects of hypertension can lead to the development of the following often fatal conditions:

1. Congestive heart disease or cardiac failure, a condition where cardiac output fails to meet the needs of the body, which develops due to the excessive workload placed on the heart as a result of the increased preload. This can lead ultimately to death by heart attack.

2. Stroke. The term *blood pressure* is actually defined as the force exerted by the blood against any unit area of the blood vessel wall. A mean arterial pressure of 110 mm Hg is therefore sufficient to raise a column of mercury to a height of 110 mm. A consequence of this increase in force is that it can rupture major blood vessels in the brain. This can lead to death or serious cerebral injury leading to disorders such as paralysis, blindness, epilepsy and dementia.

1.5.5 Excitation-contraction coupling in vascular smooth muscle

Anatomically, vascular smooth muscle cells are arranged in circular layers around arteries and in a single circular layer around arterioles. Consequently the diameter of the artery or arteriole and therefore blood flow and preload is proportional to the contractile status of this encircling smooth muscle 'jacket'. If the smooth muscle cells are contracted then the diameter of the vessel is reduced, resistance and preload are increased and conductance is reduced.

The smooth muscle layer in arteries and arterioles therefore constitutes a therapeutic target when attempting to dilate blood vessels and reduce the preload. The concentration of free intracellular Ca^{2+} is the single most important determinant of the degree of tone of vascular smooth muscle. This is because the free intracellular Ca^{2+} concentration in vascular smooth muscle cells ultimately determines the degree of excitation-contraction coupling in vascular smooth muscle cells and hence crossbridge cycling, contractility and ultimately the diameter of the blood vessel.

The events described below, and illustrated in the cartoons in figures 1.vi and 1.vii, occur following membrane depolarisation and a subsequent increase in the free cytoplasmic [Ca^{2+}] of vascular smooth muscle, principally due to Ca^{2+} influx through voltage-gated Ca^{2+}-channels (for recent comprehensive reviews of the mechanisms of smooth muscle contraction see Somlyo & Somlyo, 1994; Horowitz *et al.*, 1996). An increase in Ca^{2+} influx raises the intracellular free [Ca^{2+}] from a submicromolar concentration to ~1-10 μM. It should be noted however, that although the free [Ca^{2+}] in smooth muscle is very low, the total cellular [Ca^{2+}] is far higher, typically several millimolar. This is due to the presence of Ca^{2+} buffering molecules, such as calsequestrin and calreticulin, which are localised in the sarcoplasmic reticulum, and calmodulin which is present in the cytoplasm. Upon entering the cell Ca^{2+} ions bind to calmodulin, a small cytoplasmic protein capable of binding up to 4 Ca^{2+} ions, forming the Ca^{2+}-calmodulin complex. The Ca^{2+}-calmodulin complex is a versatile signalling molecule in biology and can combine with, and activate a number of different enzymes in a variety of systems producing Ca^{2+}-dependent activation. A number of examples of Ca^{2+}-dependent activation involving the Ca^{2+}-calmodulin complex are illustrated periodically throughout this thesis. In smooth muscle cells the Ca^{2+}-calmodulin complex associates with the regulatory subunit of the enzyme myosin light chain kinase (MLCK), activating the enzyme. Activated-MLCK is a very specific protein kinase, with only one recognised physiological substrate, a serine residue located at position 19 on the 20 kDa regulatory *light chain* of the enzyme myosin. Smooth muscle myosin is a hexamer and consists, following dissociation with detergents or denaturation, of 1 pair of heavy chains and 2 pairs of light chains which are covalently linked to form functional myosin. The light chains can be further distinguished on the basis of their respective molecular weights. One pair is 20 kDa (LC_{20}), the other 17 kDa (LC_{17}). It is LC_{20} which is phosphorylated at serine 19 by MLCK and this phosphorylation is the 'on-switch' which enables the ATPase activity of myosin to be activated by actin.

Actin is a globular, filamentous protein found in the contractile thin filaments, which are mostly aligned along the longitudinal axis of smooth muscle cells. In the cytoplasm, actin polymerises to form a two stranded helical thin filament which is long, rigid and insoluble. In smooth muscle cells myosin is also filamentous, forming so-called thick filaments. What is striking about each myosin molecule is that one end of the molecule is composed of a long rigid insoluble 'tail' and the other end consists of two 'head' structures, with one polypeptide from each set of light chains associated with each of these 'heads'. Small groups of 3-5 thick filaments are aligned, surrounded by many thin filaments which are connected to cytoplasmic and membrane-bound 'dense bodies'. These dense bodies are analogous to the Z-disks found in striated muscle cells, as they contain α-actinin and act as attachment points for numerous thin actin filaments. An extensive network of intermediate filaments composed of polymers of the protein desmin interconnect the dense bodies, forming a cytoskeletal network that transmits contractile force to the plasma membrane. The apparent anatomical arrangement of the thick, intermediate and thin filaments in smooth muscle cells is illustrated in figure 1.vii. The actual contraction of smooth muscle is produced by the 'sliding filament' mechanism. Very simply this involves each 'head' from the myosin molecule forming a 'crossbridge' by linking to an adjacent actin filament, forming the actomyosin complex. As mentioned above phosphorylated myosin when bound to actin is an ATPase and catalyses the hydrolysis of ATP to ADP and inorganic phosphate. The energy released following the breaking of the phosphate bond is harnessed and produces a conformational change in the crossbridge causing the thick and thin filaments to 'interdigitate' and pulling the thin actin filament along the thick myosin filament, producing cellular contraction and development of force.

A fall in the $[Ca^{2+}]_i$ leads to the inactivation of MLCK, allowing myosin light chain phosphatase to *dephosphorylate* the serine residue at position 19 on the regulatory light chain of MLC. Dephosphorylated myosin light chain heads do not form crossbridges with thick filaments, consequently no interdigitation of thick and thin filaments occurs leading to a slow relaxation of the smooth muscle.

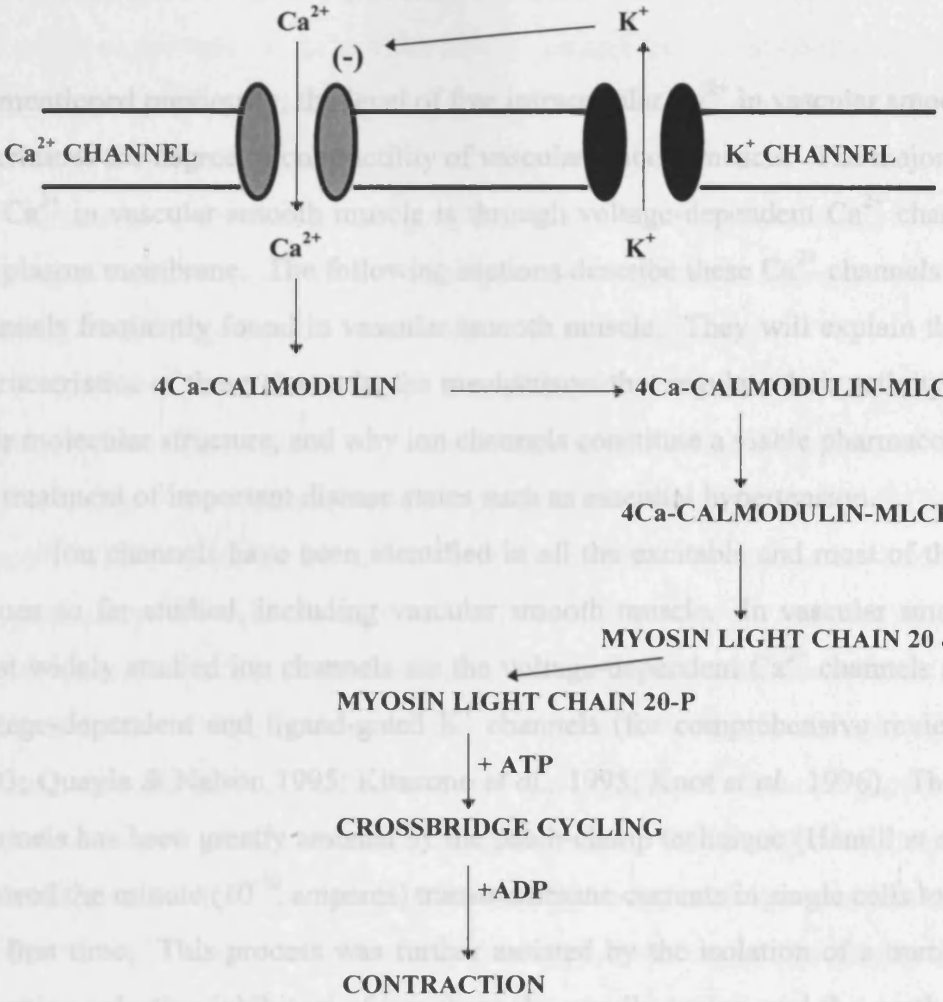


Figure 1.vi Simplified mechanism of excitation-contraction coupling in vascular smooth muscle

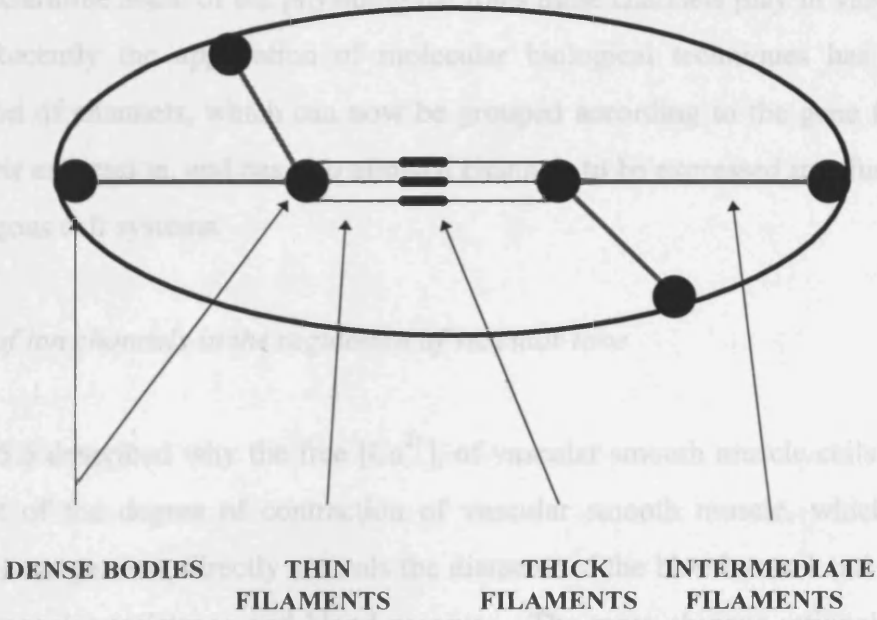


Figure 1.vii Apparent anatomical organisation of the cytoskeleton and myofibrils in smooth muscle cells.

1.6 Ion channels in vascular smooth muscle

As mentioned previously, the level of free intracellular Ca^{2+} in vascular smooth muscle cells determines the degree of contractility of vascular smooth muscle. The major influx pathway for Ca^{2+} in vascular smooth muscle is through voltage-dependent Ca^{2+} channels located in the plasma membrane. The following sections describe these Ca^{2+} channels and also the K^+ channels frequently found in vascular smooth muscle. They will explain the physiological characteristics of these channels, the mechanisms that regulate their activity with respect to their molecular structure, and why ion channels constitute a viable pharmacological target in the treatment of important disease states such as essential hypertension.

Ion channels have been identified in all the excitable and most of the non-excitable tissues so far studied, including vascular smooth muscle. In vascular smooth muscle the most widely studied ion channels are the voltage-dependent Ca^{2+} channels and a variety of voltage-dependent and ligand-gated K^+ channels (for comprehensive reviews see Nelson, 1993; Quayle & Nelson 1995; Kitazono *et al.*, 1995; Knot *et al.*, 1996). The study of these channels has been greatly assisted by the patch-clamp technique (Hamill *et al.*, 1981) which allowed the minute (10^{-12} amperes) transmembrane currents in single cells to be recorded for the first time. This process was further assisted by the isolation of a number of naturally occurring selective inhibitors of ion channels, usually toxins, and the synthesis of selective channel blocking drugs. A combination of patch-clamp and functional techniques has helped to determine some of the physiological roles these channels play in vascular smooth muscle. Recently the application of molecular biological techniques has assisted the identification of channels, which can now be grouped according to the gene family which encodes their expression, and has also allowed channels to be expressed in a functional state in heterologous cell systems.

1.6.1 Role of ion channels in the regulation of vascular tone

Sections 1.5.5 described why the free $[Ca^{2+}]_i$ of vascular smooth muscle cells is the major determinant of the degree of contraction of vascular smooth muscle, which, due to its anatomical arrangement, directly controls the diameter of the blood vessel and therefore the degree of vascular resistance and blood pressure. The most obvious rational approach to produce vasorelaxation is to actually stop or reduce the entry of Ca^{2+} into vascular smooth muscle cells.

The first group of drugs developed to inhibit Ca^{2+} entry were discovered over twenty years ago and are now referred to as the *calcium antagonists*. This chemically diverse group of drugs has a common mode of action, namely they reduce the amount of calcium entering vascular smooth muscle cells by blocking the major influx pathway for Ca^{2+}, plasmalemmal Ca^{2+} channels. By referring to figure 1.vi it is obvious that reducing the amount of Ca^{2+} entering the cell reduces the free $[Ca^{2+}]_i$, which will inhibit phosphorylation of MLC-20, reducing crossbridge formation and smooth muscle contraction.

In recent years a number of chemically diverse vasodilator compounds have been described whose mechanism of action involved increasing the efflux of K^+ from smooth muscle cells. Collectively termed potassium channel openers (KCO) and initially exemplified by a series of compounds synthesized by Beecham Research Ltd, these compounds were demonstrated to produce vasorelaxation by opening ATP-dependent K^+ (K_{ATP}) channels located in the smooth muscle cell membrane (for reviews see Cook 1988; Quast & Cook, 1989; Longman & Hamilton, 1992). How does the opening of K^+ channels produce vasodilation? To understand why the opening K^+ channels produces vasodilation, it is necessary to first understand what influences the gating of voltage-activated Ca^{2+} channels present in vascular smooth muscle cells, and the relationship between the likelihood that these Ca^{2+} channels are open, their *open probability* (P_{open}) and the membrane potential of the smooth muscle cells.

1.6.2 Calcium channels, the resting membrane potential and electromechanical coupling

Of the many different Ca^{2+} channels so far identified (see Hille, 1992), the channel most commonly found, and widely studied in vascular smooth muscle has three main characteristics:

1. It inactivates slowly following activation.

2. The P_{open} of this Ca^{2+} channel is reduced by 'classic' organic Ca^{2+} channel antagonists such as nifedipine (a dihydropyridine), verapamil (a phenylalkylamine) and diltiazem (a benzothiazepine).

3. It has a large single channel conductance (see table 1.i).

For the above reasons these channels are commonly referred to as L-type (long lasting or large conductance) or dihyropyridine (DHP)-sensitive Ca^{2+} channels, and this section will concentrate only on these channels.

The P_{open} of DHP-sensitive Ca^{2+} channels increases steeply with membrane depolarisation's between -60 and ~-10 mV. *E*-fold increases in channel P_{open} per 5-10 mV depolarisation from -60 mV for example, has been reported for DHP-sensitive Ca^{2+} channels in vascular smooth muscle (Nelson *et al.*, 1990; Smirnov & Aaronson, 1992b). Consequently only relatively small depolarisations of the cell membrane can substantially increase the P_{open} of DHP-sensitive Ca^{2+} channels, triggering Ca^{2+}-influx and smooth muscle contraction. This type of regulation of Ca^{2+} influx and tone by the membrane potential is an example of *electromechanical* coupling.

The resting membrane potential (RMP) of most vascular smooth muscle, which is a product of the activity of ion channels and certain ionic carrier mechanisms, is stable at rest, between -60 and -40 mV (Nelson & Quayle, 1995). With either physiological Ca^{2+} (1.5-2 mM) or Ba^{2+} (10-110 mM) extracellularly, various studies have reported that DHP-sensitive Ca^{2+} channels in vascular smooth muscle cells isolated from rabbit ear artery, rabbit basilar artery, human mesenteric arteries and rat cerebral arteries are open over a range of potentials equivalent (-60 to -40 mV) to those the cells would experience physiologically (Aaronson *et al.*, 1988; Nelson *et al.*, 1990; Smirnov & Aaronson, 1992b; Langton & Standen, 1993). The opening of these DHP-sensitive channels when the smooth muscle is at rest produces small, sustained, non-inactivating *window* currents, which occur as a result of the RMP lying in a 'voltage window' where inactivation of DHP-sensitive Ca^{2+} channels is incomplete and a small degree of channel activation subsequently occurs. This small steady-state Ca^{2+} influx therefore contributes to the basal level of contractile force and hence to the development of vascular tone. The studies performed by Smirnov and Aaronson (1992b) and Langton & Standen (1993) illustrated the presence of these window currents in human mesenteric and rat basilar arterial smooth muscle cells respectively. When steady state currents were recorded over a range of membrane voltages corresponding to the RMP (-58 to -38 mV), inward non-inactivating currents of ~5-8 pA through DHP-sensitive Ca^{2+} channels were detected (Smirnov & Aaronson, 1992b; Langton & Standen, 1993). Reducing Ca^{2+} influx by blocking or inhibiting this window current therefore constitutes a therapeutic target to decrease Ca^{2+} entry and produce vasorelaxation. The calcium antagonists obviously block the influx of Ca^{2+} but another mechanism to produce essentially the same effect uses the voltage dependence of DHP-sensitive Ca^{2+} channels.

The P_{open} of DHP-sensitive Ca^{2+} channels is reduced upon raising or *hyperpolarising* the membrane potential. Hyperpolarisation of vascular smooth muscle is possible because the RMP of vascular smooth muscle is positive to the K^+ equilibrium potential (E_K) (which is ~ -85 mV under physiological conditions). Hyperpolarisation can be produced by opening K^+ channels located in the plasma membrane. The resulting efflux of K^+, due to the existing favourable electrochemical gradient, produces membrane hyperpolarisation due to the net loss of positive charge from the cytoplasm of the smooth muscle cell. Raising the membrane potential from ~-60 mV nearer to E_K directly reduces the P_{open} of DHP-sensitive Ca^{2+} channels, in effect blocking the steady state 'window' Ca^{2+} current. This obviously reduces Ca^{2+} entry, lowers the free $[Ca^{2+}]_i$, ultimately producing relaxation of vascular smooth muscle due to inhibition of the Ca^{2+}-calmodulin dependent enzyme, MLCK.

To summarise, in the absence of other factors, Ca^{2+} influx through voltage-activated, DHP-sensitive Ca^{2+} channels is the major pathway controlling Ca^{2+} influx and hence the free-cytosolic $[Ca^{2+}]$ in vascular smooth muscle. Ca^{2+} entry can be reduced directly by blocking DHP-sensitive Ca^{2+} channels or indirectly by membrane hyperpolarisation which reduces the P_{open} of DHP-sensitive Ca^{2+} channels and blocks the window current through these channels.

It is unsurprising that numerous studies have demonstrated that manipulation of the membrane potential of vascular smooth muscle significantly influences the contractility of blood vessels *in vitro,* and that raising the membrane potential towards E_K, by for example activating K^+ channels, is a powerful mechanism to lower blood pressure by vasodilation (for reviews see Quayle & Nelson, 1995; Knott *et al.,* 1996). Charybdotoxin (CTX), a blocker of large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}), for example, was reported to depolarise and constrict rabbit cerebral arteries which had developed myogenic tone in response to an increase in the intraluminal pressure (Brayden & Nelson, 1992), whereas the K_{ATP} channel opener, cromakalim, hyperpolarised and relaxed similar arteries. These effects occur as a direct result of the electromechanical relationship between the membrane potential of smooth muscle and the degree of arterial tone. Changes in the membrane potential of only a few millivolts for example have been reported to significantly alter the free $[Ca^{2+}]_i$ of vascular smooth muscle and hence smooth muscle contractility and blood vessel diameter (Nelson *et al.,* 1990). A ± 3 mV change in the membrane potential, for example, has been reported to increase or decrease Ca^{2+} influx up to twofold (see Quayle & Nelson, 1995; Knot *et al.,* 1996).

1.6.3 Potassium channels in vascular smooth muscle: Physiology; pharmacology and molecular biology

To understand which K^+ channels affect the membrane potential of vascular smooth muscle one has to be aware of the different K^+ channels usually present in vascular smooth muscle, the range of potentials over which they are open, their putative physiological roles and important channel properties such as voltage-dependence, pharmacology and molecular structure. The following sections briefly describe these characteristics which are also summarised below in table 1.i.

	L-type Ca^{2+}	K_V	K_{ATP}	BK_{Ca}	K_{IR}
VOLTAGE DEPENDENCE	✓	✓	×	✓	✓
INHIBITORS & BLOCKERS	Nifedipine	4-AP K_D 200 μM-1.1 mM	Glibenclamide K_D 20-100 nM	IbTX and CTX K_D 1-10 nM	Ba^{2+} K_D 2 μM at -60 mV
ACTIVATORS	BAY K 8644, SDZ(+) 202-791	VIP?	Cromakalim Pinacidil, Nicorandil.	NS004, NS1619, DHS-I	None
UNITARY CONDUCTANCE	20-25pS with 110 mM Ba^{2+} externally	Small Conductance K_V Channel ~8 pS Large Conductance K_V Channel 50-70 pS	Small Conductance K_{ATP} Channel ~20 pS and ~50 pS Large Conductance K_{ATP} Channel ~100 pS	250 pS in high symmetrical K^+	?

Table 1.i. Summary of some of the Characteristics of the Channels Commonly Found in Vascular Smooth Muscle

1.6.4 Voltage-dependent K^+ channels or K_V channels

Results from numerous studies suggest that vascular smooth muscle preparations contain at least one K^+ channel which is activated by depolarisation of the cell membrane.

These channels are usually referred to as delayed rectifier K^+ (K_V) channels after Hodgkin and Huxley, who in their groundbreaking description of the current underlying the nerve action potential described an outwardly rectifying K^+ current which took longer to develop following depolarisation of the squid giant axon relative to the Na^+ current, hence *delayed rectifier* (for a review of K_V channels in vascular smooth muscle see Nelson & Quayle, 1995). K^+ efflux through K_V channels increases with depolarisation due to the voltage-dependent increase in channel P_{open} and also because depolarisation increases the electro-chemical driving force for K^+ efflux from the cell, therefore increasing the unitary conductance of the channels.

1.6.4.1 Role in repolarisation and regulation of the membrane potential

In excitable cells K_V channels are important during the repolarisation phase of the action potential. However most vascular smooth muscle cells do not generate action potentials, but respond to stimulation with graded changes in membrane potential. What physiological roles therefore do K_V channels play in vascular smooth muscle? K_V channels are thought to limit membrane depolarisation in vascular smooth muscle, opening as the membrane is depolarised and functioning as a physiological 'brake' ensuring that a vessel does not 'overcontract'. In some vascular beds, such as the rabbit basilar artery, K_V channels have been shown to be open at potentials in the same range as the RMP, and may therefore play an active role in setting and maintaining the RMP (Robertson & Nelson, 1994). Evidence for this latter role came from the demonstration that the K_V channel blocker, 4-aminopyridine (4-AP), reduced K_V currents (Robertson & Nelson, 1994) and depolarised and constricted rabbit cerebral arteries with pressure-induced myogenic tone (Knot & Nelson, 1995). An additional role for K_V channels has been proposed in the pulmonary circulation. Uniquely the pulmonary arterial bed initially constricts under hypoxic conditions (for a review of hypoxic pulmonary vasoconstriction see Weir & Archer, 1995). The basis of this constriction is thought to be the inhibition of K_V channels by hypoxia, leading to membrane depolarisation, Ca^{2+} influx through DHP-sensitive Ca^{2+} channels and constriction.

1.6.4.1 Pharmacology

No openers of K_V channels currently exist, although a number of studies have claimed that vasoactive intestinal peptide (VIP) and other activators of adenylyl cyclase such as forskolin and isoprenaline increased 4-aminopyridine (4-AP)-sensitive currents from smooth muscle cells isolated from the canine colon and rabbit portal vein (Aiello *et al.*, 1995; Shuttleworth *et al.*, 1996). K_V channel blockers on the other hand do exist and are employed both clinically as anti-arrhythmic drugs and experimentally. The most frequently used blocker of K_V currents in electrophysiological experiments is 4-AP, which is used to help confirm the identity of unknown currents or block K_V currents allowing the isolation of other voltage-activated currents. K_D values for the blocking effects of 4-AP on K_V channels of between 200 μM and 1.1 mM have been reported (see Knot *et al.*, 1996). 4-AP is however a non-specific compound and has pronounced effects on other ion channels frequently found in vascular smooth muscle such as BK_{Ca} channels (K_D >5 mM), ATP-dependent K^+ (K_{ATP}) channels (K_D ~200 μM) and inward rectifier K^+ (K_{IR}) channels (10 % inhibition at 1 mM) (Nelson & Quayle, 1995).

1.6.4.3 K_V channel sub-types

A body of evidence, based on pharmacological and single channel studies, suggests that the K_V currents in arterial smooth muscle are composed of currents through multiple types of K_V channels. The demonstration, for example, that 4-aminopyridine (4-AP) only inhibits a portion of the voltage-activated K^+ currents in many vascular smooth muscle cells suggests a 4-AP-insensitive K_V channel may exist (Smirnov & Aaronson, 1992a; Robertson & Nelson, 1994). Alternatively it may simply reflect the shortcomings of 4-AP as a K_V channel blocking agent. Additional evidence for the existence of multiple K_V channels was provided using measurements of the unitary channel conductance of K_V channels. Data from these studies suggested that K_V channels could be sub-divided into 2 groups:

1. Small conductance K_V channels with a unitary conductance of 5-8 pS when measured using the on-cell configuration of the patch-clamp technique, with a quasi-physiological K^+ concentration extracellularly (Beech & Bolton, 1989; Volk *et al.*, 1993; Robertson & Nelson, 1994).

2. Large conductance K_V channels with unitary conductances of 57 and 70 pS were reported which were also measured using the on-cell configuration but with 140 mM K^+ extracellularly (Gelband & Hume, 1992).

1.6.4.4 Molecular biology

The precise molecular structure of the K_V channel (s) expressed in vascular smooth muscle is currently unknown. However gene products encoding several Kv channel subunits have been identified in vascular and visceral smooth muscle (Roberds & Tamkun, 1991; Overturf *et al.*, 1994). Interestingly, when one member of the Kv channel family, Kv 1.5, isolated from canine colonic smooth muscle or rabbit portal vein, was expressed in *Xenopus* oocytes or mouse L cells, the resulting whole cell and single channel currents closely resembled native vascular smooth muscle K_V currents (Overturf *et al.*, 1994; Clement-Chomienne *et al.*, 1997). Channels composed entirely of Kv 1.5 subunits, like all native K_V channels were activated by depolarisation, they were half inactivated by a holding potential of ~-20 mV and their unitary channel conductance was 9.8 pS in symmetrical 140 mM K^+ (Overturf *et al.*, 1994), values similar to previous studies of native K_V channels in vascular smooth muscle (Beech & Bolton 1989; Volk *et al.*, 1991).

While the actual molecular identity of vascular K_V channels is currently unknown, although studies with Kv 1.5 subunits make it a possible candidate, the molecular study of K_V channels is in itself a scientific success story. This is because different techniques to those previously used so successfully to isolate and examine the structure of voltage-gated Na^+ and Ca^{2+} channels and the nicotinic acetylcholine receptor were employed to determine the molecular structure of K_V channels. Tissues correspondingly rich in K_V channels could not be found and agents that bound to the channel with the specificity and affinity of tetrodotoxin or α-bungarotoxin were unavailable. Of all things the humble fly provided the answer. A behavioural mutant of the fruit fly *Drosophila* exists which shakes its legs when anaesthetised with ether. This characteristic led to the mutant being christened *Shaker*. The mutation responsible for this behavioural characteristic was localised to a particular point on the X chromosome and the flight muscle cells of flies with the *Shaker* mutation were found using voltage-clamp techniques to possess modified voltage-activated K^+ currents. Cloning of the gene responsible for the *Shaker* mutation enabled the amino-acid sequence of the putative K^+ channel which it encoded to be predicted.

The hydrophobicity profile of the *Shaker* gene product, as discussed previously and illustrated in figures 1.iii and 1.iv, was similar to one of the four domains reported to comprise the main α subunit of voltage-activated Na^+ and Ca^{2+} channels, consisting of six putative transmembrane spanning regions termed S1-S6. The fourth of these regions, S4, was found to be highly positively charged and probably acts as a voltage sensor, moving in response to membrane depolarisation leading to channel opening. A number of related genes encoding Kv channel subunits have been subsequently identified in various tissues from a number of vertebrates. These genes have been grouped together as the *Shaker*-related gentetic subfamily because of their homology with the *Shaker* gene and are identified by the prefix Kv (see Chandy, 1991).

1.6.5 Other K^+ channels present in arterial smooth muscle

The work presented in this thesis concentrates almost entirely on BK_{Ca} channels, although some experiments were performed on K_V and DHP-sensitive Ca^{2+} channels. This introduction will therefore concentrated on these particular channels. Two additional K^+ channels, however, have also been identified in isolated arterial smooth muscle cells: the inward rectifier K^+ (K_{IR}) channel and the adenosine triphosphate dependent (K_{ATP}) K^+ channel (for recent reviews see Nelson & Quayle, 1995; Quayle *et al.*, 1997). The following sections briefly describe these channels.

1.6.6 ATP-dependent K^+ channels

During the 1960s a group of chemically heterogeneous vasodilator compounds were found to relax pre-contracted blood vessels by a then unknown mechanism of action. In a series of studies performed in the mid 1980s, Arthur Weston, in collaboration with Beecham Research Ltd, reported that the smooth muscle relaxant effects of these compounds was related to their ability to open plasmalemmal K^+ channels (Weir & Weston, 1986a; Weir & Weston, 1986b). The resulting K^+ efflux hyperpolarised vascular smooth muscle producing vasorelaxation by inhibition of Ca^{2+} entry through DHP-sensitive Ca^{2+} channels.

The K^+ channel opened by these drugs was later identified as the K_{ATP} channel and generated an enormous amount of interest in the succeeding years, as the pharmacological opening of K_{ATP} channels represented a novel vasodilator mechanism which could be exploited therapeutically (for reviews see Cook, 1988; Cook & Quast, 1989; Longman & Hamilton, 1992).

The K_{ATP} channel is a ligand-gated ion channel which is closed when the intracellular ATP concentration is raised. In smooth muscle cells, K_i values for half inhibition of the channel by ATP concentrations between 29 and 200 μM have been reported (Quayle *et al.*, 1997). K_{ATP} channels were first identified in cardiac myocytes (Noma, 1983) and have subsequently been identified in a number of systems including vascular smooth muscle (for review see Nelson & Quayle, 1995; Quayle *et al.*, 1997).

A degree of controversy still exists however as to the biophysical characteristics of the vascular smooth muscle K_{ATP} channel (reviewed by Quayle *et al.*, 1997), with several reports claiming to have identified an ATP-dependent K^+ channel which possessed a single channel conductance similar to that of the BK_{Ca} channel (100 to 250 pS), while others have reported K_{ATP} channels with far smaller single channel conductances in symmetrical high K^+ of 29 pS (Ottalia & Toro, 1996) and 20-24 pS and ~50 pS (Zhang & Bolton, 1995; Zhang & Bolton, 1996).

Vascular K_{ATP} channels are essentially voltage independent. Whole cell K_{ATP} currents activated, for example, following the addition of calcitonin gene related peptide (CGRP) and nicorandil to rabbit mesenteric and porcine coronary artery smooth muscle cells did not exhibit any voltage-dependence (Quayle *et al.*, 1994; Holland unpublished observations).

1.6.6.1 Physiological roles

The main physiological role of K_{ATP} channels in vascular smooth muscle appears to be to regulate local blood flow by linking the metabolic status of the cell to the membrane potential. Both hypoxia and the metabolic poison dinitrophenol have been reported to induce glibenclamide-sensitive currents in vascular smooth muscle cells, presumably by depleting the sub-membrane [ATP] leading to an increase in the P_{open} of K_{ATP} channels (Conway *et al.*, 1994; Dart & Standen, 1995; Zhang & Bolton, 1995).

K_{ATP} channel opening and K^+ efflux will raise the membrane potential, leading to a direct reduction in the P_{open} of DHP-sensitive Ca^{2+} channels and inhibition of electromechanical coupling leading to vasorelaxation, vasodilation and increased blood flow to areas where the supply of oxygen, sugars and other metabolic substrates were previously insufficient.

A number of supplementary roles for K_{ATP} channels in the vasculature have also been proposed (for a recent reviews see Nelson & Quayle 1995; Knot *et al.*, 1996; Quayle *et al.*, 1997). These include regulation of basal tone and as a putative target for some endogenous vasodilators and vasoconstrictors (see Quayle *et al.*, 1997).

1.6.6.2 Pharmacology

1.6.6.3 Openers

Antihypertensive potassium channel openers (KCOs), for example pinacidil and levcromakalim, act by opening K_{ATP} channels in vascular smooth muscle. The vasorelaxation to all these KCOs is inhibited by anti-diabetic sulphonylurea drugs, such as glibenclamide, which reduce the P_{open} of K_{ATP} channels. A problem incumbent with many drugs which target K^+ channels is the ubiquitous nature of the target channel. This effectively ensures that KCOs used as antihypertensives, although effective, will act throughout the cardiovascular system, producing a variety of unwanted side-effects including hypotension.

1.6.6.4 Inhibitors

Inhibitors of K_{ATP}, for example the sulphonylureas glibenclamide and tolbutamide, are widely used therapeutically in the treatment of non-insulin dependent *diabetes mellitus* rather than essential hypertension. The P_{open} of K_{ATP} channels in pancreatic β cells regulates the release of insulin by setting the resting membrane potential (RMP). The sulphonylureas reduce the P_{open} of K_{ATP} channels by binding to the sulphonylurea receptor (SUR) (Aguilar-Bryan *et al.*, 1995) which co-assembles with a weakly inwardly rectifying channel to form functional K_{ATP} channels (Inagaki *et al.*, 1995). Blocking K_{ATP} channels and K^+ efflux leads to membrane depolarisation and the subsequent opening of voltage-dependent Ca^{2+} channels.

The resulting influx of Ca^{2+} ions increases the intracellular free [Ca^{2+}], which then stimulates exocytosis of insulin-containing secretory vesicles leading to the release of insulin into the extracellular space.

As experimental tools sulphonylureas selectively inhibit K_{ATP} channels in vascular smooth muscle, K_D values between 20 and 200 nM have been reported in a variety of vascular preparations (reviewed by Nelson & Quayle, 1995; Quayle *et al.*, 1997). This selectivity makes sulphonylureas, especially glibenclamide, the drugs of choice when characterising K_{ATP} channels.

1.6.7 Inward rectifier potassium (K_{IR}) channels

This channel is so-called because K^+ influx at membrane potentials negative to E_K is greater than K^+ efflux when the membrane potential is positive to E_K. The major, and perhaps in some tissues, only role of K_{IR} channels is to contribute to setting the membrane potential.

1.6.7.1 Physiological roles

As mentioned above one of the well established roles for K_{IR} channels is in regulating the membrane potential. It is reasonable to assume therefore that K_{IR} channels may play a similar role in vascular smooth muscle and help to set the RMP of vascular smooth muscle. Several studies have reported that Ba^{2+} ions, which block K_{IR} channels, significantly depolarise the membrane potential of arterial smooth muscle (reviewed by Quayle *et al.*, 1997), perhaps indicating K_{IR} channels are important regulators of the membrane potential in some arterial beds.

An additional role for K_{IR} in vascular smooth muscle has been proposed which, while incompletely understood, is potentially extremely interesting. This role of K_{IR} channels is in the local regulation of blood flow. Vasodilation in response to small elevations of extracellular [K^+] from 3-5 mM to 10 mM have been observed in a small (100-200 μM in diameter) cerebral and coronary arteries, although the exact mechanism responsible for this K^+-induced vasodilation is currently unknown. Two mechanisms have been proposed to account for this response:

1. Activation of the electrogenic Na^+-K^+ pump. The increase in the extracellular $[K^+]$ increases the activity of Na^+-K^+ ATPase located in the plasma membrane of vascular smooth muscle cells, which extrudes 3 Na^+ ions in exchange for 2 K^+ ions. The net loss of positive charge raises the membrane potential and could produce vasodilation by reducing the P_{open} of DHP-sensitive Ca^{2+} channels. An auxiliary effect of reducing the intracellular $[Na^+]$ is that driving force for Na^+ entry into the cell is increased, which increases the activity of Na^+-Ca^{2+} exchange therefore increasing the removal of intracellular Ca^{2+}. A study in 1990 reported that increasing the extracellular $[K^+]$ in the range 1-5 mM produced a transient dilation of small cerebral arteries (McCarron & Halpern, 1990). This dilation was abolished by ouabain, a cardiac glycoside which inhibits Na^+-K^+ ATPase, suggesting that over this concentration range in this vascular bed, raising the $[K^+]_o$ may dilate arteries by increasing the activity of Na^+-K^+ ATPase leading to hyperpolarisation.

2. Activation of K_{IR} channels. The role of K_{IR} in potassium-induced dilations was initially proposed in the late 1980s where increasing the external $[K^+]$ from 5 mM to 15 mM was reported to hyperpolarise certain sections of rat cerebral arteries (Edwards *et al.*, 1988). This effect was regional, only affecting distal sections of the middle cerebral artery, and was inhibited by Ba^{2+} ions (Edwards *et al.*, 1988), which are routinely used to block K_{IR} channels.

How does the activation of an *inward* rectifier K^+ channel, which conducts only small outward currents, produce hyperpolarisation, especially considering that a likely outcome of increasing the $[K^+]_o$ would be to reduce E_K, therefore depolarising the membrane potential and activating DHP-sensitive Ca^{2+} channels? A current model (for an illustration of the proposed mechanism see Nelson & Quayle 1995) makes use of a unique gating property of K_{IR} channels, namely that K_{IR} channel gating shifts with $[K^+]_o$, and proposes that even though the K_{IR} channel in smooth muscle is a strong inward rectifier, at potentials positive to E_K some K^+ efflux does occur. When the $[K^+]_o$ is increased slightly the current-voltage relation is moved to the right, as a consequence the increased outward K^+ currents through K_{IR} now hyperpolarise the membrane and produce vasodilation.

1.6.8 Molecular biology of K_{ATP} and K_{IR}

The next section very briefly deals with the molecular biology of K_{ATP} and K_{IR} channels.

Due to their obvious similarities the genes encoding these channels have been grouped together to form a new gene family the 'inward rectifiers'. The gene products encoding the inward rectifier channel family contain the prefix Kir.

The first functional K_{ATP} channel was produced following co-expression of the Kir6.2 gene, which encodes a weak inwardly rectifying K^+ channel, isolated from pancreatic islets and glucose-responsive insulin-secreting cell lines, together with the sulphonylurea receptor (SUR), an ATP-binding cassette protein (Agilar-Bryan *et al.*, 1995). This resulted in the expression of weakly inwardly rectifying K^+ channels, whose P_{open} was increased by 100 μM diazoxide, a K_{ATP} channel opener, and reduced by both 100 nM glibenclamide and by increases in the cytosolic [ATP] (Inagaki *et al.*, 1995).

Studies of the structure of inwardly rectifying K^+ channels revealed marked differences from the Kv channel family. Hydrophobicity plots of Kir channels predicted two rather than six putative membrane spanning regions, designated M1 and M2, flanking a highly conserved pore analogous to the H5 pore region in K_V channels (for review see Doupnik *et al.*, 1995). The pore region of Kir however, did show a high degree of homology to the H5 region of Kv channels, strongly indicating that this region of the Kir subunit may form part of the channel pore. By analogy with Kv channels, functional Kir channels have also been proposed to exist as tetramers (Kubo *et al.*, 1993). A recent study for example reported that functioning K_{IR} channels were expressed when four Kir subunits were linked together as a single polypeptide chain (Yang *et al.*, 1995), thus providing the first direct evidence that functional Kir channels are tetramers. To date the subunits which constitute vascular K_{ATP} and K_{IR} channels have not been isolated and identified, probably because of the relatively low density of these channels in vascular smooth muscle. Estimates for example of the number of K_{ATP} channels in vascular smooth muscle cells range between 300-500 channels per cell (Quayle *et al.*, 1997).

1.7 Large conductance calcium activated K^+ or BK_{Ca} channels

Large-conductance Ca^{2+}-activated K^+ channels were first identified in chromaffin cells by Alain Marty in 1981, although increased membrane permeability and membrane hyperpolarisation due to K^+ efflux following intracellular Ca^{2+} injection has been well documented since the turn of the 1970s. Marty identified a channel with a large unitary conductance (~180 pS) in symmetrical 140 mM K^+ which was activated by increasing the cytosolic [Ca^{2+}] (Marty, 1981).

Hence 'big' conductance Ca^{2+}-activated K^+ channel, or as it will be referred hereafter, the BK_{Ca} channel (for comprehensive reviews of BK_{Ca} channels see Blatz & Magleby, 1987; Latorre *et al.*, 1989; McManus, 1991; Kaczorowski *et al.*, 1996).

BK_{Ca} channels are widely distributed and have been found in virtually all excitable and non-excitable tissues, with the exception of the heart (Wallner *et al.*, 1996), including all of the vascular smooth muscle preparations so far studied (Nelson, 1993). The channel itself is highly selective for K^+ over other cations and has a conductance that approaches the limit theoretically expected for a pore (Hille 1992). BK_{Ca} channels are activated by membrane depolarisation and by increasing the cytosolic Ca^{2+} concentration, characteristics which make the BK_{Ca} channel somewhat unusual amongst ion channels, in being both voltage- and ligand-gated channels. A considerable amount of effort has been invested in attempting to determine the relationship of these two modulatory mechanisms in relation to BK_{Ca} channel opening. An early study performed by Barry Pallotta at the University of Miami reported that *N*-bromoacetamide removed the Ca^{2+}-dependent component from BK_{Ca} channels contained in membrane patches excised from skeletal muscle, rendering the BK_{Ca} channel purely voltage-dependent (Pallotta, 1985). What was interesting about this study was removal of the Ca^{2+}-dependence greatly reduced the BK_{Ca} channel P_{open}, perhaps indicating that of the two modulatory mechanisms, the cytosolic $[Ca^{2+}]$ exerted the stronger influence upon channel opening and without this Ca^{2+}-dependence the channel functioned as a weak voltage-activated channel. This assumption has been supported by a number of recent studies using various cloned BK_{Ca} or *slo* channels, which have reported that increasing the cytosolic $[Ca^{2+}]$ exerts a greater influence on channel P_{open} than voltage, and when the cytosolic $[Ca^{2+}]$ falls below ~100 nM BK_{Ca} channels become purely voltage-dependent and are only activated at very depolarised potentials (>100 mV) (Cox *et al.*, 1996; Toro *et al.*, 1996; Strobaek *et al.*, 1996; Meera *et al.*, 1996). An attempt to place these findings in the context of BK_{Ca} channel regulation proposed that Ca^{2+} binding to the BK_{Ca} channel α subunit decreases the energy required to displace the S4 voltage sensor, leading to channel activation at less depolarised membrane potentials (Diaz *et al.*, 1997).

The Hill co-efficient for activation of BK_{Ca} channels by Ca^{2+} has been reported to be ~4, perhaps indicating that 1 Ca^{2+} ion binds to each of the four subunits proposed to form the functioning BK_{Ca} channel (see section 1.8), with 'full' channel activation being produced when all four putative binding sites are occupied by Ca^{2+} ions (Barrett *et al.*, 1982, McManus & Magleby, 1991; Kaczorowski *et al.*, 1996) possibly by Ca^{2+} binding reducing the energy required to move the voltage sensor, effectively changing the channel from a weakly voltage-activated channel to one more sensitive to voltage. This hypothesis potentially explains why increasing the cytosolic [Ca^{2+}] increases BK_{Ca} channel opening at less depolarised membrane potentials, as the Ca^{2+} binding sites become occupied which decreases the energy required to open the channel. When cytosolic Ca^{2+} is reduced below a critical concentration, channel opening becomes solely voltage-dependent, as insufficient Ca^{2+} is present to fill the binding sites and more energy is required to move the S4 region to open the channel.

1.7.1 Regulation of vascular tone

The functional role of BK_{Ca} channels has been extensively studied in vascular smooth muscle, where the channel activation profile indicated that these channels may be involved in the regulation of vascular tone (Brayden & Nelson, 1992; Nelson, 1993). Increased intravascular pressure in resistance arteries depolarises smooth muscle and produces a degree of vasoconstriction. This is the so-called 'myogenic' response and the resulting increase in vascular tone is an important autoregulatory feature of the circulatory system, especially in the cerebral circulation, which maintains constant rates of blood flow. When vessels develop myogenic tone the membrane potential decreases i.e. the smooth muscle cells become depolarised. This depolarisation and the associated increase in the intracellular [Ca^{2+}] provide conditions favourable for the activation of BK_{Ca} channels. Indeed the involvement of BK_{Ca} in the regulation of cerebral myogenic tone was first proposed by Joe Brayden and Mark Nelson in a report published in 1992. In this study BK_{Ca} channel blockers, including tetraethylammonium (TEA), charybdotoxin (CTX) and iberiotoxin (IbTX) depolarised and further constricted cerebral arteries with pressure-induced myogenic tone (Brayden & Nelson, 1992). Upon reducing the intravascular pressure the membrane hyperpolarised, leading to a reduction in myogenic tone and an attenuation of the effects of BK_{Ca} channel blockers (Brayden & Nelson, 1992).

One of the functions of BK_{Ca} channels in vascular smooth muscle therefore appears to be to provide a negative feedback pathway, activated by membrane depolarisation and increased $[Ca^{2+}]_i$, which may serve to limit the degree of contraction of vascular smooth muscle.

The Smooth Muscle Pharmacology Group in Vermont in collaboration with John Lederer in Baltimore have recently proposed an additional role for BK_{Ca} channels in the regulation of vascular smooth muscle tone. They reported that sub-membrane 'Ca^{2+}-sparks', resulting from the release of Ca^{2+} from the sarcoplasmic reticulum, simultaneously activate a large number of BK_{Ca} channels (~85 channels per spark Nelson *et al.*, 1995). The resulting currents, which were originally described by Chris Benham and Thomas Bolton in 1986 and designated 'Spontaneous Transient Outward Currents' or STOCs (Benham & Bolton, 1986; for review see Bolton & Imaizumi, 1996), were claimed to hyperpolarise vascular smooth muscle and produce vasorelaxation. This idea is interesting, although certain key questions remained unanswered by the initial report. Nelson and his co-workers reported that when the intracellular Ca^{2+} stores were depleted with caffeine, STOC activity decreased with time, but surely this decrease in STOC activity should have been preceded by a large transient increase in STOC activity as the Ca^{2+} stores were emptied and the sub-membrane Ca^{2+} concentration rose activating BK_{Ca} channels, as has previously been reported (Komori & Bolton, 1989). Also the actual physiological relevance of STOCs is still questionable and may simply represent an attempt by isolated vascular smooth muscle cells to reduce Ca^{2+} overloading (Komori & Bolton, 1989).

1.7.2 Potential role of BK_{Ca} channels in vasorelaxation produced by activators of protein kinase G and protein kinase A

A number of modulatory mechanisms have been described which influence the activity of ion channels. These include what could be designated direct effects, which are exemplified by the binding of a ligand to an extracellular receptor or to intracellular binding sites resulting in altered channel activity. More recently it has been recognised that the activity of ion channels can be influenced by 'indirect' processes. An example of this latter effect is the putative modulation of BK_{Ca} channels in vascular smooth muscle by the cGMP signal transduction pathway, which will be the main focus of this section.

The first evidence that K^+ channels in smooth muscle were subject to indirect agonist-induced modulation by the products of a second messenger cascade was reported almost thirty years ago. β-adrenergic stimulation was reported to hyperpolarise various smooth muscle preparations, including some vascular smooth muscle preparations (Johansson *et al.*, 1967; Diamond & Marshall, 1969; Somlyo *et al.*, 1970). The discovery of selective K^+ channel blocking agents allowed the identification of K^+ channels activated by smooth muscle relaxants such as β-agonists and nitrovasodilators. The putative role of the BK_{Ca} channel in cyclic-nucleotide mediated relaxation has been particularly well studied in airway smooth muscle, where CTX and IbTX, selective inhibitors of BK_{Ca} channels, have both been reported to produced rightward shifts in the relaxation curves of β_2-agonists and cGMP elevators against carbachol-contracted guinea pig trachea (Jones *et al.*, 1990; Jones & Charette, 1990). These data therefore indicate that BK_{Ca} channels are activated in cyclic nucleotide-induced tracheal relaxation (for a recent review see Kotlikoff & Kamm, 1996).

In smooth muscle cells both β-agonists and nitrovasodilators stimulate the activity of cyclic nucleotide-dependent protein kinases. Stimulation of β_2-adrenoceptors leads to the activation of adenylyl cyclase and the generation of cAMP. Briefly, following the binding of a β agonist such as isoprenaline to a β_2 receptor, a conformational change in the receptor occurs activating a G-protein complex which is associated with the β_2 receptor. Following the activation of the G-protein complex, the affinity of the α subunit of the G-protein complex for GDP is reduced, allowing GTP, which is present in excess intracellularly, to displace GDP and bind to the G-protein α subunit. This leads to the activation of the α subunit which is inactive when bound to GDP. The activated α-GTP subunit dissociates from the βγ subunits, binding to and activating adenylyl cyclase, which catalyses the production of cAMP from ATP. The increase in intracellular levels of cAMP then leads to the activation of protein kinase A and ultimately smooth muscle relaxation.

An early patch-clamp study, performed using isolated tracheal myocytes, reported that following the addition of the non-specific β adrenergic receptor agonist, isoprenaline, to isolated tracheal myocytes BK_{Ca} channels were activated (Kume *et al.*, 1989). This study further claimed that the effects of isoprenaline were augmented by the protein phosphatase inhibitor okadaic acid and were mimicked by activated protein kinase A catalytic subunits, an effect which was dependent on the presence of ATP (Kume *et al.*, 1989).

These authors concluded that the mechanism responsible for this effect was the result of a 'conventional' phosphorylation reaction involving the transfer of a phosphate from ATP by protein kinase A to a putative phosphorylation site, most probably a serine or threonine residue, located on the BK_{Ca} channel itself or on a closely associated regulatory site. Indeed, following the sequencing of the smooth muscle BK_{Ca} channel a consensus protein kinase A phosphorylation site has been identified on the β subunit (Kaczorowski *et al.*, 1996). Once phosphorylated the P_{open} of the BK_{Ca} channel is increased relative to its control value at all potentials by a currently unknown mechanism which may contribute to the vasorelaxation produced by β-agonists. Protein kinase A catalytic subunits have been reported to increase the P_{open} of *hslo* channels composed of α subunits alone but decrease the P_{open} if the β-subunit was co-expressed (Dworetzky *et al.*, 1996). These data would appear to explain the results obtained by Kume *et al.*, (1989), although BK_{Ca} channel β-subunits were initially identified in airway smooth muscle (Knaus *et al.*, 1994a)!

Agents which activate guanylyl cyclase, such as the clinically useful nitrovasodilator drug sodium nitroprusside, relax smooth muscle by a mechanism involving an increase in the intracellular cGMP concentration. Unlike β-agonists, nitrovasodilators do not produce their effects by binding to a defined membrane bound receptor, but release nitric oxide (NO) which activates soluble guanylyl cyclase (GC-S), an enzyme found in the cytosol of smooth muscle cells. Activated GC-S converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which then activates cGMP-dependent protein kinase (protein kinase G). Protein kinase G appears to reduce the intracellular Ca^{2+} concentration by a number of different mechanisms covered in detail in chapter 5. One of these mechanisms may involve the opening of K^+ channels, with the subsequent membrane hyperpolarisation reducing Ca^{2+} entry. A number of functional studies, for example, have reported that the vasorelaxant effects of nitrovasodilators were attenuated by specific K^+ channel blockers, including specific BK_{Ca} channel blockers (Tare *et al.*, 1991; Taniguchi *et al.*, 1992; Khan *et al.*, 1993; Cowen *et al.*, 1993; Bolotina *et al.*, 1994; Archer *et al.*, 1994; Koh *et al.*, 1995; Simonson *et al.*, 1995). Several patch-clamp studies also reported that nitrovasodilators and NO lead to the activation of BK_{Ca} channels in vascular smooth muscle (Williams *et al.*, 1989; Robertson *et al.*, 1993; Archer *et al.*, 1994; Bolotina *et al.*, 1994; Chen & Rembold, 1996).

The activation of BK_{Ca} channels may occur via a mechanism involving the activation of protein kinase G, as protein kinase G catalytic subunits activate BK_{Ca} channels when applied to the cytosolic surface of inside-out membrane patches excised from canine coronary artery smooth muscle cells (Taniguchi *et al.*, 1992), rabbit cerebral artery smooth muscle cells (Robertson *et al.*, 1993), rat basilar artery smooth muscle cells (Chapter 6 of this thesis) and bovine tracheal smooth muscle cells (Zhou *et al.*, 1996).

The actual mechanism by which protein kinase G catalytic subunits lead to the opening of BK_{Ca} channels is currently uncertain, although the effect appears to be dependent on cytosolic ATP and Ca^{2+} (Chapter 6 of this thesis). A recent study reported that the effect of protein kinase G subunits was attenuated by okadaic acid at a concentration (10 nM) which selectively inhibits protein phosphatase 2A (PP 2A) (Zhou *et al.*, 1996). This led Zhou and co-workers to propose that activated protein kinase G catalytic subunits phosphorylate and activate PP 2A which is associated with the BK_{Ca} channel. Once phosphorylated PP 2A is activated and dephosphorylates the BK_{Ca} channel, resulting in an increase in the channel P_{open}. Recent studies of the gene responsible for expression of BK_{Ca} channels, the *slo* gene, have revealed a consensus phosphorylation site for protein kinase A located on the N-terminus of the regulatory β subunit of the BK_{Ca} channel (see section 1.8.2 and figure 1.viii and Kaczorowski *et al.*, 1996). This implies that the phosphorylated BK_{Ca} channel has a lower P_{open} than the dephosphorylated channel. Evidence which may support this proposal was potentially provided in a study by Dworetzky and co-workers, who reported that phosphorylation of channels composed of *hslo* α and β subunits by protein kinase A catalytic subunits decreased the channel P_{open}, while the P_{open} of channels composed of α subunits alone was increased (Dworetzky *et al.*, 1996). This suggests that protein kinase A phosphorylation of a site on the β subunit may decrease the P_{open} of BK_{Ca} channels rather than increase the P_{open} as was previously reported (Kume *et al.*, 1989; Kume *et al.*, 1992). Activation of protein kinase G, by NO-induced increases in the intracellular cGMP concentration, could ultimately lead to the phosphorylation of PP 2A and the subsequent dephosphorylation of BK_{Ca} channels which increases the P_{open} of these channels which may ultimately contribute to the relaxation of smooth muscle.

The overall importance of BK_{Ca} channel activation in response to NO-induced vasorelaxation is however still uncertain.

A study using sharp electrode impalement to record cell membrane potential, reported that full relaxation of pre-contracted rabbit and guinea pig coronary arteries was produced by NO without any accompanying change in the membrane potential (Parkington *et al.*, 1995), and the EC_{50} for membrane hyperpolarisation of these vessels by NO was ~forty times higher than the EC_{50} for full relaxation (Parkington *et al.*, 1995). The findings from this study suggested that the hyperpolarisation in response to NO may only occur when large amounts of NO are produced. A condition where this may occur is when inducible nitric oxide synthase (see chapter 5 section 5.1.1) contained in vascular smooth muscle cells and endothelial cells, is activated during pathophysiological conditions such as sepsis or endotoxic shock. Indeed a recent patch-clamp study reported that NO produced by stimulating the inducible form of nitric oxide synthase with endotoxin activated BK_{Ca} channels in cultured vascular smooth muscle cells (Miyoshi & Nakaya, 1994). The activation of BK_{Ca} channels may therefore partly explain the extensive hypotension which occurs during such an episode, frequently leading to death due to circulatory collapse.

1.7.3 Pharmacology

1.7.4 Openers

The first electrophysiological studies regarding the effects of agents which directly open BK_{Ca} channels have been published recently (for reviews see Olesen, 1994; Edwards & Weston, 1996) and include several studies which were conducted using vascular smooth muscle cells. The compounds described include structures as diverse as the substituted benzimidazolones NS004 and NS1619 (Olesen, 1994; Olesen *et al.*, 1994a; Olesen *et al.*, 1994b; Edwards *et al.*, 1994; Sellers & Ashford 1994; McKay *et al.*, 1994; Holland *et al.*, 1996), substituted diphenylureas (Strobaek *et al.*, 1996), fenamates (Ottalia & Toro, 1994; Greenwood & Large, 1995), triterpene glycosides (McManus *et al.*, 1993) and the dihydroxylisoprimane diterpene termed '*Maxikdiol*' (Kaczorowski *et al.*, 1996), as well as endogenous molecules such as the fatty acids arachidonic and myristic acid (Kirber *et al.*, 1992).

The most potent BK_{Ca} channel activator currently described is the triterpene glycoside dehydrosoyasaponin I (DHS I), which has been reported to activate smooth muscle BK_{Ca} channels reconstituted in planar lipid bi-layers at concentrations as low as 10 nM (McManus *et al.*, 1993). NS1619 and other benzimidazolones activate BK_{Ca} channels in the μM range (Edwards *et al.*, 1994; Sellers & Ashford, 1994; Holland *et al.*, 1996).

The actual mechanism of action of BK_{Ca} channel openers is currently unclear and is almost certainly heterologous. NS1619, for example, has been reported to activate BK_{Ca} channels composed entirely of *slo* α subunits (Gribkoff *et al.*, 1996; Dworetzky *et al.*, 1996) and co-expression of a β-subunit did not alter the effects of NS1619 (Dworetzky *et al.*, 1996). DHS I on the other hand failed to activate cloned BK_{Ca} channels unless the β subunit was additionally co-expressed (Kaczorowski *et al.*, 1996) and is only effective when applied to the cytosolic surface of membrane patches (McManus *et al.*, 1993). NS004 and NS1619 however activated BK_{Ca} channels when applied to either the external or cytosolic sides of the membrane. The effects of NS1619 and the fenamates have also been reported to require the presence of cytosolic Ca^{2+} (Holland *et al.*, 1996; Ottalia & Toro, 1994), possibly implying an effect on the Ca^{2+}-sensitivity of the channel, while the effects of NS1608, a substituted diphenylurea, are reportedly Ca^{2+}-independent (Strobaek *et al.*, 1996). The effects of some BK_{Ca} channel activators may also involve altering the conformation of the channel. DHS-I, for example, inhibited [^{125}I-CTX] binding to smooth muscle membranes (McManus *et al.*, 1993), implying that following DHS-I binding the channel conformation is changed and this change may be involved in BK_{Ca} channel activation by DHS-I.

Potential therapeutic roles for BK_{Ca} channel openers are also unclear, although DHS-I was isolated from *Desmodium adscendens,* a medicinal herb which is used in Ghana in the treatment of asthma. The effects of DHS-I could be due to an effect purely at the level of the bronchial smooth muscle cells, activating BK_{Ca} channels leading to hyperpolarisation and brochodilation. Alternatively, DHS-I could hyperpolarise nerve endings within airways smooth muscle, also via BK_{Ca} channel activation, and limit depolarisation-dependent release of bronchoconstrictors and mediators involved in the response to asthma such as acetylcholine and tachykinins. Theoretically at least, activators of BK_{Ca} channels could have similar uses as relaxants of smooth muscle and could potentially have a role in the treatment of asthma, essential hypertension and other disorders of smooth muscle function.

At the present time it is difficult to determine if BK_{Ca} channel opening by itself is a viable and novel mechanism of producing vasodilation, due to the lack of specificity of the available compounds. NS1619, for example, blocked voltage-activated Ca^{2+} channels in cerebral arterial smooth muscle with a K_D of ~8 μM (Holland *et al.*, 1996), as well as having profound effects on other vascular K^+ channels (Edwards *et al.*, 1994; Holland *et al.*, 1996). The functional effects of NS004 were also previously explained as resulting from the block of Ca^{2+} channels in cardiac smooth muscle rather than the activation of BK_{Ca} channels (Sargent *et al.*, 1993).

In conclusion, a number of drugs have been described recently which activate BK_{Ca} channels, however compounds with a greater degree of selectively are required before BK_{Ca} channel activation can be proven to be a viable mechanism to produce vasorelaxation and relaxation of smooth muscle.

1.7.5 Blockers

1.7.5.1 Charydotoxin

The identification of functional roles of BK_{Ca} channels has been enormously assisted by the availability of specific channel blockers. The best known of these compounds is the peptide CTX, which was isolated in the mid 1980s from the venom of the scorpion *Leiurus quinquestriatus hebraeus* (Miller *et al.*, 1985). CTX is positively charged in solution (net charge +5 at physiological pH) and binds to negatively charged residues in the external mouth of the channel pore. It spans several of the subunits of the expected tetrameric BK_{Ca} channel complex, physically occluding the channel pore and blocking the channel by preventing ion conduction (Miller *et al.*, 1985; Anderson *et al.*, 1988). In vascular smooth muscle CTX is a potent blocker of BK_{Ca} channels, K_D values of between 1-10 nM have been reported (Nelson & Quayle, 1995). A degree of care however should be taken when interpreting results obtained using CTX, as CTX is not entirely BK_{Ca} specific and affects channels composed of Kv 1.3 and 1.2 subunits (Lewis & Cahalan, 1988; Price *et al.*, 1989; Swanson *et al.*, 1990; Deutsch *et al.*, 1991; Grissmer *et al.*, 1994). Channels composed of these subunits are not present in most arterial smooth muscle so CTX can be used as a selective blocker of BK_{Ca} channels in vascular preparations. CTX has also been invaluable in the study of smooth muscle BK_{Ca} channels, initially as a probe to purify the native channel and later to obtain information about BK_{Ca} channel structure and function.

1.7.5.2 Iberiotoxin

In an attempt to discover more selective BK_{Ca} channel blocking agents, the research team headed by Maria Garcia at Merck Research laboratories in New Jersey conducted an extensive screening program. They assessed the ability of crude scorpion venom extracts to displace [^{125}I]CTX binding in smooth muscle membranes. This task led to the isolation of the peptide iberiotoxin (IbTX) from the old world scorpion, *Buthus tamulus* (Galvez *et al.*, 1989). IbTX, despite possessing 68 % homogeneity to CTX and blocking BK_{Ca} channels by a similar mechanism to CTX, does not affect CTX-sensitive K^+ channels other than BK_{Ca} channels. This selectivity therefore makes IbTX the drug of choice when characterising BK_{Ca} channels in any preparation. In vascular smooth muscle the K_D of IbTX is similar to that of CTX (<10 nM).

1.7.5.3 TEA and tremorgenic indole alkaloids

TEA is another commonly used (and far cheaper!) alternative to the peptide toxins when characterising BK_{Ca} channels. The TEA^+ ion blocks BK_{Ca} channels in vascular smooth muscle with a K_D of ~200 μM when applied to the external surface of the channel (Langton *et al.*, 1991). At these concentrations TEA^+ ions selectively block BK_{Ca} channels with a characteristic 'flickery block', due to the rapid blocking and unblocking rate constants of TEA because of its relatively low affinity for its binding site on the BK_{Ca} channel. More recently a series of potent non-peptidyl BK_{Ca} channel blockers were discovered by Merck Research Laboratories, again using the displacement of [^{125}I]CTX binding from membranes as an indication of BK_{Ca} channel blocking ability. This group of compounds includes penitram A, paxilline, aflatrem and verruculogen, collectively termed the tremorgenic indole alkaloids, as they produce neurological disorders in experimental animals (Knaus *et al.*, 1994b). These compounds potently block BK_{Ca} channels in excised membrane patches, with K_D values of 0.1-10 nM. They are however non-specific and have additional effects on other ion channels, such as GABA-gated channels, which produces the undesirable neurological side effects (Knaus *et al.*, 1994b).

1.8 Molecular biology: BK_{Ca} channel structure and function

Molecular techniques have provided an enormous amount of information about BK_{Ca} channel structure and function. These techniques may ultimately identify sites on the channel which can be exploited therapeutically and may provide answers to questions concerning BK_{Ca} channel function and the dual modulation of the BK_{Ca} channel by both membrane depolarisation and the cytosolic Ca^{2+} and the relationship between these two factors..

1.8.1 α Subunit

The first report concerning the molecular identity of Ca^{2+}-activated K^+ channels predicted that the product of the so-called *slowpoke* (*slo*) gene, isolated from *Drosophila* flight muscle which led to the gene product being christened *dslo*, encoded a Ca^{2+}-activated K^+ channel (Atkinson *et al.*, 1991). The first direct electrophysiological evidence to support this idea was provided when RNA encoding the *dslo* locus was injected into *Xenopus* oocytes (Adelman *et al.*, 1992). Inside-out patches from these oocytes contained Ca^{2+}-activated K^+ channels with a unitary channel conductance of 126 pS in symmetrical 120 mM K^+. Rather surprisingly CTX did not affect *dslo* channel currents, indicating that they were unlike vertebrate BK_{Ca} channels expressed in skeletal muscle or smooth muscle. However, 100 μM-10 mM TEA applied to the external surface did block the channel (Adelman *et al.*, 1992). *Dslo* channels were also less Ca^{2+}-sensitive than native vertebrate BK_{Ca} channels, cytosolic Ca^{2+} concentrations between 30-100 μM, for example, were required to produce robust levels of channel activation (Adelman *et al.*, 1992).

The first functional mammalian BK_{Ca} channels were produced following the identification and isolation of the *slo* gene from murine brain and skeletal muscle preparations, which by analogy to *dslo* was termed *mslo*. Following the injection of *mslo* mRNA into *Xenopus* oocytes, electrophysiological studies revealed a Ca^{2+}- and voltage-dependent K^+ channel with a unitary conductance of 272 pS in symmetrical 156 mM K^+ which was sensitive to external CTX, IbTX and TEA, typical characteristics of native BK_{Ca} channels (Butler *et al.*, 1993). Like *dslo, mslo* channels were also less Ca^{2+}-sensitive than native BK_{Ca} channels present in smooth muscle, cytosolic Ca^{2+} concentrations between 10 and 100 μM, for example, were required to produce significant increases in channel P_{open} (Butler *et al.*, 1993).

Analysis of the predicted sequences and hydrophilicity profiles revealed that the gene products encoded by *dslo* and *mslo* were structurally related to Kv channels (compare figure 1.iv with figure 1.viii) (Adelman *et al.*, 1992; Butler *et al.*, 1993), with each possessing a S1-S6 'core' domain. This core domain contains all 6 putative transmembrane segments, with a 'string' of regularly spaced positive charges in the S4 region and a highly conserved region between S5 and S6, termed H5, which defines part of the pore. However the predicted primary sequence of *mslo* was approximately double the length of the gene product predicted by Kv DNA. This additional length was partly due to an additional peptide specific to *slo* which is located on the C-terminus of the core domain and illustrated in figure 1.viii. This region is composed of a large intracellular loop culminating in 4 additional hydrophobic regions termed S7-S10 which links with high affinity to the S1-S6 region.

Despite *mslo* and *dslo* sharing considerable sequence homology the two channels differed considerably with respect to their single channel conductance values and pharmacological properties, perhaps indicating that this S7-S10 'tail' profoundly influences channel function. An elegant study performed by Wei *et al.*, (1994) reported that functional BK_{Ca} channels were only expressed when the S1-S6 'core' construct was co-injected with the S7-S10 'tail' construct. By producing functional chimeras and exploiting biophysical differences between *mslo* and *dslo* variants, such as unitary channel conductance of the two channels, they were able to determine that the 'core' determined properties such as the channel conductance and voltage-dependence, whereas the 'tail' determined Ca^{2+} sensitivity (Wei *et al.*, 1994). Detailed analysis of the predicted amino acid sequence of the α subunit revealed a number of putative modulatory sites, including phosphorylation sites for protein kinase C which may be important in channel regulation and a protein kinase A recognition site (Kaczorowski *et al.*, 1996).

Following the isolation of *dslo* and *mslo*, genes encoding for BK_{Ca} channels were identified from bovine aortic and tracheal smooth muscle (*bslo*) and human smooth muscle (*hslo*). However problems persisted with the *slo* channel α subunit, it was for example far less Ca^{2+}-dependent than native BK_{Ca} channels found in smooth muscle preparations. Also expression of the α subunit alone could not physically account for the *slo* gene product, which predicted a 135 kDa product rather than the 62 kDa α subunit. The answer to these questions were provided by Garcia and her co-workers at Merck Laboratories (for excellent reviews see Knaus *et al.*, 1994c; Garcia *et al.*, 1995; Kaczorowski *et al.*, 1996).

Rather than identifying the gene encoding for BK_{Ca} channels expression and cloning the predicted channel using this information, Garcia and co-workers used mono-iodotyrosine charybdotoxin (^{125}I-CTX) to label native BK_{Ca} channels. Using preparations suitably rich in BK_{Ca} channels, such as bovine aortic or tracheal smooth muscle cell membranes, they isolated BK_{Ca} channels using methods analogous to those previously employed to isolate the nicotinic acetylcholine receptor and voltage-activated Na^+ channels. The labelled BK_{Ca} channels were then purified to homogeneity using biochemical techniques. Following purification two channel subunits were identified which bound CTX. These were an α subunit, *bslo* (M_r 62 kDa), with very high sequence homology to the *mslo* α subunit, and a novel β subunit (M_r 31 kDa) (Garcia-Calvo *et al.*, 1994; Knaus *et al.*, 1994a; Knaus *et al.*, 1994d;) which existed in a 1:1 stoichiometric arrangement. This information however still did not fully explain the difference in molecular weight between the isolated channel and that predicted by the *slo* gene. This problem was recently clarified and was found to be the result of a highly reproducible proteolytic cleavage of the channel protein which occurred at the chromatography stage of purification (Knaus *et al.*, 1995). When antibodies were raised against synthetic peptides encompassing the entire *slo* sequence and the product immunoprecipitated, the *in vitro* product of *slo* was a 135 kDa protein (Knaus *et al.*, 1995). Recently another region of the BK_{Ca} channel has been reported which links the regulatory β subunit (see section 1.8.2) to the α subunit of the channel complex (Wallner *et al.*, 1996). This additional region has been denoted S0 and was identified following hydrophobicity analysis of *hslo* and *dslo*. It is a putative transmembrane spanning region located at the N terminus of the α subunit close to S1 and its presence is a necessary requirement for *slo* channel activity (Wallner *et al.*, 1996). When RNA encoding for *dslo* or *hslo* channels minus the S0 region were injected into *Xenopus* oocytes no currents were produced. However co-injection of the S0 deleted RNA with RNA encoding the S0 region resulted in the expression of functional channels with characteristics expected of *slo* (Wallner *et al.*, 1996). The study by Wallner and colleagues also reported that co-expression of a human β subunit with *dslo* did not increase the channel P_{open} at a fixed cytosolic [Ca^{2+}] (3μM), whereas co-expression of *hslo* with a human β subunit resulted in a shift to the left in the half-activation potential of ~100 mV at a constant (3 μM) [Ca^{2+}] (Wallner *et al.*, 1996). This may indicate that the BK_{Ca} β subunit is not promiscuous.

1.8.2 β Subunit

The BK_{Ca} channel β-subunit was found to consist of 2 hydrophobic (putative transmembrane) domains and an additional binding site for CTX (Knaus *et al.*, 1994a; Knaus *et al.*, 1994d; Knaus *et al.*, 1994e). The β-subunit may not be an intrinsic part of the CTX / IbTX receptor, as illustrated by the reduced potency of IbTX on BK_{Ca} channels composed of α and β-subunits (Dworetzky *et al.*, 1996), but the demonstration that one CTX molecule cross-linked α and β-subunits implied that the β-subunit existed in close proximity to the channel pore of the α-subunit (Munujos *et al.*, 1995). Garcia and her co-workers proposed a model of the smooth muscle BK_{Ca} channel, where the native channel exists as a tetrameric complex of 4 α-subunits, with each α-subunit closely (<12 Å), and non-covalently bound to a β-subunit. When these 2 subunits were co-expressed, channels with identical properties (unitary conductance, ion selectivity, voltage and Ca^{2+}-dependence and pharmacology) to native BK_{Ca} channels were produced (Garcia *et al.*, 1995; Wallner *et al.*, 1995). Recent studies have reported that channels expressed in *Xenopus* oocytes following the injection of DNA encoding for both the α and β-subunits were more sensitive to activation by both voltage and cytosolic Ca^{2+} than channels resulting from the injection of DNA encoding the α subunit alone (McManus *et al.*, 1995, Meera *et al.*, 1996; Wallner *et al.*, 1996). The effects of α and β subunit co-expression was startling, resulting in a shift in the current / voltage relationship of the expressed channels ~100 mV to the left when compared to channels composed entirely of α subunits, an effect comparable to a 10 fold increase in cytosolic Ca^{2+} (McManus *et al.*, 1995; Wallner *et al.*, 1996). When the *hslo* α subunits were co-expressed with a β subunit obtained from human myometrium, functional BK_{Ca} channels reportedly ten-fold more Ca^{2+}-sensitive than channels formed from the α subunit alone were produced (Meera *et al.*, 1996). What this latter study further illustrated was that at Ca^{2+} concentrations ≤100 nM, comparable to the resting Ca^{2+} concentration, the α/β complex was somehow 'uncoupled', and the resulting channel functioned as a Ca^{2+}-independent, purely voltage-activated channel. However, when the Ca^{2+} concentration was increased above 100 nM, a situation which occurs following cellular depolarisation, the channel was 'switched' into a Ca^{2+}-dependent mode. When switched into this mode the channel responded far more efficiently to both Ca^{2+} and voltage, with shifts in both $K_{1/2}$ and $V_{1/2}$, when compared to cells expressing the α subunit alone (Meera *et al.*, 1996).

The BK_{Ca} β subunit also contains a protein kinase A phosphorylation site which is situated cytoplasmically on the N-terminus of the β subunit and may be involved in the regulation of BK_{Ca} channel function (Kaczorowski *et al.*, 1996).

The discovery of the β subunit is an important one in terms of BK_{Ca} channel pharmacology as it may represent a target distinct from the α subunit, therefore providing a site which may be exploited to selectively target BK_{Ca} channels. As mentioned previously the BK_{Ca} channel opener DHS-I did not activate channels composed of α subunits alone but did when the β subunit was present, suggesting that the mechanism of action of DHS-I involves the β subunit (McManus *et al.*, 1995). The presence or absence of β subunits may also explain the different characteristics of BK_{Ca} channels observed in different tissues. Toro and her co-workers at UCLA in collaboration with Merck laboratories recently conducted an extensive screening program where they used Northern blot analysis to detect β subunit RNA in a variety of animal and human tissues (Wallner *et al.*, 1995; Jiang *et al.*, 1996). Signals corresponding to high levels of β subunit expression were found in uterine and visceral smooth muscle preparations, while only weak signals were detected from skeletal muscle preparations (Wallner *et al.*, 1995; Jiang *et al.*, 1996). This pattern of β subunit distribution may explain an earlier observation that BK_{Ca} channels tended to fall into two groups, high Ca^{2+} sensitive BK_{Ca} channels present for example in visceral and vascular smooth muscle (Benham *et al.*, 1986) and lower Ca^{2+} sensitive BK_{Ca} channels present in skeletal muscle (McManus & Magleby, 1991).

To summarise the information in this section, BK_{Ca} channels in smooth muscle appear to consist of two non-covalently bound subunits termed α and β. The α-subunit is analogous to the α-subunit of Kv channels, consisting of six putative transmembrane spanning sections termed S1-S6. The α subunit of *dslo*, *mslo*, *hslo* and also their bovine counterpart, *bslo*, contain an additional polypeptide located on the C-terminus of the channel. This peptide is composed of four additional putative membrane spanning sections termed S7-S10 which are covalently bound to S1-S6. This 'tail' region is thought to confer a degree of Ca^{2+}-sensitivity to the α-subunit (Wei *et al.*, 1994). More recently another putative membrane spanning region has been identified on the α subunit of *dslo* and *hslo* termed S0. This region, which may be specific to BK_{Ca} channels, appears to be involved in binding the β subunit to the α subunit. The β-subunit consists of 2 hydrophobic regions, which presumably span the membrane, and a large extracellular loop which contains a secondary CTX-binding site. It is located close to the N-terminus of the α subunit to which it is non-covalently bound.

The function of the β subunit appears to act as a 'switch' which increases Ca^{2+} and voltage-dependence of the channel. BK_{Ca} channels probably exist as a multimer of 4 α and 4 β subunits and alternate splicing of *slo* α subunits or tissue specific β subunits may give rise to functionally diverse BK_{Ca} channels. Supporting experimental evidence for this model is that α and β subunits exist in a 1:1 ratio in purified channel preparations using smooth muscle as the source of BK_{Ca} channels, and the mass of the CTX receptor measured by sucrose density gradient centrifugation is ~600 kDa (4(135α + 31β) = 664 kDa).

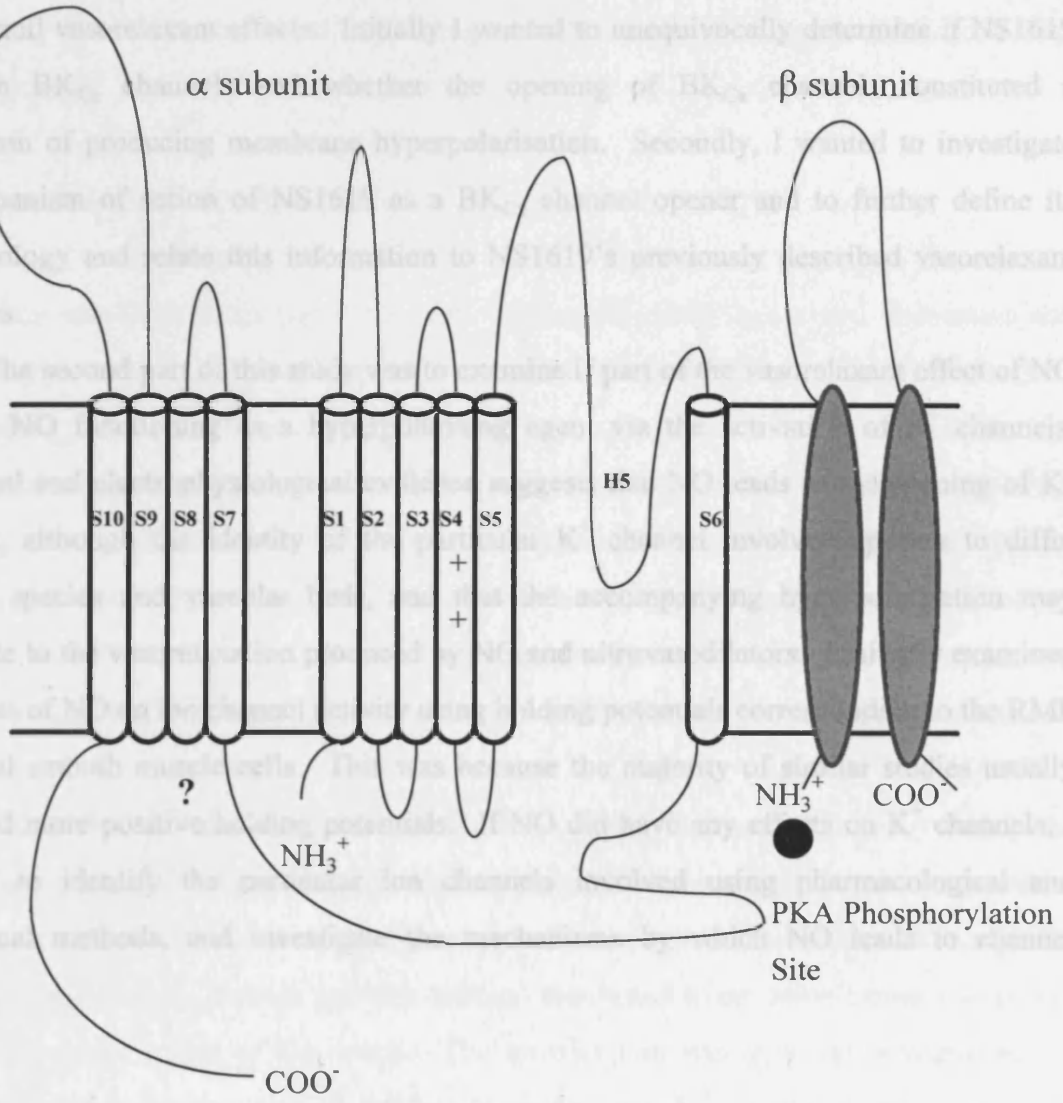


Figure 1.viii Proposed transmembrane topology of the BK_{Ca} channels α and β subunits. Adapted from Knaus *et al.*, (1994c).

1.9 Aims

The aims of the present study were to investigate the effects of NS1619, a putative BK_{Ca} channel opener and vasodilator compound (Olesen *et al.*, 1994a), and the NO / cGMP signalling pathway (see figures 5.i and 5.ii), one of the principal endogenous regulators of vascular tone, on ion channel activity using isolated arterial smooth muscle cells. Both NS1619 and the NO / cGMP signalling pathway have been reported to open K^+ channels in arterial smooth muscle, albeit by different mechanisms, which may in part contribute to their overall vasorelaxant effects. Initially I wanted to unequivocally determine if NS1619 did open BK_{Ca} channels and whether the opening of BK_{Ca} channels constituted a mechanism of producing membrane hyperpolarisation. Secondly, I wanted to investigate the mechanism of action of NS1619 as a BK_{Ca} channel opener and to further define its pharmacology and relate this information to NS1619's previously described vasorelaxant properties.

The second part of this study was to examine if part of the vasorelaxant effect of NO involves NO functioning as a hyperpolarising agent via the activation of K^+ channels. Functional and electrophysiological evidence suggests that NO leads to the opening of K^+ channels, although the identity of the particular K^+ channel involved appears to differ between species and vascular beds, and that the accompanying hyperpolarisation may contribute to the vasorelaxation produced by NO and nitrovasodilators. I initially examined the effects of NO on ion channel activity using holding potentials corresponding to the RMP of arterial smooth muscle cells. This was because the majority of similar studies usually employed more positive holding potentials. If NO did have any effects on K^+ channels, I intended to identify the particular ion channels involved using pharmacological and biophysical methods, and investigate the mechanisms by which NO leads to channel opening.

CHAPTER TWO Materials and methods

2.1 Preparation

The mammalian brain is well supplied with blood from several major sources. One of these sources, the basilar artery, is formed by the fusion of the two vertebral arteries. The vertebral arteries travel through the upper vertebrae and enter the skull through the *foramen magnum*, forming the basilar artery on the dorsal side of the brain stem. All the experiments described in this thesis were conducted using smooth muscle cells freshly dissociated from the basilar artery.

2.1.1 Dissection of the rat basilar artery

Male Wistar rats (200-300g) were killed by stunning followed by cervical dislocation and decapitation. A longitudinal cut was made through the skin covering the head allowing the skin covering the skull to be removed. The fine point of a pair of scissors was inserted into the foramen magnum and parallel cuts were made down the right and left sides of the skull, taking great care not to cut into the brain. This allowed the top of the skull to be removed revealing the cerebral hemispheres. The brain was carefully extracted from the skull by lifting the brain with a spatula, cutting the optic nerves, then carefully levering the intact brain out into a dissecting dish containing ice cold buffer (see Solutions Appendix at the end of this chapter).

The brain was pinned dorsal side down exposing the ventral face. Viewing the preparation with the aid of a dissecting microscope and fibre optic light source, the posterior end of the basilar artery was located originating at the base of the brain stem. It was gently held with watchmaker's forceps and fine scissors were used to cut away excess connective tissue, freeing the length of the artery. The anterior end was then cut proximal to the anterior cerebellar arteries, freeing the basilar artery, which was then transferred for 10 minutes to a small universal tube containing approximately 1 ml of the buffer solution described in the solutions appendix, except that the calcium (Ca^{2+}) concentration was reduced to 0.1 mM.

2.2 Cell isolation procedure

The cell dissociation procedure used in all these experiments was adapted from a method previously described by Quayle *et al.*, (1994) for isolating smooth muscle cells from rabbit mesenteric arteries. The basilar artery was incubated at 37 °C for 30 minutes in a 0.1 mM Ca^{2+} buffer additionally containing (mg ml^{-1}): papain 1.0 (Sigma), dithiothreitol 1.0 (Sigma) and bovine serum albumin (BSA) 1.0 (essentially fatty acid free Sigma). The artery was then transferred to fresh 0.1 mM Ca^{2+} buffer which additionally contained (mg ml^{-1}): collagenase type F 1.5 (Sigma) 1.5, BSA 1.0 and hyaluronidase type I-S 1.0 (Sigma) and incubated at 37°C for a further 10 minutes. Finally the digested artery was transferred to fresh 0.1 mM Ca^{2+} buffer containing BSA 1.0 mg ml^{-1}.

Single smooth muscle cells were dispersed from the digested artery by gentle trituration with a flamed Pasteur pipette. The mouth of this pipette had a wide bore, ~3 mm, which was produced by cutting the pipette near its shank and flaming the cut end. Cells were stored at 4°C and used within 8 hours. A couple of drops of buffer containing dissociated cells were then transferred to a 35 mm petri dish (Nunclon, Denmark). Cells were left for a period of approximately 30 minutes to adhere to the bottom of the dish. This method was found to be a very effective way of dissociating cells from the basilar artery, reproducibly producing a high yield of relaxed, spindle-shaped, densely phase-bright cells.

2.3 Solutions

All solutions used in the experiments described within this thesis were prepared using milliQ water. All chemicals were purchased from Sigma and added in solid form except $CaCl_2$ and $MgCl_2$ which were added in the appropriate volume from 1M stock solutions. Before use, all solutions were passed through a 0.2 μm filter. For a comprehensive description of the chemical composition of the solutions used see the appendix section at the end of this chapter.

2.4 Preparation of experimental solutions

2.4.1 Nitric Oxide

Nitric oxide (NO) is relatively hydrophobic but dissolves to a limited extent forming an ~2 mM saturated solution at standard temperature and pressure. Any protocol used to produce solutions of NO must take into account that when NO is introduced into solution it is rapidly oxidised initially by even trace amounts of superoxide, O_2 or redox metals, for example Fe^{3+}, to peroxynitrite and then subsequently to nitrites and nitrates. It was therefore of the utmost importance that the following procedures were followed to remove all traces of O_2 and other contaminants (see Feelish, 1991).

Solutions used in the preparation of NO were passed through a 0.2 μm filter to remove traces of redox metals followed by bubbling for ~60 minutes with a noble gas such as helium or argon to de-aerate the solution. Bubbling the de-aerated solution at room temperature and one atmosphere for 4-5 minutes with NO gas (minimum 97% pure) produces a saturated solution of NO containing a concentration of NO in the low milli-molar range (~1-2 mM). Once produced the saturated solution of NO was kept on ice in darkness in custom made gas tight vials. When used, NO was delivered into the recording bath using gas tight syringes and low permeability tubing to avoid oxidative decomposition before the NO reached the target.

2.4.2 SIN-1A

A stock solution of SIN-1 was also made in de-aerated superfusion solution (Feelish, 1991). It was then warmed to 37 °C for 5 minutes to activate SIN-1 to SIN-1A.

2.4.3 Other solutions

All other solutions were freshly made up from stock solutions. Glibenclamide and NS1619 were made up in DMSO (Sigma, Tissue culture grade), apamin was made as stock solution in 0.1 mM acetic acid and IbTX was made as a stock solution in milliQ water. Solutions were made such that the final concentration of vehicles, such as DMSO, was kept as low as possible, usually <1% of the solution.

2.5 Perfusion system

The experiments described in this thesis required a perfusion system that allowed rapid exchange of test solutions, while minimising the consumption of expensive toxins or drugs with limited availability. Consequently, using a gravity perfusion system, where relatively large volumes of test solution are used and a relatively slow rate of solution exchange can be achieved, was inappropriate.

The perfusion system that best suited the type of experiments performed in this thesis, and the one that was used in all the experiments described in later chapters, was designed and built by Dr Phil Langton. This system consists of 7 separate 2 ml reservoirs. Flow from these reservoirs is assisted by positive pressure provided by a diaphragm pump and the outflow from the reservoirs is controlled by electrically switched solenoid valves (Lee Instac Ltd Bucks), each one with an individual outflow. Each outflow is connected to a single multi-channel superfusion tip by Teflon tubing (Cole Parmer) with an inside diameter of 0.5 mm. This superfusion tip had a single outflow which, when placed close to the cell under study, allows the application of up to 7 different test solutions. For a detailed description of the construction of superfusion tips see the appendix section at the end of the of this chapter. The switches controlling flow from the reservoirs are controlled by an external control box and the power to drive the electric switches is provided by a 12 V battery. Flow rates from the system were in the order 0.25 to 0.5 ml min^{-1} so the rate of solution exchanged was limited by the volume of the dead space of the perfusion tip. I estimated this volume to be in the order of 5 to 10 µl, allowing complete exchange of solutions within approximately 3-5 seconds.

2.6 Bath perfusion

Bath perfusion was provided by a drip-feed reservoir suspended within the Faraday cage and connected to the Petri dish with a length of Teflon tubing. The flow rate was controlled with an artery clip giving a rate of flow of approximately 2 ml min^{-1}. The bath volume was maintained at approximately 5 ml with the aid of a vacuum pump connected to a modified syringe.

2.7 Patch-clamp apparatus and methods

2.7.1 Electrodes

Patch pipettes used for recording whole-cell currents and membrane potential were constructed from thin walled borosilicate glass (o.d. 1.5 mm, Clarke Electromedical). Patch pipettes used for recording single channel currents using excised membrane patches were constructed from thick walled borosilicate glass (o.d. 1.5 mm, Clarke Electromedical). All pipettes were pulled on the day of the experiment using a two stage Narashige vertical pipette puller (Narashige Instruments Ltd., Tokyo, Japan) and the electrode tips were coated with sticky dental wax (Kemdent, Swindon) to reduce capacitance, and fire-polished to the desired shape and resistance using a Narashige fire-polisher.

2.7.2 Liquid junction potentials

In patch clamp recording, the choice of experimental solutions is often dictated by the individual demands of the experiment. This usually involves using pipette and bath solutions containing different ionic concentrations and can lead to the development of 'liquid junction' potentials (E_L) due to a Donnan effect. E_L develops as a result of the different ionic mobility's of the ions at the interface between two solutions, with more mobile ions diffusing more rapidly across the concentration gradient at the interface. This can lead to the pipette gaining charge and the development of E_L. A simple rule which can help at a glance to determine if a E_L will develop is that larger ions are more mobile in solution and move quicker. Consequently if two solutions are being used and the pipette solution contains a greater concentration of large cations relative to the bath solution, then the formation of E_L is likely.

It is standard practice when performing patch-clamp experiments to zero the E_L prior to attempting to make a seal. The amplifier is used to apply a potential equal but opposite to the E_L, termed here E_L^*, such that $E_L + E_L^* = 0$ mV. Problems can arise following seal formation when E_L disappears, as a barrier now exists between pipette solution and the bath solution; but E_L^*, applied to compensate for the E_L, is still being applied and will contribute to the cell membrane potential (V_M), so that any current measured by the amplifier (I) thought to be produced by that particular membrane voltage (V_M), is actually equal to $V_M + E_L^*$.

This will distort the true value of any command potential ($V_{COMMAND}$) such that I is not produced by $V_{COMMAND}$ but by $V_{COMMAND} + E_L^*$. To correct for the E_L^*, the value corresponding to it must be subtracted from the $V_{COMMAND}$. To correct for E_L it must first be measured. This was achieved using the following method:

The amplifier was set in current clamp mode enabling the amplifier to the follow the pipette potential. The pipette, filled with the appropriate solution, was placed in a bath containing normal bath solution and any E_L or tip potential was compensated. The solution was substituted for the flow solution, if this differed from the bath solution, using the perfusion system. The E_L was displayed on the LED meter on the amplifier. This process was repeated several times on different days enabling the calculation of a mean value. No significant (>±3 mV) E_L developed when recording K^+ currents. However, when recording Ba^{2+} currents, which involved using CsCl in the pipette solution and NaCl externally, a large negative E_L developed. This is because Cs^+ is a larger ion than Na^+ (ionic radii of Cs^+ ~1.7 Å; ionic radii of Na^+ ~1 Å) and is less hydrated and has a higher ionic mobility. Consequently Cs^+ diffuses out of the pipette quicker than a corresponding amount of Na^+ moves into the pipette solution, the subsequent loss of positively charged ions causes a negative charge to build up on the pipette. Using the method described above, the mean E_L was -8 mV (n=4) and the Henderson equation predicted that the pipette would become 5.2 mV more negatively charged with respect to the bath. Therefore, using standard patch-clamp techniques, E_L was corrected prior to seal formation and then disappeared after successfully gaining whole cell access, but a +8 mV potential was still present in addition to any command potential so distorting the actual $V_{COMMAND}$. To compensate for this discrepancy, -8 mV was routinely added to all applied command potentials when analysing voltage-activated Ba^{2+} currents.

2.7.3 Seal formation

The first obstacle which has to be overcome when performing experiments using patch clamp techniques is the formation of a high resistance seal between the tip of the glass recording pipette and the cell membrane of the cell under study. These high resistance 'gigaseals', so called because their resistance is in the range 10^9 Ω, are the main reason why the small currents associated with ion channel opening can be resolved above the background noise level.

This is because some of the noise, so-called Johnson noise, arises from thermal agitation of electrical charges and is inversely proportional to the resistance of the current source (which is mainly the combination of the feedback resistor in the amplifier, the seal resistance and the resistance of the preparation itself) according to Equation 6. In other words as resistance between the cell membrane and recording pipette increases the current noise in the recording decreases.

$$\sigma^2_i = 4kTB / R_s \qquad [6]$$

where R_s is the seal resistance, k is Boltzmann's constant, T is temperature in degrees kelvin and B is the filter bandwidth.

The probability that successful seal formation occurs can be increased by using 'clean' solutions i.e. solutions that have been passed through a 0.2 μm filter and by enzymatically cleaning the surface of the cells. This second requirement was obviously met by the cell isolation procedure, which subjected the artery to the action of enzymes which may have aided seal formation by cleaning the cellular surface. Positive pressure was applied to the inside of the pipette to keep the pipette tip free from debris and also to help 'clean' the surface of the cell prior to seal formation being attempted. As the pipette was advanced towards the cell the pipette resistance was monitored by constantly applying a 1 or 5 mV potential to the interior of the pipette. This produced a small current which allowed the resistance of the pipette to be calculated using Ohm's Law (see Equation 2 chapter 1).

This practice was particularly useful as it enabled pipettes to be discarded if the resistance was unsuitable for a particular experiment. Typical values for the resistance of the pipettes when filled with electrolyte in response to the 1mV test pulse were: 1-7 MΩ for pipettes used in whole cell recordings and 7-15 MΩ for excised patch recordings. Contact of the pipette with the cell membrane was characterised by a sudden increase in tip resistance. Application of gentle suction to the interior of the pipette then draws an Ω shaped section of cell membrane into the lumen of the pipette and seal formation is visualised as a dramatic increase in the tip resistance, such that virtually no current can pass between pipette and the electrode measuring bath current. This is because the distance between the cell membrane and the rim of the pipette has fallen to around 1 Å and the electrical resistance is typically in the order of 10^9 Ω.

This configuration now corresponds to the on cell configuration illustrated in figure 2.iii and from this configuration the various other configurations of the patch clamp technique can be achieved, each of which has its own advantages and disadvantages when used in certain experimental situations.

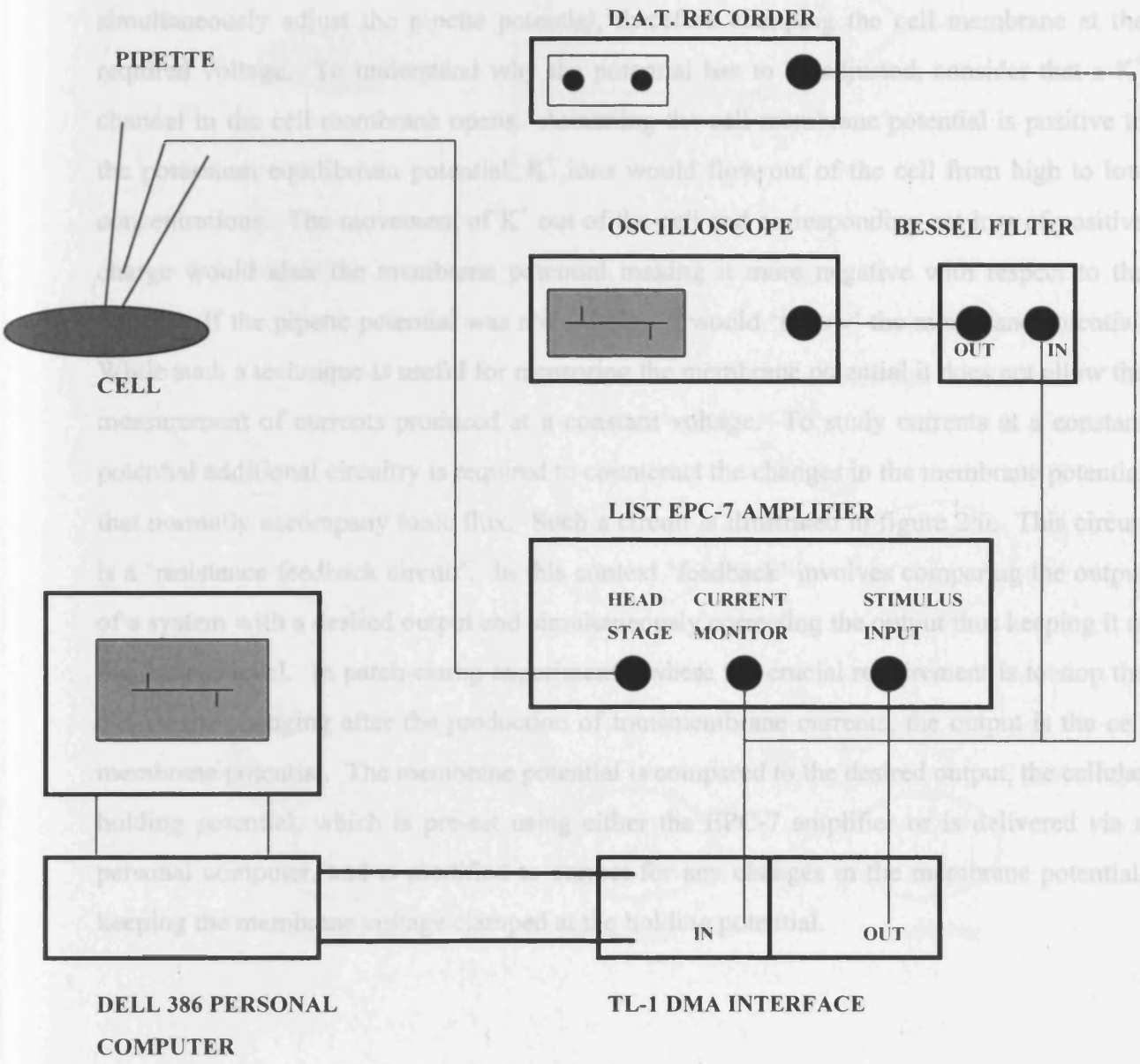


Figure 2.i Stylised diagram of patch clamp set-up used in the experiments described in this thesis

2.7.4 Patch-clamp electronics

All the experiments described in this thesis were conducted using an EPC-7 (List-Electronic) patch-clamp amplifier and List EPC-7 headstage (both Adams & List Associates, Darmstadt, Germany) using the set-up illustrated in figure 2.i. This apparatus allows the amplification and accurate measurement of small cellular currents and simultaneously adjust the pipette potential, therefore clamping the cell membrane at the required voltage. To understand why the potential has to be adjusted, consider that a K^+ channel in the cell membrane opens. Assuming the cell membrane potential is positive to the potassium equilibrium potential, K^+ ions would flow out of the cell from high to low concentrations. The movement of K^+ out of the cell and corresponding net loss of positive charge would alter the membrane potential making it more negative with respect to the outside. If the pipette potential was not adjusted it would 'follow' the membrane potential. While such a technique is useful for measuring the membrane potential it does not allow the measurement of currents produced at a constant voltage. To study currents at a constant potential additional circuitry is required to counteract the changes in the membrane potential that normally accompany ionic flux. Such a circuit is illustrated in figure 2.ii. This circuit is a 'resistance feedback circuit'. In this context 'feedback' involves comparing the output of a system with a desired output and simultaneously correcting the output thus keeping it at the desired level. In patch-clamp experiments, where the crucial requirement is to stop the membrane changing after the production of transmembrane currents, the output is the cell membrane potential. The membrane potential is compared to the desired output, the cellular holding potential, which is pre-set using either the EPC-7 amplifier or is delivered via a personal computer, and is modified to correct for any changes in the membrane potential, keeping the membrane voltage clamped at the holding potential.

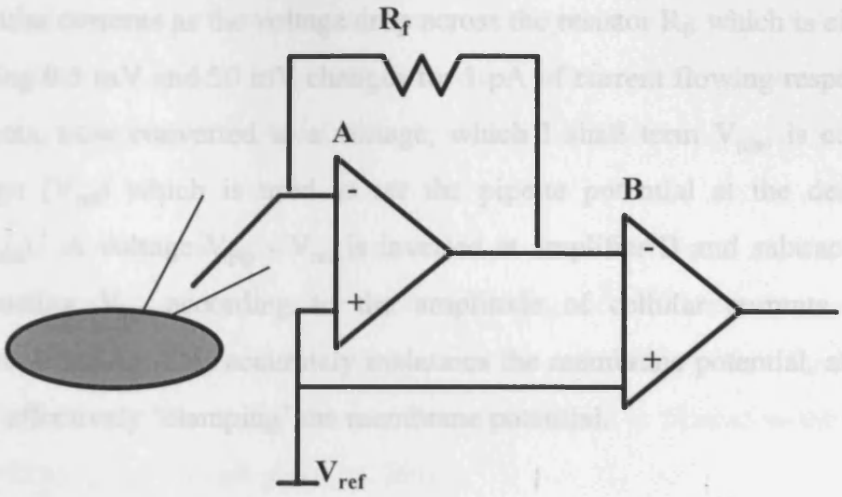


Figure 2.ii Simplified Circuit Diagram of the EPC-7 headstage

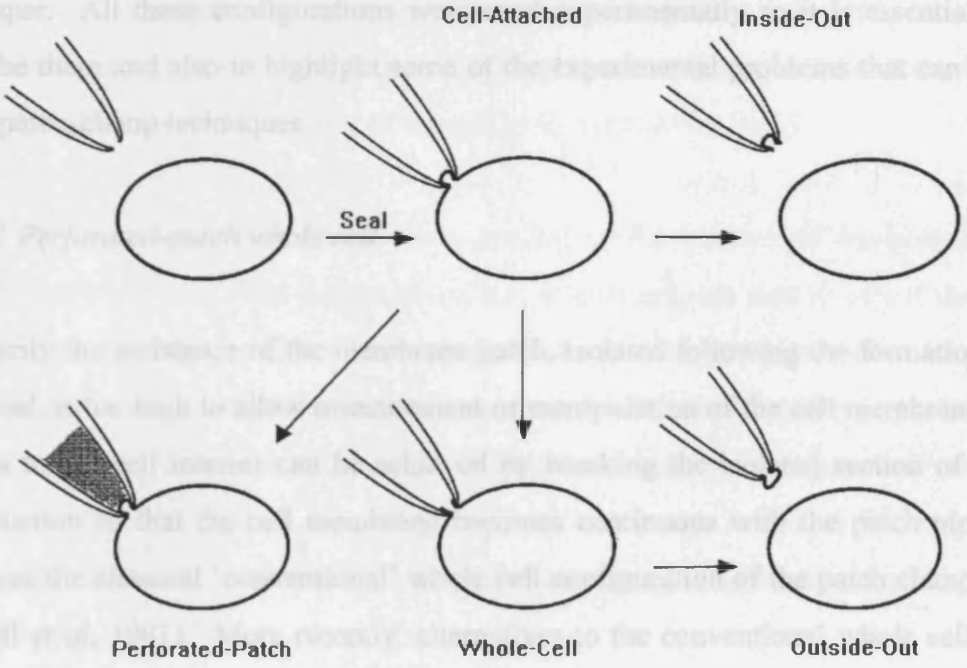


Figure 2.iii. Schematic Diagram of Patch Clamp Configurations. Adapted from Rudy & Iverson.

Figure 2.ii illustrates the basic electronic circuit that allows both the measurement of cellular currents and control of membrane potential.

In very basic terms the operational amplifier in the headstage, marked A in figure 2.ii, amplifies cellular currents as the voltage drop across the resistor R_f, which is either 500 MΩ or 5 GΩ, giving 0.5 mV and 50 mV changes for 1 pA of current flowing respectively. The cellular currents, now converted to a voltage, which I shall term V_{pip}, is compared to a battery voltage (V_{ref}) which is used to set the pipette potential at the desired holding potential (V_{hold}). A voltage V_{pip} - V_{ref} is inverted at amplifier B and subtracted from V_{ref} therefore adjusting V_{ref} according to the amplitude of cellular currents measured at operational amplifier A. This accurately maintains the membrane potential, at the required voltage V_{hold}, effectively 'clamping' the membrane potential.

2.7.5 Patch-clamp configurations and potential sources of error

Figure 2.iii illustrates the most frequently used configurations of the patch clamp technique. All these configurations were used experimentally so it is essential to briefly describe them and also to highlight some of the experimental problems that can arise when using patch-clamp techniques.

2.7.5.1 Perforated-patch whole cell

Ordinarily the resistance of the membrane patch, isolated following the formation of a giga ohm seal, is too high to allow measurement or manipulation of the cell membrane potential. Access to the cell interior can be achieved by breaking the isolated section of membrane with suction so that the cell membrane becomes continuous with the patch pipette. This produces the classical 'conventional' whole cell configuration of the patch clamp technique (Hamill *et al*, 1981). More recently, alternatives to the conventional whole cell technique have been described. One of these methods involve incorporating the polyethene antibiotic amphotericin B (Rae *et al.*, 1991) in the pipette solution. This antibiotic incorporates into cell membranes and forms membrane spanning channels which allow the movement across the membrane of monovalent ions or uncharged molecules smaller than approximately 0.8 nm. As the number of channels perforating the cell membrane increases, hence perforated-patch, the resistance of the isolated cell membrane patch is systematically reduced. This eventually produces access to the interior of the cell, enabling the manipulation of the cell voltage. The following method was routinely used and enabled perforated patch whole cell recordings to be made:

1. Amphotericin B (Sigma) was dissolved in DMSO (tissue culture grade Sigma) (6 mg $100\mu l^{-1}$ stock solution). It was sometimes necessary to sonicate this solution to ensure that all the amphotericin B was dissolved. This stock solution was then stored at -20°C and if made up at the beginning of the week remained potent for the duration of that week.

2. 20 μl of the stock solution was added to 5 ml of filtered pipette solution and briefly sonicated, giving a final concentration of 200 μg ml^{-1}. This solution was stored in darkness, as amphotericin B is light sensitive, at 0°C, and used within 4 hours. Once the amphotericin has been added to the pipette solution it cannot be filtered as the amphotericin B molecule will not pass through a 0.2 μm filter.

3. When the pipette had been filled with the amphotericin B pipette solution a continuous 1 mV voltage step was applied.
This allowed calculation of the pipette resistance using Ohm's law (Equation 2). The electrode was then moved onto the cell and a seal formed. I then turned off the light source to ensure that minimal breakdown of the antibiotic by light occurred.

4. A test pulse of 10 mV was applied to the interior of the pipette to evoke capacitative transients. The fast transient, due to charging up, then decay of the charge on the glass pipette was compensated using the fast capacitance compensation circuitry built into the patch clamp amplifier, producing the on cell configuration. As the antibiotic incorporates into the membrane access begins to occur, appearing, usually within five minutes, as the development of a slow transient. As access improves this event becomes faster, until the access resistance resembles that produced using the conventional whole cell configuration.

2.7.5.2 Inside-out patch

By rapidly withdrawing the pipette from the cell following seal formation, an isolated patch of membrane can be torn off. The resulting patch is 'inside-out' with the cytoplasmic membrane surface facing the bath solution. One problem often encountered when excising inside-out patches is the formation of 'vesicles'. These occur when the patch of excised membrane seals over, enclosing a small volume of bath solution.

Vesicles could be identified as distinct from 'true' inside-out patches as the current amplitude of any channels present in the vesicle were reduced and possessed a distinctive 'rounded' appearance. Occasionally vesicles could be ruptured restoring the inside-out patch configuration by briefly exposing the pipette tip to air, although loss of the patch was the usual result of this action. Vesicle formation could be reduced by excising patches into a solution containing a low concentration of Ca^{2+}.

2.7.5.3 Conventional whole cell

Following seal formation it is possible to rupture the isolated section of cell membrane by applying suction to the interior of the pipette. This produces electrical and chemical continuity between the pipette and the cell. Briefly whole cell access can be produced in the following way:

1. A 10 mV hyperpolarising voltage pulse is continuously applied which produces a fast capacity transient due to patch pipette being charged up, and can be eliminated using the fast capacity compensation circuitry built into the patch clamp amplifier.

2. The pipette potential is set at -40 or -60 mV so that when access is achieved the cellular membrane potential is clamped at a potential close to the RMP of these cells.

3. Strong suction produces access by rupturing the cell membrane isolated in the lumen of the patch pipette. Access is visualised as a sudden increase in a capacitative current in response to the 10 mV voltage pulse, as the whole of the cell membrane, not just the isolated patch, is being charged up, with little or no change in the holding current.

4. Allow the access to stabilise and dialysis of the interior of the cell to occur by leaving the cell for approximately 1-2 minutes before the slow capacitance, representing the access resistance and the membrane capacitance, is compensated using the slow capacitance compensation circuitry.

One very frustrating problem that I frequently encountered when using isolated vascular smooth muscle cells was 'sealing over' of the electrode tip with cell membrane after successfully establishing whole cell access. Applying positive pressure after gaining access sometimes helped to overcome this problem.

2.7.5.4 Series resistance (R_{SERIES})

Potentially important sources of error associated with whole-cell patch clamping are series resistance (R_{SERIES}) and space clamp. An excellent account of the importance of these problems is given by Armstrong & Gilly, (1992)

Series resistance can originate from two sources:

1. The access resistance of the patch pipette and the 'hole' in the cell membrane together constitute the access resistance (R_{ACCESS}). The diameter of the patch-pipette tip, which is the major determinant of the resistance of the pipette, is essentially a trade off between having a pipette diameter small enough to allow frequent seal formation but large enough not to contribute to a large R_{ACCESS}.

2. The resistance of the bath electrode and the electrolyte solution to the flow of current from the pipette to the bath electrode ($R_{EXTERNAL}$).

Large R_{SERIES} values have 2 main consequences:

1. It slows the changing of the cell membrane potential in response to command potentials.

2. More importantly, it can lead to an underestimation or overestimation of the current as the membrane potential is not clamped at the required voltage.

The following example illustrates this important point. If a command potential V, 500 ms in length activates no channels, then because of the high electrical resistance of the cell membrane very little current will flow across the cell membrane and be measured by the patch-clamp amplifier.

Consequently little current will be injected to clamp the cell potential at V for the duration of the command pulse. If however, another command potential V` activates many $K^+$ channels in the cell membrane, then, assuming V` is positive to E_K, K^+ will flow out of the cell. If the current activated by V` is very large, then problems can occur. This is because the cellular currents measured by the patch-clamp amplifier and inverted and injected via the pipette into the cell to 'clamp' the membrane potential is correspondingly large. But because of the resistance to the flow of this clamping current due to R_{ACCESS} this current will not clamp the cell at the correct $V_{COMMAND}$ but at $V_{COMMAND}$-V_{DROP}, where V_{DROP} is equal to the voltage drop of the clamping current that occurs across R_{ACCESS}.

If R_{ACCESS} = 10 MΩ, a not unreasonable value in patch-clamp experiments, and the outward current activated by a 500 ms command potential of the value V is 1 pA, V_{DROP} = 0.01 mV. If the other membrane potential V` activates an outward current of 1 nA then a current will have to be injected into the cell to clamp the membrane potential at V`. If 1 nA of current were injected a V_{DROP} of 10 mV across a 10 MΩ resistor would occur. The clamping current therefore will not be large enough to clamp the cellular membrane potential at the required voltage V` for the duration of the command potential but at a potential equal to V` - 10 mV. The currents activated by the command potential V` will therefore be not be an accurate estimation of the current that would actually be activated by the 'true' $V_{COMMAND}$.

In the experiments described in this thesis steady state currents were normally <1000 pA. Assuming mean R_{ACCESS} values were 13 MΩ and 25 MΩ, not unreasonable values when using the conventional whole cell and perforated patch whole cell configurations respectively, and an outward K^+ current of 750 pA was activated, voltage drops due to R_{SERIES} of 9.75 mV and 18.75 mV would be predicted if no R_{SERIES} compensation was used. To compensate for the voltage drop R_{SERIES} compensation (40 to 60 %) was routinely used when such large currents were activated.

2.7.5.5 Space clamp

When manipulating the membrane potential using patch-clamp techniques, an investigator assumes that the whole of the cell membrane is maintained at the same potential as the command voltage ($V_{COMMAND}$) i.e. the cell membrane is isopotential. This assumption however probably only applies to small spherical cells.

If a cell is not small and spherical, the potential of the whole of the cell membrane in response to a command voltage will not be identical throughout the cell but will decay exponentially with distance from the point of current injection. In electronics, the point in a cable where the voltage produced following an injection of current falls to half its original level is termed the 'electrical half-distance'. Space clamp problems can also provide potential sources of error when making whole cell recordings, as the assumption that $V_{MEMBRANE}$ throughout the whole of the cell equals $V_{COMMAND}$ may be incorrect if the length of the cell greatly exceeds the electrical half distance.

The smooth muscle cells used in the experiments described in this thesis were relaxed and cylindrical. However, they were relatively small (approximately 100 μM in length and ≤10 μM in diameter) and at rest had a high input resistance (>5 GΩ). Therefore, assuming the cells had a low internal resistance (R_i), ~250 Ωcm^{-1}, as has previous been reported for smooth muscle cells (Abe & Tomita, 1968), then the electrical half distance (λ) can be calculated using Equation 7:

$$\lambda = \sqrt{(d / 4 * R_m / R_i)} \qquad [7]$$

Where d equals the diameter of the cell (~4), R_m equals the specific membrane resistance, which is the product of the input resistance (5 GΩ) and the area of the cell (capacitance ~20 pF / assumed specific membrane capacitance 1 $\mu F/cm^2 = 0.2 \times 10^{-4}$ cm^2), which equals 100 KΩ cm^2, a value similar to previous estimates of R_m (Bolton *et al.*, 1985; Lang, 1989; Toro & Stefani, 1987), and R_i equals 250 Ω cm^{-1}. λ therefore equals ~0.2 cm, a value similar to previous measurements (Lang, 1989; Smirnov & Aaronson, 1992a). As the patch pipette was routinely positioned in the middle of cells (~100 μM) then the membrane potential of these cells would be virtually isopotential at rest as λ exceeds 50 μm by 40 times. Even if a whole cell current of ~1000 pA flowed, which would decrease the input resistance from 5 GΩ to ~0.06 GΩ, then λ would equal 0.0219 cm a value still approximately four-fold greater than 50 μM. It is therefore likely that space clamp was maintained even following the activation of whole cell currents by NS1619 or NO.

2.7.5.6 Outside-out patch

If the pipette is withdrawn from the cell after achieving conventional whole cell access, a section of membrane is torn from the cell which sometimes reseals to form an outside out patch.

2.8 Data collection and analysis

A Dell 386 or 486 personal computer was used to control the patch-clamp amplifier and also to generate command potentials and voltage ramps. Currents resulting from these commands were stored directly onto either digital audio tape (D.A.T.) or the computer hard disk for analysis. The software described in this thesis was written by Dr Noel Davies and I would like to thank him for the help and assistance he has given me. It consists of a suite of programs developed using the AxoBASIC subroutine library. The following programs were regularly used:

-STIMTOR: This program generates command potentials and voltage ramps.

-TAPE: This program allows the transfer of information recorded on digital audio tape to the computer hard disk.

-TRACAN: A trace analysis and manipulation program.

2.8.1 Command potentials

As discussed in detail above, many channels are activated by changes in the cellular membrane potential. Command potentials were therefore used to manipulate the membrane potential and any currents resulting from this new membrane potential were written directly to the hard disk.

Coupled with the current activated by the change in membrane potential may be a 'leak current'. When recording for example voltage-activated Ca^{2+} currents, which may have small peak inward currents of <30 pA, it is imperative that any outward leak component is measured and corrected so as to maximise the amplitude of the inward currents.

Digital leak subtraction was performed by producing an average leak current trace at each command potential. This was achieved by applying a train of 12 voltage pulses 1/6 the amplitude of the command pulse after the command pulse. This was then averaged and subtracted from the 'raw' current data giving leak subtracted data. When measuring Ba^{2+} currents through Ca^{2+} channels leak subtraction was performed using an external solution of 2 mM $CoCl_2$. Co^{2+} ions reversibly blocked any ion channels but left any leak current intact allowing the construction of a leak current pulse which was then subtracted from 'raw' data giving the leak-subtracted current.

2.8.2 Voltage ramps

Whole cell voltage ramps were frequently used to activate whole cell currents. The resulting currents were low pass filtered at 1 kHz, sampled at 5 kHz and written directly to the hard drive.

2.8.3 Steady state data

Data from steady state recordings from either whole cell experiments or excised membrane patch experiments were recorded at 10 kHz directly onto digital audio tape using a modified D.A.T. recorder (Sony). Data were replayed for analysis through an 8-pole bessel filter of variable shut-off frequency and digitised using a TL-1 DMA interface (Axon Instruments) and Dell 386 / 486 computer and written onto the hard drive.

2.8.4 Amplitude histograms

Knowledge of the unitary current amplitude of an ion channel can help channel characterisation, as in many cases this value is a 'fingerprint' for a particular channel. A commonly used method of measuring unitary current is to plot data points corresponding to channel open and closed events as a histogram. To measure single channel opening events satisfactorily the noise level has to be as low as possible (<1 pA). Noise can be decreased by increasing the resistance of the current source (which is mainly the combination of the feedback resistor in the amplifier, the seal resistance and the resistance of the preparation itself). The smooth muscle cells used in this study had very high input resistance values, typically >5 GΩ at -60 mV.

This high input resistance, coupled with the high resistance giga-seals which form between the pipette tip and the cell membrane, and the large unitary current amplitude of BK_{Ca} channels sometimes allowed measurements of the single channel amplitude of BK_{Ca} channels to be made by constructing amplitude histograms.

Data containing numerous channel open and closed events, from excised patches or whole cell recordings were low pass filtered at 1 kHz and sampled at 5 kHz. The distribution of the data gave peaks corresponding to open and closed channel levels. Each peak was fitted with a Gaussian distribution of the form:

$$\mathbf{f(x) = a / \sqrt{2\pi\sigma^2}\ exp\ [-(x-\mu)^2 / 2\sigma^2]} \qquad [8]$$

where σ^2 equals varience, σ equals standard deviation, μ equals the mean and a is the relative area under the peak.

2.8.5 Channel open probability

For measurements of channel open probability (P_{open}) data were filtered at 1 kHz and sampled at 5 kHz. P_{open} values were obtained by measuring the time spent at current levels corresponding to 0, 1, 2,...N channels with the threshold for a channel opening set at the 50 % level of a full opening using Equation 9:

$$\mathbf{P_{open} = (\sum_{j=1}^{N} tj.j) / NT} \qquad [9]$$

Where T equals the duration of the recording, usually between 30 seconds and 3 minutes, N equals the maximum number of channels open in the patch, tj equals the time spent at current levels corresponding to j= 0,1,2.....channels open.

2.9 Appendix

2.9.1 Construction of superfusion system tips

The tip was fashioned from polypropylene tubing (o.d. 1 mM, i.d. 0.5 mM Portex) and yellow Eppendorf (20-200 µl) pipette tips using the following method.

1. Flame (using a small yellow Bunsen flame) 30 cm sections of polypropylene tubing in the middle and pull gently apart so that the middle section forms a fine filament approximately 10 cm in length.

2. Cut the filament in the middle to provide the 2 flow lines. Repeat until there are 7 flow lines.

3. Bunch the flow lines together, ensuring that the point where the lines thin are roughly lined up.

4. Cut the tips off an Eppendorf, leaving a hole that all the lines will just fit through. Then cut off the back of the tip leaving a length of 1.5 cm. Thread the lines through the modified Eppendorf and tie them in position with stretched polypropylene tubing.

5. Fill the Eppendorf with Sylgard 184 elastomer and leave in a warm place to promote curing.

6. To make a capping tip that provides the single outflow for the perfusion system; flame the tip of a yellow Eppendorf until it begins to soften. Then pull the softened area with forceps until a filament approximately 5 cm long is pulled. Cut back the filament so the aperture of the tip is approximately 0.5 mm in diameter. Cut off the tip so that the dead space is as small as possible.

7. Cut back the Eppendorf containing the polypropylene tubes fixed in position with Sylgard so that the tip fits tightly on the end.

8. Attach the perfusion tip to the Teflon tubing with short lengths of silicon tubing.

2.9.2 Buffer solution

Composition of buffer used when dissecting out the basilar artery (mM):
NaCl 136, 4-(2-hydroxy-ethyl)-1-piperazineethanesulphonic acid (HEPES) 10, KCl 5.6, $NaHCO_3$ 4.17, $CaCl_2$ 2.6, $MgCl_2$ 1, NaH_2PO_4 0.44 and $NaHPO_4$ 0.42, adjusted to pH 7.4.

An identical buffer was used when the artery was stored or when undergoing digestion except the Ca^{2+} concentration was 0.1 mM

2.9.3 Bath solution

Once dissociated, cells were bathed in a solution containing (mM):
NaCl 136, glucose 10, HEPES 10, KCl 6 , $MgCl_2$ 1, and $CaCl_2$ 0.2. The pH of this solution was adjusted to 7.4 such that the final concentration of Na^+ was 140 mM.
This particular Ca^{2+} concentration was used as I found that cells bathed in it remained relaxed and therefore viable for longer periods compared to cells bathed in a similar solution containing 2mM Ca^{2+}.

2.9.4 Perforated patch solutions

The pipette solution contained (mM):KCl 136, HEPES 10, $MgCl_2$ 1 and EGTA 0.5. the pH of this solution was adjusted to 7.2 with KOH such that the final K^+ concentration was 140 mM. The bath solution was identical to the basic bath solution described above.

2.9.5 Solutions for recording K_V currents

The conventional configuration of the patch clamp technique is produced by disrupting the patch of cell membrane isolated following the formation of a giga-seal. This produces electrical and chemical continuity between pipette and cell and obviously allows intracellular dialysis with the pipette solution. This allows manipulation of the intracellular contents, thus aiding the selective activation of discrete channel types. For example, K_V and BK_{Ca} channels exhibit voltage-dependence and are activated by membrane depolarisation as described in the introduction.

Therefore, to minimise the level of BK_{Ca} channel activation when recording K_V currents in response to depolarising command potentials the following solutions were used:

The extra-cellular solution was identical to the bath solution described above except that 100 nM of IbTX was included. I found that this concentration of toxin produced rapid, near complete block of BK_{Ca} channels.

The pipette solution contained (mM): KCl 140, glucose 10, HEPES 10, EGTA 5, and $MgCl_2$ 0.5 and was adjusted to pH 7.2 with NaOH. Na_2ATP 2 and LiGTP 0.5 were added on the day of the experiment and the pH of the solution readjusted to 7.2.
Using these conditions the dominant outward K^+ current activated in response to whole cell voltage steps was due to the activation of K_V channels.

2.9.6 Solutions for recording cell membrane potential

The composition of the bath solution was identical to that described above and the pipette solution was identical to that for recording voltage-activated K^+ currents except that EGTA was omitted.

2.9.7 Solutions for recording Ca^{2+} currents

The conventional whole cell configuration of the patch clamp technique was used to measure whole cell Ca^{2+} currents. Ba^{2+} ions rather than Ca^{2+} ions were used as the transmembrane charge carrier. This is because Ca^{2+} ions have a higher affinity for the binding site within the channel pore than Ba^{2+} (see chapter 1) resulting in the activation of smaller currents than when Ba^{2+} is employed as the charge carrier.

The composition of the bathing (external) solution was (mM): NaCl 130, $BaCl_2$ 10, glucose 10, HEPES 10, KCl 5.4, $MgCl_2$ 1.0 and EGTA 0.1. The pH was adjusted to 7.4 such that the final bath concentration of K^+ was 6 mM.

The composition of the pipette (internal) solution was (mM): CsCl 130, glucose 10, EGTA 5 and $MgCl_2$ 1.0. The pH was adjusted to 7.2 with CsOH. Na_2ATP and LiGTP were added on the day of the experiment and the pH readjusted to 7.2 with TEAOH or CsOH.

2.9.8 Solutions for recording single BK_{Ca} channel currents

Single channel recordings were made using excised inside-out and outside-out cell membrane patches (Hamill *et al.*, 1981). For inside-out patches, the cytoplasmic face of the patch was bathed with a solution containing either 500nM free-Ca^{2+}, 1 μM free-Ca^{2+} or nominally Ca^{2+}-free. The solution containing 500 nM free-Ca^{2+} had the following composition (mM): KCl 140. glucose 10, HEPES 10, EGTA 5, $CaCl_2$ 3.74 and $MgCl_2$ 1.0 pH was adjusted to 7.2 with NaOH. This gave a calculated free Ca^{2+} concentration of 500 nM using the MaxChelator program, which uses the buffering constants described by Martell and Smith. The solutions containing 1 μM free-Ca^{2+} and nominally Ca^{2+}-free were identical in composition to the above solution except the solution containing 1 μM-free Ca^{2+} contained 4.3 mM Ca^{2+} and the Ca^{2+}-free solution contained no added Ca^{2+} and the EGTA concentration was increased to 10 mM. When studying the effects of cGMP-dependent protein kinase on BK_{Ca} channel activity ATP 1 mM, cGMP 0.5 mM and cGMP-dependent protein kinase (Bovine recombinant isoform 1α Calbiochem) were added to test solutions and their pH readjusted to 7.2

The pipette (extra-cellular) solution was identical in composition to the bath solution detailed above.

For outside-out patches the bath (extra-cellular) solution was identical in composition to the bath solution described above and the pipette (internal) solution contained the 500 nM free Ca^{2+} solution described above.

2.9.9 Solutions for recording whole cell BK_{Ca} currents

The cells were bathed in the ordinary bath solution described above until conventional access was achieved. The solution superfusing the cell under study was then changed, using the pressure superfusion system previously described in the methods chapter, to a solution containing (mM): KCl 140, glucose 10, HEPES 10, $MgCl_2$ 1.0, $CaCl_2$ 0.2 and superoxide dismutase 50 units ml^{-1} (Sigma).
The pH of the solution was adjusted to 7.4 with NaOH such that the final concentration of Na in the solution was approximately 5 mM.

The pipette (internal) solution contained (mM): KCl 140, glucose 10, HEPES 10, EGTA 1.0, Na_2ATP 1.0 and LiGTP 0.5. The pH of the solution was adjusted to 7.2 with NaOH such that the final concentration of Na in the solution was approximately 4 mM.

CHAPTER THREE

Effects of the substituted benzimidazole NS1619 (1-(2′-hydroxy-5′-trifluoromethylphenyl) trifluoromethyl-2-(3H)benzimidazolone) on BK_{Ca} channels in single rat basilar artery myocytes

3.1 INTRODUCTION

As discussed in some detail in chapter 1 the regulation of vascular smooth muscle contraction is dependent on the concentration of intracellular Ca^{2+}, which in turn is partly regulated by the membrane potential, an example of electromechanical coupling. The realisation that the membrane potential was an important regulator of vascular tone arose following the discovery that a chemically diverse group of drugs relaxed vascular smooth muscle by a mechanism involving the opening of K^+ channels located in the cell membrane (for reviews see Cook, 1988; Cook & Quast, 1988; Longman & Hamilton, 1992; Nelson & Quayle, 1995). This group of compounds, typified by pinacidil, and cromakalim, produce hyperpolarisation by opening K_{ATP} channels located in the cell membrane, leading to a reduction in the $[Ca^{2+}]_i$ and vasorelaxation. Activation of other types of K^+ channel could also produce hyperpolarisation and ultimately relaxation of vascular smooth muscle. One promising candidate is the BK_{Ca} channel which has been found in all of the vascular smooth muscle preparations that have so far been studied (Nelson, 1993). Opening of this channel is increased by depolarisation of the cell membrane and also by raising the Ca^{2+} concentration at the cytoplasmic surface of the channel. These properties strongly suggests a role for this channel in the regulation of smooth muscle tone, and indeed the BK_{Ca} channel blocker, charybdotoxin (CTX), was found to depolarise a pressurised artery by 7 mV and constrict the vessel (Brayden & Nelson, 1992). Because of the large unitary conductance of BK_{Ca} channels and their high density in vascular smooth muscle (Nelson & Quayle, 1995), drugs which open BK_{Ca} channels should theoretically be effective vasodilator compounds. A number of chemically diverse compounds have been recently described which activate BK_{Ca} channels (see chapter 1). One of these, the substituted benzimidazole (1-(2′-hydroxy-5′-trifluoromethylphenyl) trifluoromethyl-2-(3H)benzimidazolone, or NS1619, was reported to hyperpolarise cultured bovine aortic smooth muscle cells (Olesen *et al.*, 1994a). The initial aims of this study were therefore to investigate the effects of NS1619 on single smooth muscle cells acutely dissociated from the rat basilar artery using the various configurations of the patch-clamp technique. This was achieved by examining the effects of NS1619 on the membrane potential of smooth muscle cells, identifying any channels affected by NS1619 and then attempting to determine the mechanism of action of NS1619.

3.2 RESULTS

3.2.1 Effects of NS1619 on cellular membrane potential

When a physiological potassium concentration gradient exists across a cell membrane, the K^+ equilibrium potential (E_K), as predicted by the Nernst equation, is approximately -80 mV. This contrasts with the resting membrane potential (RMP) of smooth muscle cells, which ranges between -40 to -60 mV when measured *in vitro* from arteries and arterioles subjected to normal levels of intraluminal pressure (Harder *et al.*, 1987; Nelson *et al.*, 1990; Brayden & Nelson 1992; Knott & Nelson 1995), -40 to -55 mV *in vivo* (Hirst & Edwards, 1989; Nelson *et al.*, 1990) and ~-40 mV from isolated vascular smooth muscle cells (Smirnov & Aaronson, 1992a). Therefore if K^+ channels open, K^+ will move out of the cell due to a favourable electrochemical gradient and the efflux of positively charged ions would produce hyperpolarisation, moving the membrane potential, in the absence of other factors, towards E_K. If NS1619 was capable of opening BK_{Ca} channels it should produce cell membrane hyperpolarisation. The effects of NS1619 on the membrane potential of single basilar smooth muscle cells were therefore examined using the conventional whole cell configuration with 140 mM K^+ internally and the patch clamp amplifier operating in the current clamp mode. Cells selected for membrane potential recordings were relaxed and densely phase bright. After establishing whole cell access the membrane potential had to be stable for approximately 1 minute before the effects of NS1619 were assessed. Figures 3.iA and 3.iB illustrate recordings of membrane potential from 2 cells that fulfilled the above requirements. The resting membrane potentials of these examples prior to NS1619 being added (sampling rate 2Hz) were -10 mV (1A) and ~-45 mV (1B). Addition of 30 μM NS1619 to the 6 mM K^+ solution superfusing the cell raised the membrane potential to approximately -75 mV (E_K was estimated to be -79.5 mV). This occurred within ~100 seconds in the example illustrated in the upper panel and approximately 15 seconds in the example illustrated in the lower panel. This hyperpolarisation was reversed in both cases by adding 100 nM iberiotoxin (IbTX) to the superfusing solution in the continued presence of 30 μM NS1619. These experiments clearly demonstrate that 30 μM NS1619 produced membrane hyperpolarisation. The additional observation that IbTX, the selective BK_{Ca} channel blocker, reversed this effect suggests that the basis of this hyperpolarisation was the opening of BK_{Ca} channels.

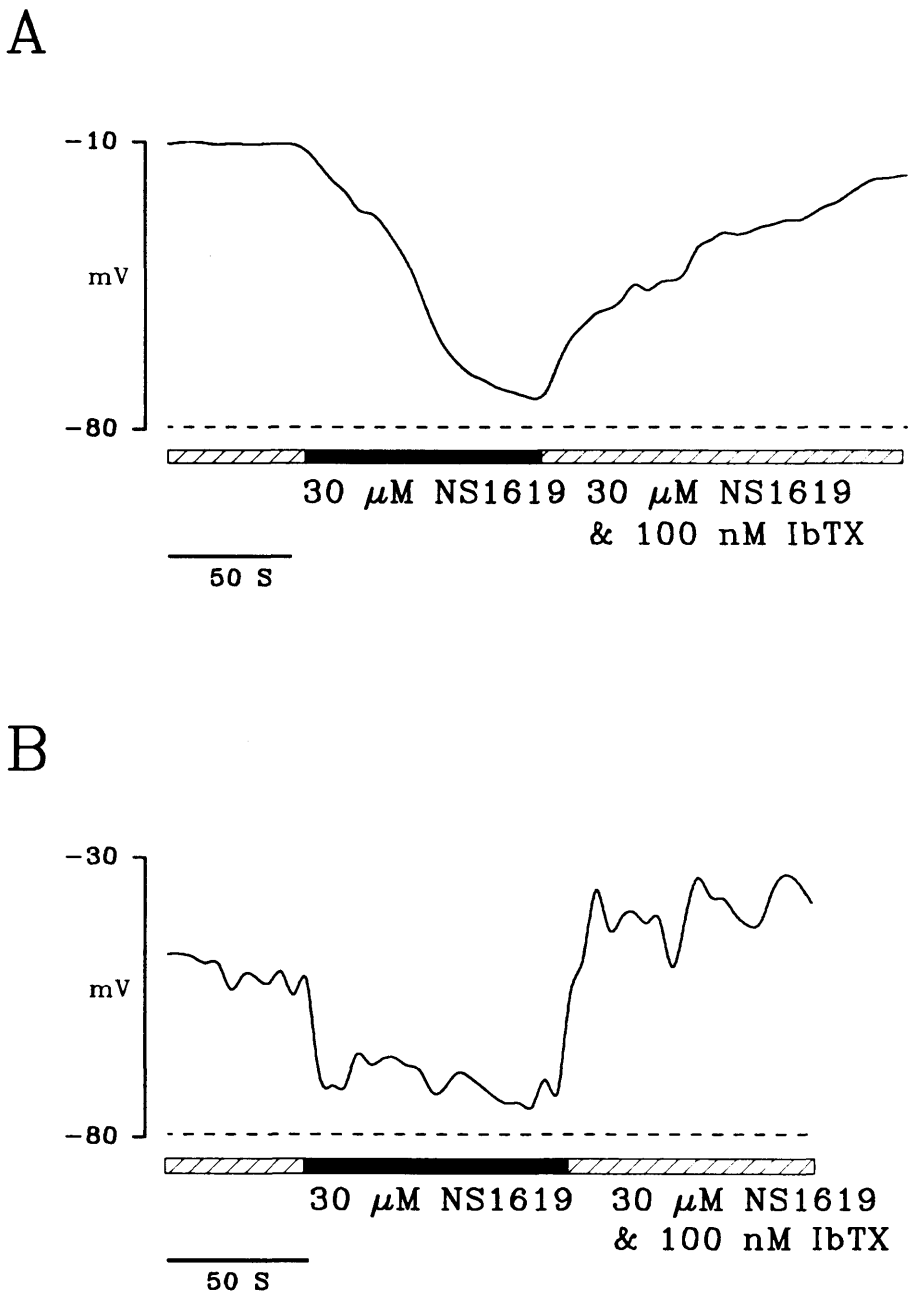


Figure 3.i. Effect of NS1619 on the cell membrane potential of rat basilar smooth muscle cells: Both panels illustrate membrane hyperpolarisation induced by 30 μM NS1619. The calculated E_K in these experiments is illustrated by the continuous dotted line. NS1619 and 100 nM IbTX were added where indicated.

This concentration of IbTX has previously been shown to be specific for BK_{Ca} channels and unlike the other commonly used blocker of BK_{Ca} channels, charybdotoxin (CTX), has no additional effects on voltage activated K^+ channels (Kaczorowski *et al.*, 1996). Following the addition of 100 nM IbTX to block the hyperpolarisation illustrated in figure 3.iB, the membrane potential fell below that recorded before NS1619 was added. The resting membrane potential was -45 to -50 mV before NS1619 was added and oscillated between -32 and -45 mV in the presence of 100 nM IbTX. This was a potentially interesting observation as a previously proposed physiological role for BK_{Ca} channels is that of regulating the resting membrane potential in rabbit cerebral vessels (Brayden & Nelson, 1992). BK_{Ca} channels may therefore have a similar role in the rat cerebral circulation.

3.2.2 Activation of whole cell currents by NS1619 in intact rat basilar artery smooth muscle cells.

To further characterise the channels opened by NS1619, patch clamp techniques were employed to study the effects of NS1619 on steady state whole cell currents. This technique allows any currents activated by NS1619 to be observed without the normal accompanying membrane potential changes. Figure 3.ii illustrates the effect of 20 μM NS1619 on the steady state current recorded from a single basilar myocyte. Access was achieved using the perforated patch technique with amphotericin B, ensuring that the intracellular Ca^{2+} concentration and buffering were maintained as close to physiological levels as possible and that diffusible cytoplasmic constituents were not lost by diffusion into the patch pipette. The cell under study was clamped throughout at 0 mV and was continuously superfused with a physiological salt solution containing 6 mM K^+, so that the opening of K^+ channels would appear as outward currents. Prior to the addition of NS1619 the basal level of channel activity was low, which probably reflects the low global Ca^{2+} concentration known to exist in cerebral artery smooth muscle cells. One report, for example, estimated the global Ca^{2+} concentration at 0 mV to be 500 nM (Kamishima & McCarron 1995). One additional experimental detail that should be noted, before any data concerning the effects of NS1619 on DHP-sensitive Ca^{2+} channels was available, 1 μM nifedipine was routinely included in all extracellular solutions to ensure that increased activity of Ca^{2+}-dependent channels could not be attributed simply to Ca^{2+} influx due to clamping the cell at a depolarised potential. Following the inclusion of 20 μM NS1619 in the solution superfusing the cell, a large, noisy outward current was activated.

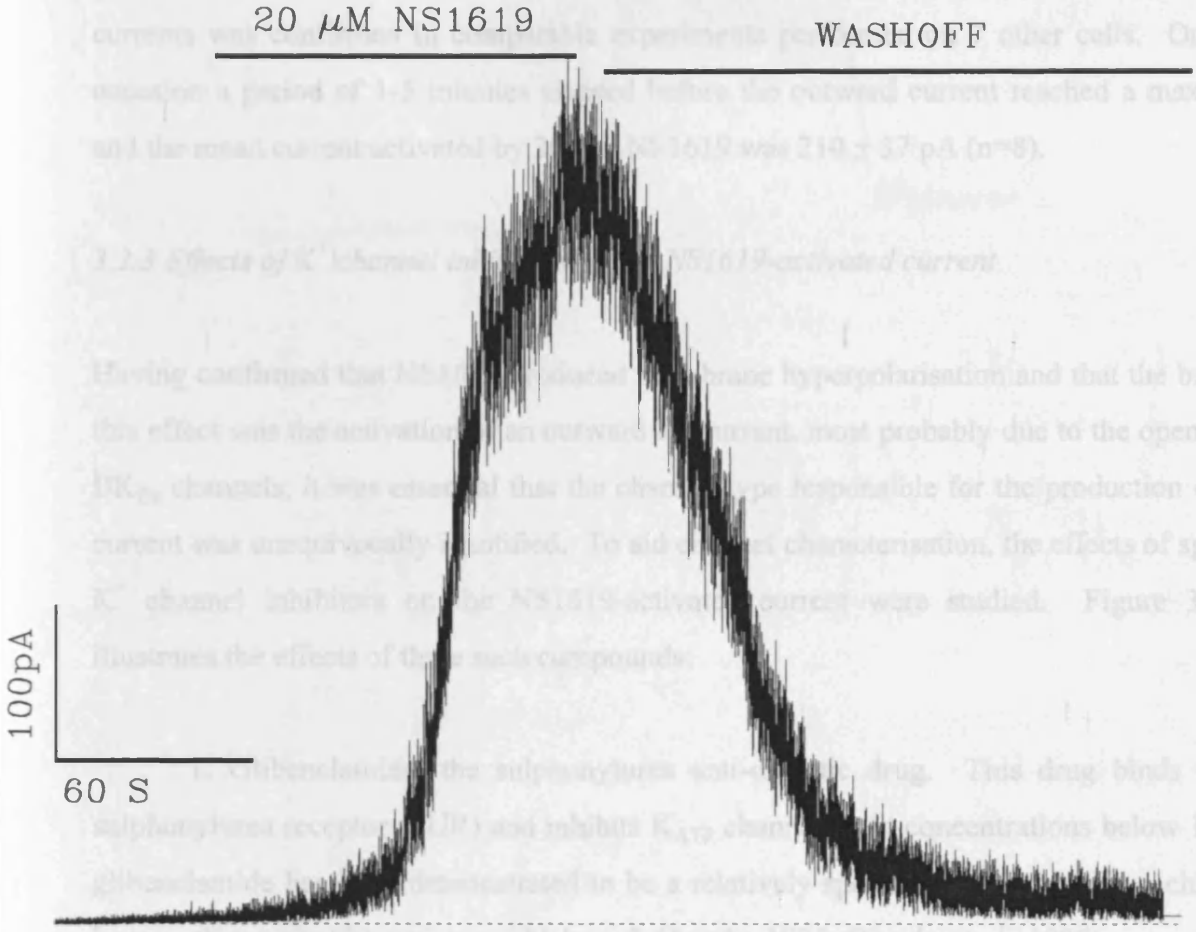


Figure 3.ii. Reversible activation of a whole cell current by NS1619. Access was gained using the perforated-patch technique with amphotericin B and the cell was held at 0 mV and bathed in 6 mM K^+ and 1 μM nifedipine throughout. The continuous dotted line indicates the zero current level and this record was filtered at 1.0 kHz and sampled at 5.0 kHz. Following the addition of 20 μM NS1619 a large outward current was activated which reversed following washout.

This current reached a steady state of approximately 500 pA after contact with NS1619 for approximately 2 minutes. When NS1619 was removed the current slowly declined. The residual level of current was still far higher than the control level even after perfusing the cell for 3 minutes with NS1619-free solution. The ability of NS1619 to activate outward currents was confirmed in comparable experiments performed on 7 other cells. On each occasion a period of 1-5 minutes elapsed before the outward current reached a maximum and the mean current activated by 20 μM NS1619 was 210 ± 37 pA (n=8).

3.2.3 Effects of K^+ channel inhibitors on the NS1619-activated current

Having confirmed that NS1619 produced membrane hyperpolarisation and that the basis of this effect was the activation of an outward K^+ current, most probably due to the opening of BK_{Ca} channels, it was essential that the channel type responsible for the production of this current was unequivocally identified. To aid channel characterisation, the effects of specific K^+ channel inhibitors on the NS1619-activated current were studied. Figure 3.iii A illustrates the effects of three such compounds:

1. Glibenclamide, the sulphonylurea anti-diabetic drug. This drug binds to the sulphonylurea receptor (SUR) and inhibits K_{ATP} channels. At concentrations below 10 μM glibenclamide has been demonstrated to be a relatively specific inhibitor of K_{ATP} channels in smooth muscle (for review see Nelson & Quayle, 1995; Quayle *et al.*, 1997)

2. Apamin, a peptide toxin extracted from bee venom which selectively blocks small conductance Ca^{2+}-activated (SK_{Ca}) channels (Hugues *et al.*, 1982).

3. Iberiotoxin, a peptide toxin extracted from scorpion venom which selectively blocks BK_{Ca} channels with a K_i of < 10 nM (Candia *et al.*, 1992; Giangiacomo *et al.*, 1992).

In figure 3.iiiA whole cell access was also gained using the perforated patch technique with amphotericin B. The cell was clamped throughout at 0 mV and superfused with saline containing 6 mM K^+, such that the opening of K^+ channels would produce outward currents.

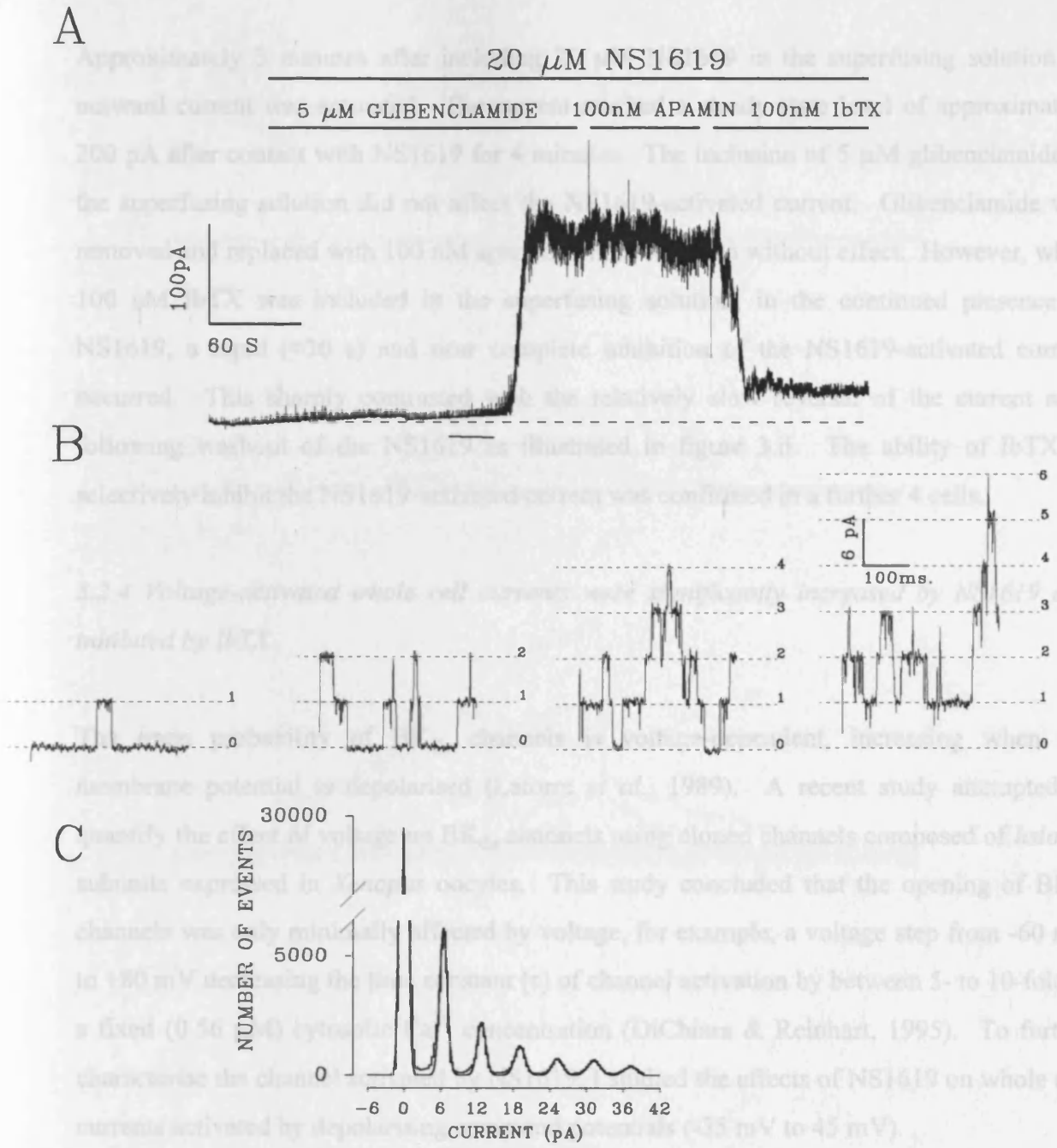


Figure 3.iii. Selective activation of BK_{Ca} channels underlay the whole cell current produced by NS1619. Access was gained using the perforated-patch technique with amphotericin B and the cell was held at 0 mV and bathed in 6 mM K^+ throughout. This recording was filtered at 1.0 kHz and sampled at 5.0 kHz. When the outward current activated by 20 μM NS1619 had reached a steady-state 5 μM glibenclamide, 100 nM apamin and 100 nM IbTX were added where illustrated, with IbTX selectively inhibiting the NS1619-activated current. Panel B shows that the opening of large-conductance channels underlay the whole cell currents activated by NS1619. These records, expanded from the section highlighted in panel A, show 500 ms sections illustrating that NS1619 selectively activated a channel with a large unitary current amplitude. The dotted lines indicate the current levels corresponding to 0, 1, 2 etc. channels open. Panel C shows a histogram of the channels activated by NS1619 from which the unitary current amplitude was measured.

Approximately 3 minutes after including 20 μM NS1619 in the superfusing solution an outward current was activated. The current reached a steady state level of approximately 200 pA after contact with NS1619 for 4 minutes. The inclusion of 5 μM glibenclamide in the superfusing solution did not affect the NS1619-activated current. Glibenclamide was removed and replaced with 100 nM apamin, which was also without effect. However, when 100 nM IbTX was included in the superfusing solution, in the continued presence of NS1619, a rapid (<30 s) and near complete inhibition of the NS1619-activated current occurred. This sharply contrasted with the relatively slow reversal of the current seen following washout of the NS1619 as illustrated in figure 3.ii. The ability of IbTX to selectively inhibit the NS1619-activated current was confirmed in a further 4 cells.

3.2.4 Voltage-activated whole cell currents were significantly increased by NS1619 and inhibited by IbTX.

The open probability of BK_{Ca} channels is voltage-dependent, increasing when the membrane potential is depolarised (Latorre *et al.*, 1989). A recent study attempted to quantify the effect of voltage on BK_{Ca} channels using cloned channels composed of *hslo* α-subunits expressed in *Xenopus* oocytes. This study concluded that the opening of BK_{Ca} channels was only minimally affected by voltage, for example, a voltage step from -60 mV to +80 mV decreasing the time constant (τ) of channel activation by between 5- to 10-fold at a fixed (0.56 μM) cytosolic Ca^{2+} concentration (DiChiara & Reinhart, 1995). To further characterise the channel activated by NS1619, I studied the effects of NS1619 on whole cell currents activated by depolarising command potentials (-35 mV to 45 mV).

Access in all cases was obtained using the perforated-patch configuration with amphotericin B and all experiments were conducted using an external saline solution which contained 6 mM K^+ A holding potential of -15 mV was used to produce voltage-dependent inactivation of K_V channels (see figure 4.v chapter 4), ensuring that BK_{Ca} channels dominated the outward currents. Figure 3.iv illustrates, reading from top to bottom, representative currents (following leak subtraction) from the same smooth muscle cell activated following a sequence of command potentials, ranging from -35 to 45 mV, before and after the addition of 10 μM NS1619, 30 μM NS1619 and 30 μM NS1619 plus 100 nM IbTX to the cell. Outward currents activated under control conditions were small. In the example illustrated a peak outward current of only 20 pA was activated following a voltage step to +45 mV.

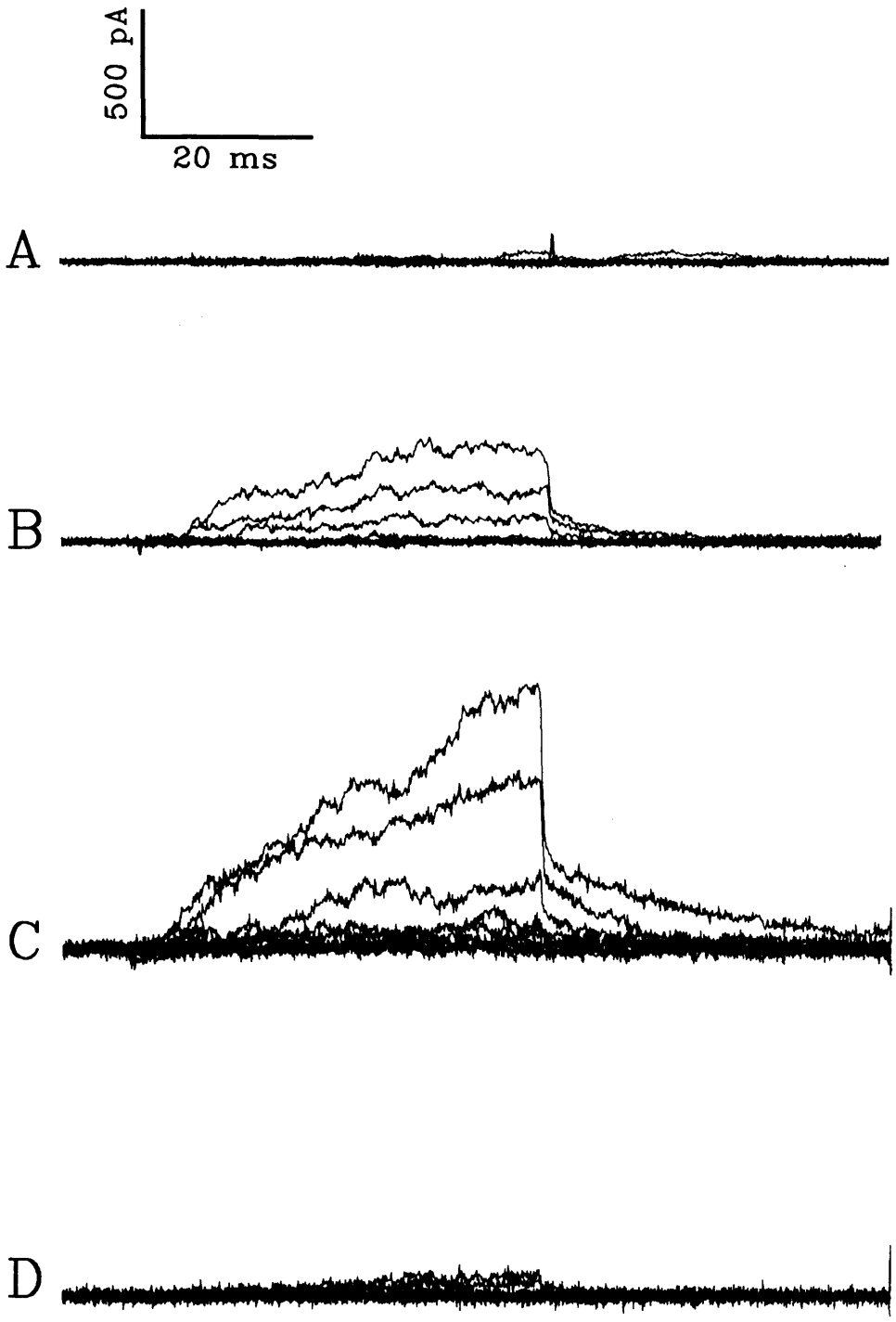


Figure 3.iv. Effects of increasing concentrations of NS1619 (10-30 μM) on whole cell currents activated by depolarising step potentials (-35 to 45 mV in 10 mV increments) from a single smooth muscle cell held at -15 mV. Access was gained using the perforated-patch technique with amphotericin B and the records illustrated were filtered at 1 kHz and leak subtracted. Panel A control currents obtained in the absence of NS1619. Panel B currents activated following the addition of 10 μM NS1619 to the cell for 1 minute. Panel C currents obtained following the addition of 30 μM NS1619 for 1 minute. Panel D currents obtained following the addition of 100 nM IbTX together with 30 μM NS1619.

After the addition of increasing concentrations of NS1619 to the cell for a period of 1 minute, large 'noisy' whole cell currents were activated. In figure 3.iv for example peak outward currents of 380 and 937 pA were activated following a voltage step to +45 mV in the presence of 10 and 30 μM NS1619 respectively. When 100 nM IbTX was included in the superfusing solution, in the continued presence of 30 μM NS1619, the peak outward current was reduced to 79 pA. Figure 3.v illustrates the mean (n=6) current-voltage relationship (I-V) where currents were measured as peak outward current following leak subtraction. Table 3.i illustrates the same data as figure 3.v numerically, with those values significantly different (Anova $p<0.05$) with respect to controls illustrated by the symbol *.

MEMBRANE POTENTIAL (mV)	CONTROL CURRENT (pA)	10 μM NS1619 CURRENT (pA)	20 μM NS1619 CURRENT (pA)	30 μM NS1619 CURRENT (pA)
5	2	3	6	41.1*
15	1	11.3	36.2	79.2*
25	3.8	32.9	107.4*	139.9*
35	9.3	99.2*	218.7*	354.8*
45 * = $p<0.05$	12	176.9*	366.8*	540.5*

Table 3.i Effects of increasing concentrations of NS1619 on mean whole cell currents activated by voltage steps

It should be noted that the whole cell currents activated by command potentials close to 0 mV in the presence of NS1619 were substantially smaller than the steady state currents activated by 20 μM NS1619 at 0 mV illustrated in figures 3.ii and 3.iiiA. This apparent anomaly is probably because currents activated by command potentials were from cells that were exposed to NS1619 for only 1 minute, whereas steady state currents were measured from cells that had been exposed to NS1619 for a longer period, typically 3-5 minutes.

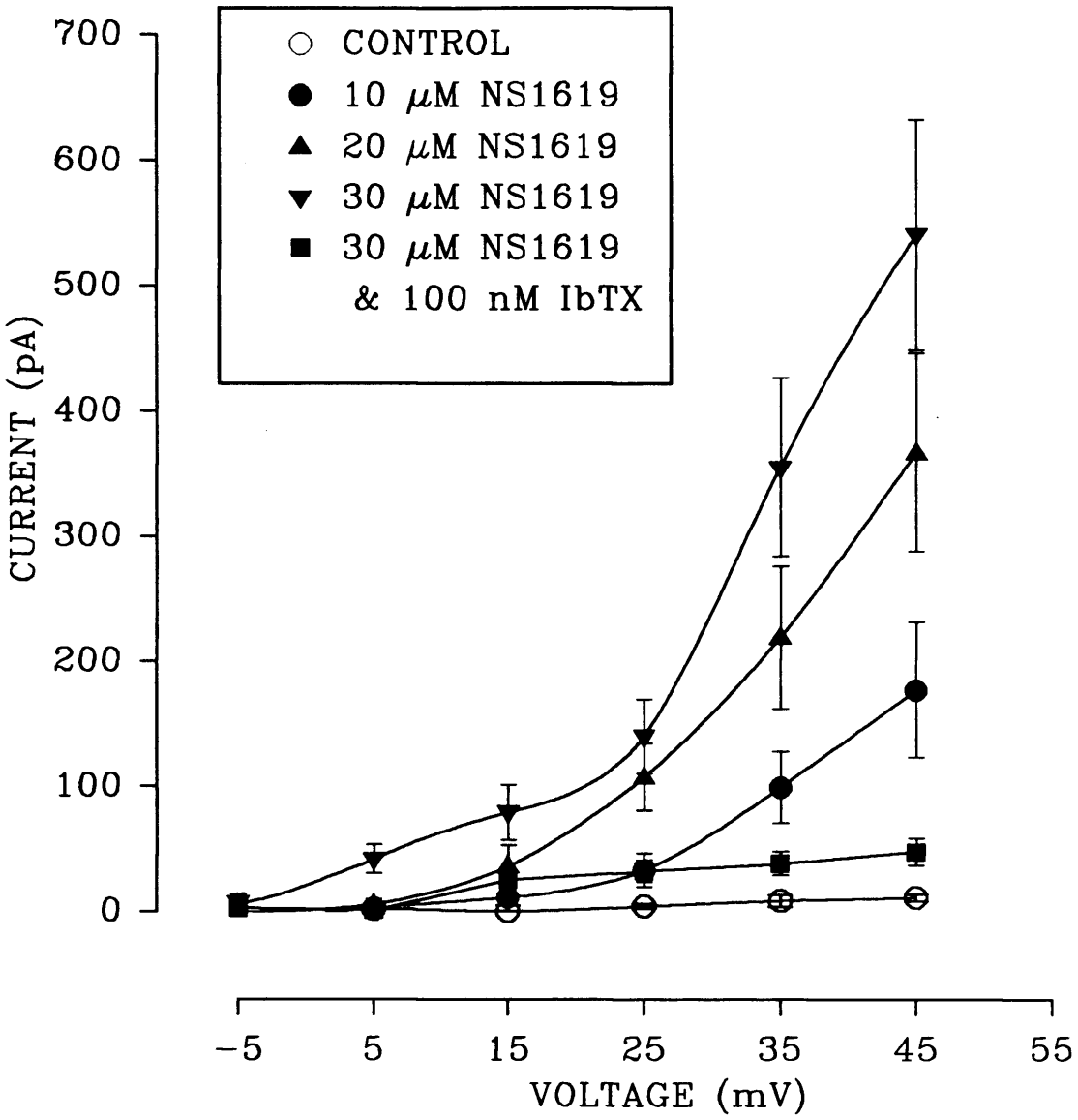


Figure 3.v. Effect of increasing concentrations of NS1619 on whole cell I-V curves. Data illustrated are the mean peak currents measured from experiments performed on 6 cells following leak subtraction after exposure to control external solution (○) and solutions containing 10 (●), 20 (▲), 30 μM (▼) and 30 μM NS1619 and 100 nM IbTX (■) respectively.

A contact time of 1 minute was simply used because it was not realistic to expect giga seals to last long enough to assess the steady state effects of each concentration of NS1619.

3.2.5 Whole cell currents activated by NS1619 flowed through large-conductance channels

The input resistance measured from single basilar artery smooth muscle cells was >5 GΩ, which, as explained in section 2.8.4 allowed the unitary current amplitude of individual channel openings to be resolved under whole-cell recording conditions. The unitary current amplitude of these channels could then be estimated by constructing an amplitude histogram of the open and closed events and measuring the unitary current amplitude from this histogram as described in chapter 2 section 2.8.4.

Knowledge of the unitary amplitude helps characterise the particular channel being studied, as this value can act as a 'fingerprint' for a particular channel. Figure 3.iii B illustrates 500 ms segments of recording expanded from the section highlighted in figure 3.iii A. These sections clearly show the progressive activation of a channel with a large unitary current amplitude by 20 μM NS1619. Because the channel open events were easily distinguished from the closed level i.e. the signal to noise ratio was high, an accurate estimate of the unitary current amplitude could be produced. Figure 3.iii C illustrates the amplitude histogram produced by analysing the data from figure 3.iii A. The unitary current amplitude of these channels, measured using the data illustrated in figure 3.iii C was 6.2 pA at 0 mV. This corresponds to a single channel conductance, using Ohms law, of 78 pS in this cell where:

conductance = unitary current/driving force E_K - holding potential [10]
($g = i/E-E_{REV}$)

The mean unitary current amplitude of the channel activated by NS1619 from similar experiments performed on 7 other cells was 5.6 ± 0.1 pA, corresponding to a mean channel conductance of 70 pS. This value agrees well with a previous study using rat portal vein smooth muscle cells, where the channel activated by NS1619, also at 0 mV and with a physiological K^+ gradient across the membrane, was found to have a conductance of 70 pS (Edwards *et al.*, 1994).

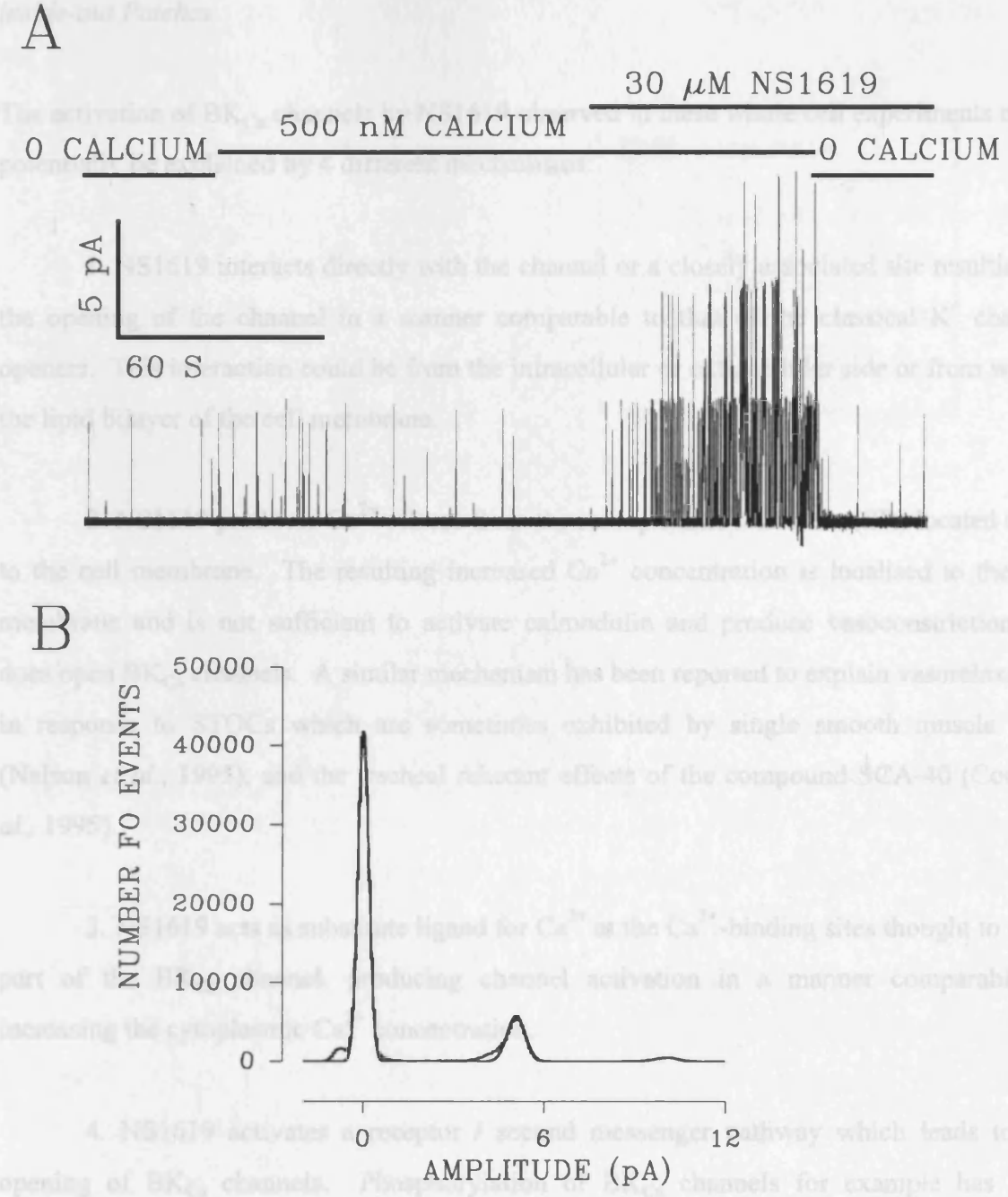


Figure 3.vi. Direct activation of BK_{Ca} channels by 30 μM NS1619 and the Ca^{2+}-dependence of this effect in an excised inside-out membrane patch. Panel A illustrates a representative record showing the rapid activation of a large-conductance channel by NS1619 and the Ca^{2+}-dependence of this effect. Panel B illustrates an amplitude histogram constructed from the data in pannel A illustrating the large unitary current amplitude of the channel opened by 30 μM NS1619. This patch was held throughout this experiment at 0 mV with a physiological K^+ gradient (140:6 mM K^+) across the membrane.

3.2.6 NS1619 rapidly activated large conductance Ca^{2+}-dependent channels in excised inside-out Patches

The activation of BK_{Ca} channels by NS1619 observed in these whole cell experiments could potentially be explained by 4 different mechanisms:

1. NS1619 interacts directly with the channel or a closely associated site resulting in the opening of the channel in a manner comparable to that of the classical K^+ channel openers. This interaction could be from the intracellular or extracellular side or from within the lipid bilayer of the cell membrane.

2. NS1619 produces Ca^{2+} release from the sarcoplasmic reticulum (SR) located close to the cell membrane. The resulting increased Ca^{2+} concentration is localised to the cell membrane and is not sufficient to activate calmodulin and produce vasoconstriction but does open BK_{Ca} channels. A similar mechanism has been reported to explain vasorelaxation in response to STOCs which are sometimes exhibited by single smooth muscle cells (Nelson *et al.*, 1995), and the tracheal relaxant effects of the compound SCA-40 (Cook *et al.*, 1995).

3. NS1619 acts as substitute ligand for Ca^{2+} at the Ca^{2+}-binding sites thought to form part of the BK_{Ca} channel, producing channel activation in a manner comparable to increasing the cytoplasmic Ca^{2+} concentration.

4. NS1619 activates a receptor / second messenger pathway which leads to the opening of BK_{Ca} channels. Phosphorylation of BK_{Ca} channels for example has been reported to produce an increase in channel P_{open} in tracheal smooth muscle cells (Kume *et al.*, 1989).

To determine which one of the above mechanisms, if any, best describes the mechanism of action of NS1619, inside-out membrane patches were used. This configuration of the patch-clamp technique also allows accurate measurements of the unitary current amplitude and single channel conductance, thus allowing a comparison with the value produced from whole cell experiments.

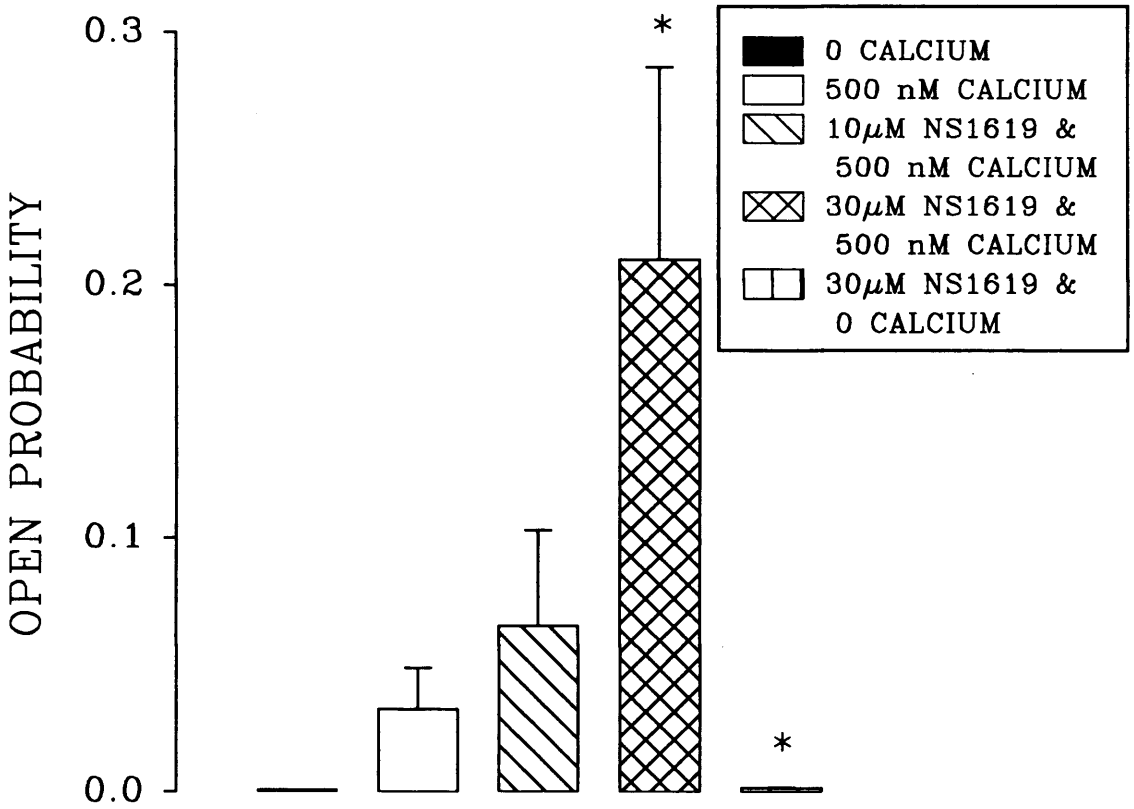


Figure 3.vii. Histogram summarising the effects of Ca^{2+} and increasing concentrations of NS1619 on channels contained in inside-out patches. The panels from left to right show channel activity when the cytosolic solution contained < 1 nM free Ca^{2+}; 500 nM free Ca^{2+}; 500 nM free Ca^{2+} & 10 μM NS1619; 500 nM free Ca^{2+} & 30 μM NS1619 and 30 μM NS1619 with < 1 nM free Ca^{2+}. Channel P_{open} was measured over 40-70s periods and significance (P<0.05 compared to control) is illustrated by *.

This configuration also allows complete control of the composition of the solution bathing the cytosolic surface of the patch, thus allowing an illustration of the Ca^{2+}-dependence of the BK_{Ca} channel and, more importantly, shed some light on the mechanism of action of NS1619. For example, if the mechanism of action of NS1619 is indirect, involving either second messenger regulation or stimulation of Ca^{2+} release from the SR then no activation should be observed in an inside-out patch. Additionally, whether the mechanism of action of NS1619 involves the drug substituting for Ca^{2+} or a phosphorylation or G-protein mediated effect can also be unequivocally determined by simply omitting Ca^{2+}, ATP or GTP from the cytosolic solution and observing if these actions affected the action of NS1619. In all excised patch experiments the cytoplasmic face of the patch was continuously superfused with 140 mM K^+ that was either nominally free from Ca^{2+} or contained 500 nM free Ca^{2+} (see solutions appendix at the end of the methods chapter). The patch pipette contained 6 mM K^+ and all recordings were made at 0 mV. Figure 3.vi A illustrates a recording from an inside-out patch excised into the nominally Ca^{2+}-free saline. In the Ca^{2+}-free solution, openings of a channel with a unitary amplitude of ~6 pA were relatively infrequent. For example, the P_{open} of this channel measured over approximately 1 minute was 0.001. Increasing the Ca^{2+} concentration to 500 nM doubled the P_{open} of this channel without affecting the unitary current amplitude, illustrating the Ca^{2+}-dependence of this channel. The addition of 30 μM NS1619 to the cytosolic surface of the patch together with 500 nM free Ca^{2+} produced a rapid and significant ($p<0.05$) increase in channel P_{open}, which increased to 0.081, a 40 fold increase with respect to the control level. This increase occurred without the presence of ATP or GTP in the cytosolic solution indicating the activation produced by NS1619 occurred independently of phosphorylation and did not involve a G-protein. When the free Ca^{2+} concentration was then reduced (nominally Ca^{2+}-free), in the continued presence of 30 μM NS1619, channel P_{open} was reduced to the control Ca^{2+}-free level, indicating that NS1619 was not acting as a substitute for Ca^{2+} at Ca^{2+} binding sites on the BK_{Ca} channel. To prove this channel was indeed a K^+ channel rather than a non-selective cation channel or a Cl^- channel, cytosolic K^+ was substituted for Na^+ with the Cl^- concentration unchanged (140 mM), this resulted in no discernible current passing through the patch (data not shown), indicating this Ca^{2+}-dependent channel was a K^+ selective channel.

Figure 3.vi B illustrates the amplitude histogram of the channel activated by 30 μM NS1619 in the presence of 500 nM-free Ca^{2+} at the cytosolic surface from figure 3.vi A.

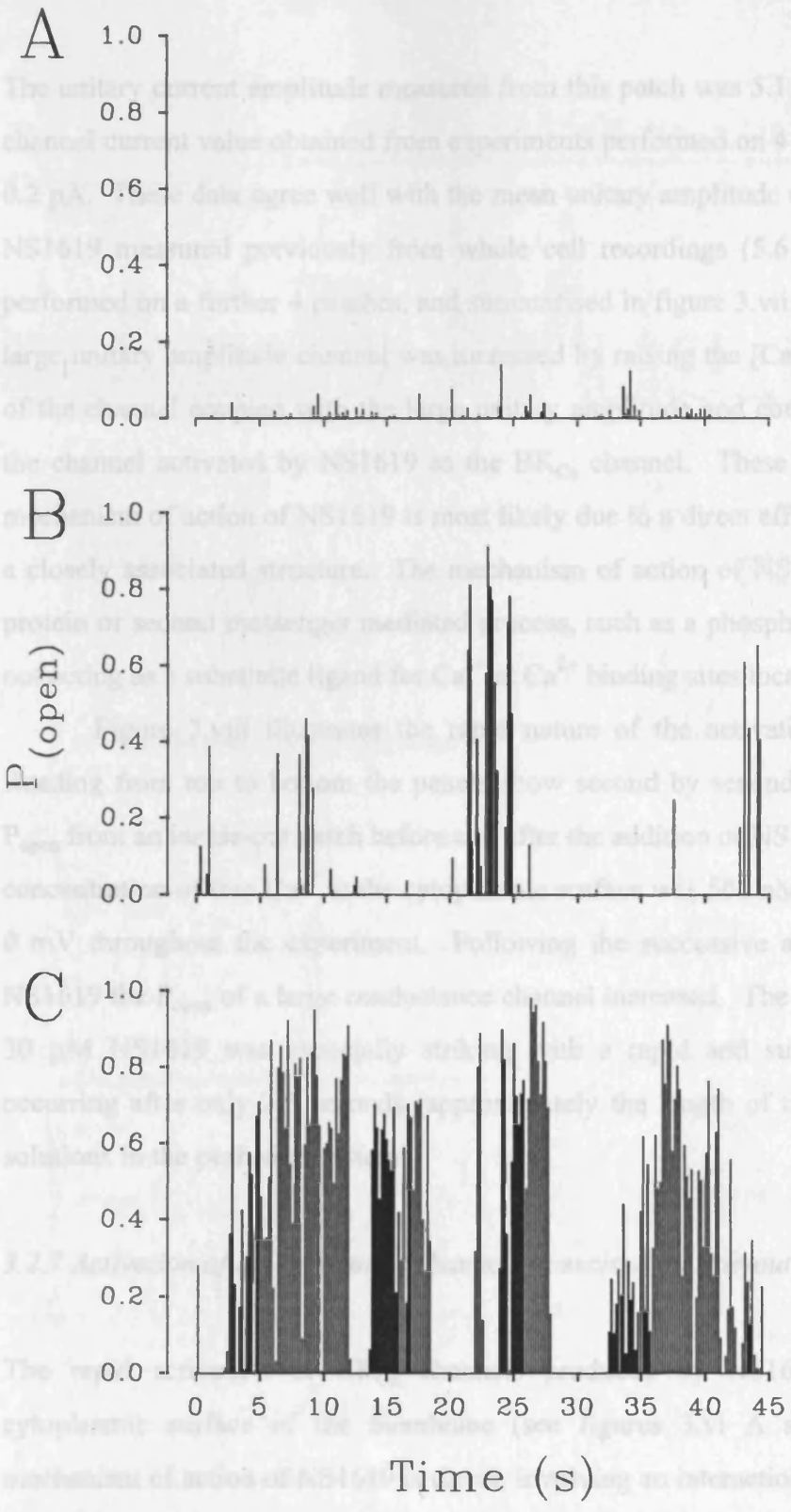


Figure 3.viii. Effects of NS1619 (10 and 30 μM) on BK_{Ca} channel P_{open} recorded from an excised inside-out membrane. The histograms illustrated in panels A, B and C show channel P_{open} calculated second by second for control data and following the addition of 10 μM and 30 μM NS1619 respectively.

The unitary current amplitude measured from this patch was 5.1 pA, while the mean single channel current value obtained from experiments performed on 4 separate patches was 5.9 ± 0.2 pA. These data agree well with the mean unitary amplitude of the channel activated by NS1619 measured previously from whole cell recordings (5.6 ± 0.1 pA). Experiments performed on a further 4 patches, and summarised in figure 3.vii, confirmed the P_{open} of the large unitary amplitude channel was increased by raising the $[Ca^{2+}]_i$. The Ca^{2+}-dependence of the channel coupled with the large unitary amplitude and conductance values identified the channel activated by NS1619 as the BK_{Ca} channel. These data also suggest that the mechanism of action of NS1619 is most likely due to a direct effect on the BK_{Ca} channel or a closely associated structure. The mechanism of action of NS1619 did not involve a G-protein or second messenger mediated process, such as a phosphorylation and NS1619 was not acting as a substitute ligand for Ca^{2+} at Ca^{2+} binding sites located on the BK_{Ca} channel.

Figure 3.viii illustrates the rapid nature of the activation produced by NS1619. Reading from top to bottom the panels show second by second measurements of channel P_{open} from an inside-out patch before and after the addition of NS1619 (10 and 30 μM). The concentration of free Ca^{2+} at the cytoplasmic surface was 500 nM and the patch was held at 0 mV throughout the experiment. Following the successive addition of 10 and 30 μM NS1619 the P_{open} of a large conductance channel increased. The increase in the presence of 30 μM NS1619 was especially striking with a rapid and substantial increase in P_{open} occurring after only 2.5 seconds (approximately the length of time taken for exchange of solutions in the perfusion system)

3.2.7 Activation of IbTX-sensitive channels in excised outside-out membrane patches

The rapid activation of BK_{Ca} channels produced by NS1619 when applied to the cytoplasmic surface of the membrane (see figures 3.vi A and 3.viii C) suggest the mechanism of action of NS1619 is direct, involving an interaction with a site on, or close to the channel, either at the cytoplasmic surface or from within the membrane. To determine if this indeed was the case, the effects of NS1619 were also examined in outside-out patches. The rationale being, if the potency of NS1619, or rate of BK_{Ca} channel activation were reduced when NS1619 was applied to the external surface, as compared to the cytoplasmic surface of the patch, then an assumption about the site of action may be made i.e. whether the site was external or cytoplasmic.

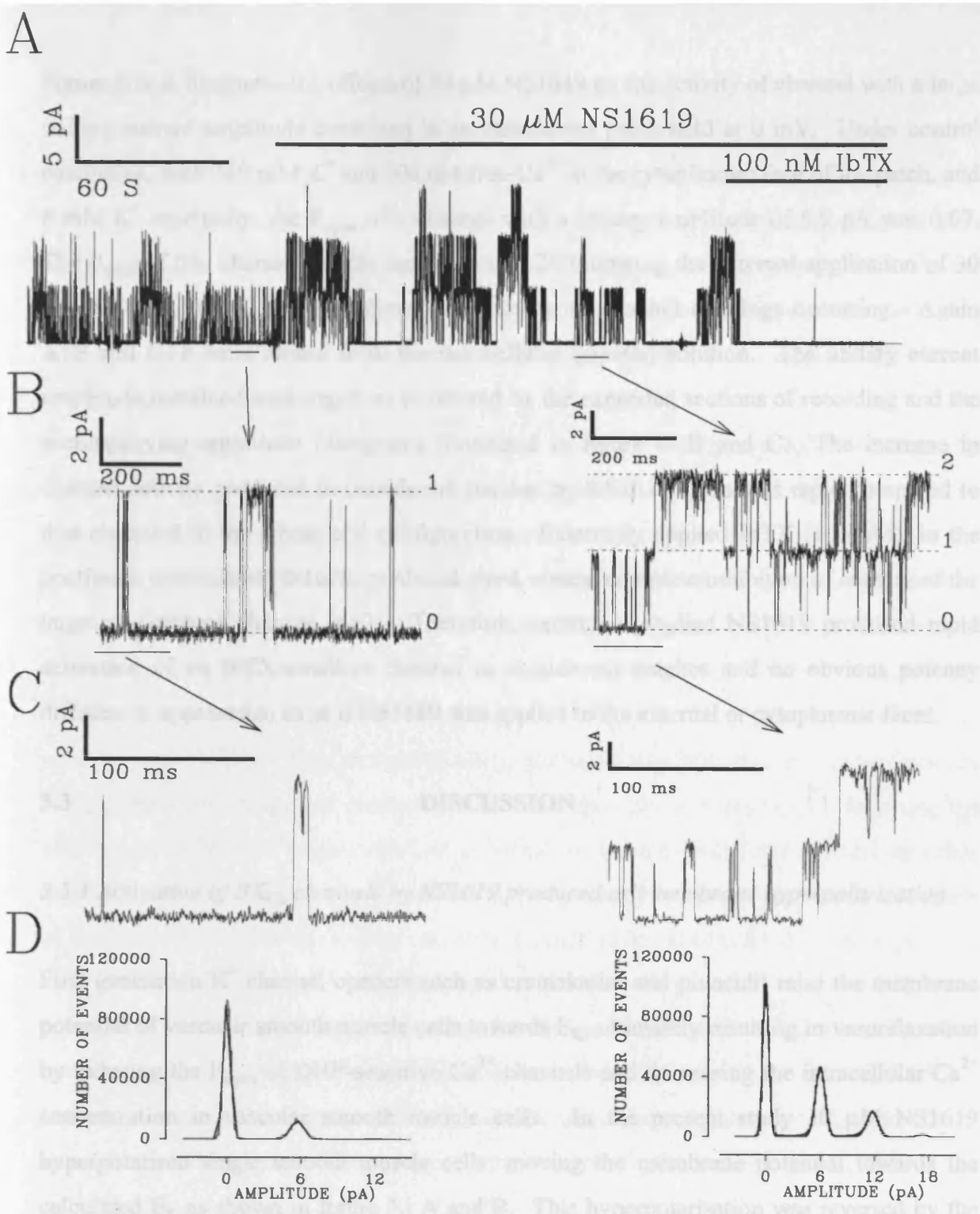


Figure 3.ix. Channel activation by NS1619 in an outside-out patch. Panel A illustrates a recording made from a patch held at 0 mV. The pipette (internal) solution contained 140 mM K^+ and 500 nM free Ca^{2+} and the bath (external) solution contained 6 mM K^+. NS1619 was added to the bath solution where indicated. Panels B & C show expanded sections of recording made before (left side) and after (right side) exposure to 30 μM NS1619. Panel C amplitude histograms constructed from the data shown in panel A before (left) and after (right) exposure to 30 μM NS1619. Note that the single channel amplitudes are not affected following exposure to NS1619.

Figure 3.ix A illustrates the effects of 30 μM NS1619 on the activity of channel with a large unitary current amplitude contained in an outside-out patch held at 0 mV. Under control conditions, with 140 mM K^+ and 500 nM free-Ca^{2+} at the cytoplasmic face of the patch, and 6 mM K^+ externally, the P_{open} of a channel with a unitary amplitude of 5.9 pA was 0.07. The P_{open} of this channel rapidly increased to 0.24 following the external application of 30 μM NS1619, with 2 and sometimes 3 simultaneous channel openings occurring. Again ATP and GTP were absent from the intracellular (pipette) solution. The unitary current amplitude remained unchanged, as illustrated by the expanded sections of recording and the accompanying amplitude histograms illustrated in figure ix B and C. The increase in channel activity produced in outside-out patches by NS1619 again was rapid compared to that observed in the whole cell configuration. Externally applied IbTX (100 nM), in the continued presence of NS1619, produced rapid, almost complete inhibition of activity of the large-conductance channel (n=2). Therefore, externally applied NS1619 produced rapid activation of an IbTX-sensitive channel in outside-out patches and no obvious potency differences appeared to exist if NS1619 was applied to the external or cytoplasmic faces.

3.3 DISCUSSION

3.3.1 Activation of BK_{Ca} channels by NS1619 produced cell membrane hyperpolarisation

First generation K^+ channel openers such as cromakalim and pinacidil raise the membrane potential of vascular smooth muscle cells towards E_K, ultimately resulting in vasorelaxation by reducing the P_{open} of DHP-sensitive Ca^{2+} channels and decreasing the intracellular Ca^{2+} concentration in vascular smooth muscle cells. In the present study 30 μM NS1619 hyperpolarised single smooth muscle cells, moving the membrane potential towards the calculated E_K as shown in figure 3.i A and B. This hyperpolarisation was reversed by the addition of IbTX, a peptide component isolated from the venom of the scorpion *Buthus tamalus*. IbTX is a potent, selective blocker of BK_{Ca} channels (Galvez *et al.*, 1989) and is the agent of choice when characterising BK_{Ca} channels as, unlike CTX, another BK_{Ca} channel blocker, it only affects BK_{Ca} channels. IbTX binds to a specific site in the external 'mouth' of the channel, physically occluding the channel and blocking the permeation of K^+ ions through the channel (Candia *et al.*, 1992; Giangiacomo *et al.*, 1992). These experiments therefore unequivocally demonstrated that NS1619 produced hyperpolarisation of smooth muscle cells.

However, the mechanism by which this occurred was different to that of the first generation K^+ channel openers, as the effects of NS1619 were inhibited by IbTX. A potentially interesting observation from figure 3.iB was, that following the addition of 100 nM IbTX to the cell to block the effects of NS1619, the membrane potential was more depolarised than under control conditions. This perhaps indicates that BK_{Ca} channels play a role in the rat cerebral circulation in controlling the RMP as has previously been proposed for the rabbit cerebral vasculature (Brayden & Nelson, 1992), though obviously too much weight should not be placed on a single observation.

Patch-clamp experiments performed on intact cells demonstrated that externally applied NS1619 activated large outward currents from cells clamped at 0 mV. The onset of activity was relatively slow (typically 1-5 minutes) and reversible following washout of the drug. The NS1619-activated currents were unaffected by the K_{ATP} channel blocker, glibenclamide and the SK_{Ca} channel blocker apamin. In contrast, 100 nM IbTX rapidly abolished the NS1619-activated current, strongly suggesting that NS1619 was selectively opening BK_{Ca} channels with no effects on either K_{ATP} or SK_{Ca} channels. Additional evidence that NS1619 was activating BK_{Ca} channels was provided by unitary current amplitude measurements and conductance values of the channel being opened following the addition of NS1619 to whole cells. As explained previously, individual channel openings could sometimes be detected in the records allowing measurements of the current amplitude of the channels. The mean unitary current amplitude of the channels activated by NS1619 at 0 mV, with 6 mM K^+ externally and 140 mM K^+ assumed to be the internal concentration, was 5.6 pA, corresponding to a channel conductance of 70 pS (n=8). This measurement of the unitary current amplitude agrees well with a previous study of NS1619, where the channels activated by NS1619 at 0 mV, with similar K^+ concentrations across the membrane, were also reported to have a conductance of 70 pS (Edwards *et al.*, 1994).

3.3.2 NS1619 increases IbTX-sensitive whole cell currents activated by depolarisation

The P_{open} of BK_{Ca} channels is voltage- as well as Ca^{2+}-dependent and increases with membrane depolarisation. Unlike other K_V channels, BK_{Ca} channels do not show voltage-dependent inactivation (Latorre *et al.*, 1989). In contrast K_V channels in smooth muscle cells are strongly inactivated by sustained, depolarised holding potentials (Volk *et al.*, 1991; Smirnov & Aaronson, 1992a).

Clamping the membrane potential at -15 mV for 1 minute before running a sequence of depolarising command potentials should inactivate K_V channels so that BK_{Ca} channel activation would dominate any currents activated. The small whole cell currents activated under control conditions shown in figure 3.iv A confirmed that inactivation of K_V channels was being produced. This point is perhaps better illustrated by comparing figure 3.iv A with representative K_V currents illustrated in figure 4.iv A in chapter 4. The small control currents also illustrated that BK_{Ca} channels appeared to be relatively voltage insensitive over this range of command potentials. NS1619 (10-30 μM) significantly increased the outward currents (see figures 3.iv B and C, figure 3.v and table 3.i), which were significantly reduced following the addition of 100 nM IbTX together with NS1619. These findings raised the following questions:

1. Was NS1619 producing BK_{Ca} channel activation by a direct or indirect mechanism?

2. Was NS1619 producing BK_{Ca} channel activation by affecting the voltage- or Ca^{2+}-dependence of the channel?

3.3.3 NS1619 directly activates BK_{Ca} channels

As mentioned previously, the activation of BK_{Ca} channels by NS1619 in intact cells could theoretically be explained by a number of different mechanisms. However, following the demonstration that NS1619 rapidly activated BK_{Ca} channels when applied to either inside-out or outside-out membrane patches, indirect mechanisms could be excluded. If NS1619 did produce BK_{Ca} channel opening by any mechanism other than a direct effect, then activation would not be seen when using excised membrane patches as vital intracellular constituents would be lost when using this configuration. Inside-out patch experiments also highlighted the requirement for cytoplasmic Ca^{2+} for NS1619 to activate BK_{Ca} channels, a result which also confirmed that NS1619 was not acting as a substitute ligand for Ca^{2+} at Ca^{2+} binding sites on the channel. This observation also suggested that the mechanism of action of NS1619 probably involves somehow changing the Ca^{2+} sensitivity of the BK_{Ca} channel rather than its voltage-sensitivity, as no activation was observed in nominally zero Ca^{2+}, a situation where BK_{Ca} channels reportedly act solely as weakly voltage-dependent channels (Cox *et al.*, 1996; Toro *et al.*, 1996; Strobaek *et al.*, 1996; Meera *et al.*, 1996).

BK_{Ca} channel activation also occurred in the absence of ATP and GTP, indicating that channel phosphorylation and G-proteins were not involved for activation of BK_{Ca} channels by NS1619. These observations agree well with some previous results performed on patches obtained from cultured bovine aortic smooth muscle cells (Olesen *et al.*, 1994a). One important point that should be made concerning excised membrane patches, is that in some preparations they have been shown not to be entirely devoid of cytoplasmic elements, as they were once thought to be, and that cytoskeletal elements and organelles may be present. Excised patches from retinal photoreceptor cells, for example, were found to contain the entire transduction pathway in addition to the cation channel (Ertel, 1990). Therefore, if excised smooth muscle patches also contained intracellular organelles such as sarcoplasmic reticulum (SR), it could be argued that the channel activation produced by NS1619 could be explained by NS1619 purging segments of SR of its Ca^{2+} which then produces BK_{Ca} channel activation. This explanation would appear unlikely however, as the channel activation produced by NS1619 did not fade with time, which would be expected if the SR was depleted of Ca^{2+}.

A recent study of cloned *hslo* and *hslo*β BK_{Ca} channels expressed in *Xenopus* oocytes and HEK 293 cells demonstrated that NS1619 activated both *hslo* and *hslo*β BK_{Ca} channels to the same extent, therefore indicating that the site of action of NS1619 was located on the α-subunit (S0-S10), possibly the S7-S10 tail region (Dworetzky *et al.*, 1996), which has previously been shown to confer some degree of Ca^{2+}-sensitivity on the channel (Wei *et al.*, 1994). Therefore in summary, the mechanism of action of NS1619 most likely involves the direct activation of the BK_{Ca} channel via an interaction with the channel or a closely associated modulatory site. This effect requires Ca^{2+}, perhaps indicating that the mechanism of action probably involves altering the Ca^{2+}-sensitivity of the BK_{Ca} channel, and does not require ATP or GTP.

3.3.4 Where is the site of action of NS1619?

If the action of NS1619 is direct, as appears extremely likely, does the drug act on the inside surface, the outside surface of the membrane or from within the bilayer? A common feature of whole cell recordings was the substantial delay of approximately 1-4 minutes, between the addition of NS1619 and the activation of an outward current.

This observation agrees well with previous reports, where contact times of cells with NS1619 of 2-6 minutes have been reported prior to full levels of activation being produced (Edwards *et al.*, 1994; Olesen *et al.*, 1994a). Also, NS004, a compound structurally related to NS1619, has also been reported to have a slow onset of action of up to 10 minutes (McKay *et al.*, 1994). The effects of NS1619 were also relatively slow to wash out as shown in figure 3.ii, a characteristic also described by Olesen and co-workers (1994a). The slow onset and offset of action of NS1619 cannot be attributed to the perfusion system used in these experiments, as the cell under study was continuously superfused from reservoirs whose outflow was always placed close (<250 μm) by, and full exchange of flow solutions was relatively quick due to the small 'dead' space of the outflow tip (<5 seconds see chapter 2). The experiments investigating the effects of NS1619 on excised patches confirmed that fast exchange of test solutions occurred. Figure 3.vi, for example, shows not only the rapid activation of BK_{Ca} channels by NS1619 but also their rapid inhibition following the removal of Ca^{2+} from the test solution (also see figure 6.i in chapter 6 for an illustration of the rapid exchange of solutions). This delay in the onset of channel activation following the addition of NS1619 to intact cells, an effect also observed when performing on-cell recordings using isolated porcine coronary smooth muscle cells (Holland unpublished observations), sharply contrasted with the rapid activation of BK_{Ca} channels in excised patches described here and by others (Olesen *et al.*, 1994a; Sellers & Ashford, 1994). A possible explanation of this difference could be that the site of action of NS1619 is intracellular. This would explain why rapid, almost instantaneous activation was seen in inside-out patches but not in whole cells and also why the effects of NS1619 were slow to reverse in whole cell experiments following washout of the drug. The delay in activation of whole cell currents may therefore represent the time taken for NS1619 to cross the cell membrane and achieve an effective intracellular concentration. This however does not explain why rapid channel activation was also seen in response to NS1619 in outside-out patches. The high lipophilicity of NS1619 coupled with a smaller diffusible 'dead' space in the patch pipette may potentially explain why rapid channel activation was also produced by NS1619 in outside-out patches but not in the whole cell configuration. In this respect NS1619 differs from the recently described soyasaponin analogues, typified by DHS-I, which only produce BK_{Ca} activation when added to the cytoplasmic surface (McManus *et al.*, 1993). The possibility exists that another mechanism may explain the activation produced by NS1619, namely that NS1619 acts within the cell membrane bi-layer.

General anaesthetics, such as halothane and ketamine, have been reported to inhibit K^+ channels (Kulkarni *et al.*, 1996), including BK_{Ca} in cerebral vascular smooth muscle (Hong *et al.*, 1994). If inhibition of BK_{Ca} channels can be produced from within the membrane could such a similar mechanism also account for the activation seen here? An answer to this interesting question would obviously be difficult to obtain, however if such a mechanism did account for the activation of BK_{Ca} channels by NS1619 surely the currents activated in whole cells would have developed more quickly than observed here, as one of the first areas affected by NS1619 would be the cell membrane. I therefore feel that an internal site of action of NS1619 would best account for the results described in this study, though obviously other mechanisms cannot be discounted without further experimentation.

3.3.5 Intracellular Ca^{2+} is required for the activation of BK_{Ca} channels by NS1619

A previous study based on conventional whole cell recordings from rat portal vein smooth muscle cells using apparently Ca^{2+}-free intracellular solutions suggested that the activation of BK_{Ca} channels produced by NS1619 was independent of cytosolic Ca^{2+} (Edwards *et al.*, 1994). The authors of this study suggested that NS1619 may therefore be acting as a substitute ligand for Ca^{2+} at Ca^{2+} binding sites on the BK_{Ca} channel, producing BK_{Ca} channel activation. This finding contrasts with a previous report (Olesen *et al.*, 1994a) using cultured bovine aortic smooth muscle cells, where the effects of NS1619 on BK_{Ca} channels in excised patches were Ca^{2+}-dependent, although no data were illustrated to support this claim. In the present study, using the inside-out patch configuration, Ca^{2+} free conditions always reversed the increase in channel activity, even in the presence of high concentrations (30 μM) of NS1619. The difference between the findings presented here and the previous report by Edwards and co-workers (1994) can probably be explained by incomplete buffering of intracellular Ca^{2+} in the region adjacent to the cell membrane when using the conventional whole cell technique. This idea is supported by data illustrated in chapter 4 examining the effects of NS1619 on K_V channels. In these experiments 30 μM NS1619 activated BK_{Ca} channels in whole cells even when the intracellular solution contained 10 mM EGTA (see chapter 4 figure 4.iii C), suggesting that even strong buffering could not completely buffer Ca^{2+} in the whole cell configuration. However, when using inside-out patches, which obviously allows a greater degree of control over the solution at the cytoplasmic surface of the membrane, 30 μM NS1619 never produced BK_{Ca} channel activation in nominally Ca^{2+}-free conditions.

The observation that the activity of NS1619 is Ca^{2+}-dependent is potentially of some importance when attempting to explain how NS1619 activates BK_{Ca} channels. BK_{Ca} channels are modulated by both the intracellular Ca^{2+} concentration and membrane voltage (Latorre *et al.*, 1989), raising the question, does NS1619 produce BK_{Ca} channel activation by increasing the sensitivity of the channels to Ca^{2+} or by shifting the channel voltage-dependence such that the channel is opened by a less depolarised membrane voltages, or both? Of the two modulators of BK_{Ca} channel activity, Ca^{2+} has been reported to have by far the stronger effects on the activity of mammalian BK_{Ca} channels, and thus is thought to be the more important regulator of BK_{Ca} channel activity (Pallota, 1985; Benham *et al.*, 1986; Dichiera & Reinhart, 1995; Cox *et al.*, 1996; Toro *et al.*, 1996; Strobaek *et al.*, 1996; Meera *et al.*, 1996). Several recent studies, for example, have reported that when the cytosolic Ca^{2+} concentration is reduced below 100 nM, BK_{Ca} channels function as purely voltage-dependent channels which are only activated by depolarised potentials greater than ~100 mV (Cox *et al.*, 1996; Toro *et al.*, 1996; Strobaek *et al.*, 1996; Meera *et al.*, 1996). The demonstration therefore that BK_{Ca} channel activation by NS1619 did not occur when the cytosolic $[Ca^{2+}]$ was nominally Ca^{2+}-free provides evidence that NS1619 was probably not activating BK_{Ca} channels by increasing the voltage-sensitivity of the channel. A recent study of BK_{Ca} opening by the fenamates proposed that the mechanism of action of these compounds was best explained by these drugs increasing the Ca^{2+}-sensitivity of the BK_{Ca} channel (Ottalia & Toro, 1994). I feel that a similar effect probably best explains the results described here, although additional experimentation would be required to provide a definitive answer to this problem. To determine for example whether NS1619 did alter the Ca^{2+}-sensitivity of the BK_{Ca} channel, its effect against a series of different cytoplasmic Ca^{2+} concentrations would have to be assessed. This could be achieved using inside-out patches and producing a dose-response curve for channel P_{open} against $[Ca^{2+}]$ at a constant holding potential in the absence and presence of NS1619. A shift to left of the dose response curves by NS1619 would provide good evidence that the Ca^{2+}-sensitivity of the BK_{Ca} channel was being increased. Alternatively the effects of NS1619 on voltage-dependence alone could be addressed using *N*-bromacetamide, a reagent which has been reported to remove the Ca^{2+}-sensitivity of the BK_{Ca} channel but leave the voltage-dependence intact (Pallotta, 1985). If NS1619 activated BK_{Ca} channels in inside-out patches treated with *N*-bromacetamide then the mechanism of action of NS1619 would involve increasing the voltage-dependence of the channel.

In conclusion, NS1619 is an effective activator of BK_{Ca} channels and this action accounts for its ability to hyperpolarise cerebral arterial smooth muscle cells. The mechanism of action of NS1619 is probably due to a direct effect of the drug with either the channel itself or a closely related site possibly located on the cytoplasmic face of the membrane. For NS1619 to activate BK_{Ca} channels cytoplasmic Ca^{2+} is required, which perhaps indicates that the channel activation produced by NS1619 involves altering the Ca^{2+}-sensitivity of the BK_{Ca} channel.

CHAPTER FOUR

Effects of NS1619 on other ion channels contained in arterial smooth muscle cells

4.1 INTRODUCTION

The vasorelaxant properties of NS1619 were initially attributed to the ability of this compound to open BK_{Ca} channels in vascular smooth muscle cells (Olesen *et al.*, 1994a), resulting in the hyperpolarisation of the smooth muscle cells. Electrophysiological and calcium imaging data published in the same report suggested that the effects of NS1619 were specific to BK_{Ca} channels and NS1619 had no effects on other ion channels, including K_{ATP} channels in rat pancreatic β-cells, K_V channels in rat cerebellar granule cells or Ca^{2+} entry in mouse cerebellar granule cells (Olesen *et al.*, 1994a). A subsequent study reported that 33 μM NS1619 almost completely inhibited DHP-sensitive Ca^{2+} currents recorded from smooth muscle cells isolated from the rat portal vein and that NS1619 also inhibited other K^+ channels including K_V channels and K_{ATP} channels (Edwards *et al.*, 1994), although NS1619 did not inhibit the K_{ATP} channel found in neurones (Sellers & Ashford, 1994). This led Edwards and co-workers to suggest that the Ca^{2+}-channel blocking effects of NS1619, rather than the opening of BK_{Ca} channels might underlie the functional vasorelaxant properties of this drug. The aims of the second part of this study were to identify ion channels other than BK_{Ca} channels present in rat basilar artery smooth muscle cells and then to determine the effects of NS1619 on these channels and, if so, to assess the concentration and voltage dependence of these effects. This would allow a direct comparison of the effects of NS1619 on smooth muscle cells obtained from phasic and tonic blood vessels and further help to define the pharmacology of NS1619.

4.2 RESULTS

4.2.1 Inhibition of currents through Ca^{2+} channels by NS1619

The vasorelaxant effect of NS1619 could be explained by direct block of Ca^{2+} channels. The effects therefore of NS1619 on Ca^{2+} channels was studied using rat basilar smooth muscle cells. The Ca^{2+} currents in these cells have previously been well characterised and are entirely due to the activation of DHP-sensitive Ca^{2+} channels (Langton & Standen, 1993; Langton, 1993). Ca^{2+} currents in basilar myocytes are also relatively small so it was important to use conditions that maximised existing currents and correct for any outward leakage current so the amplitude of inward currents was not underestimated.

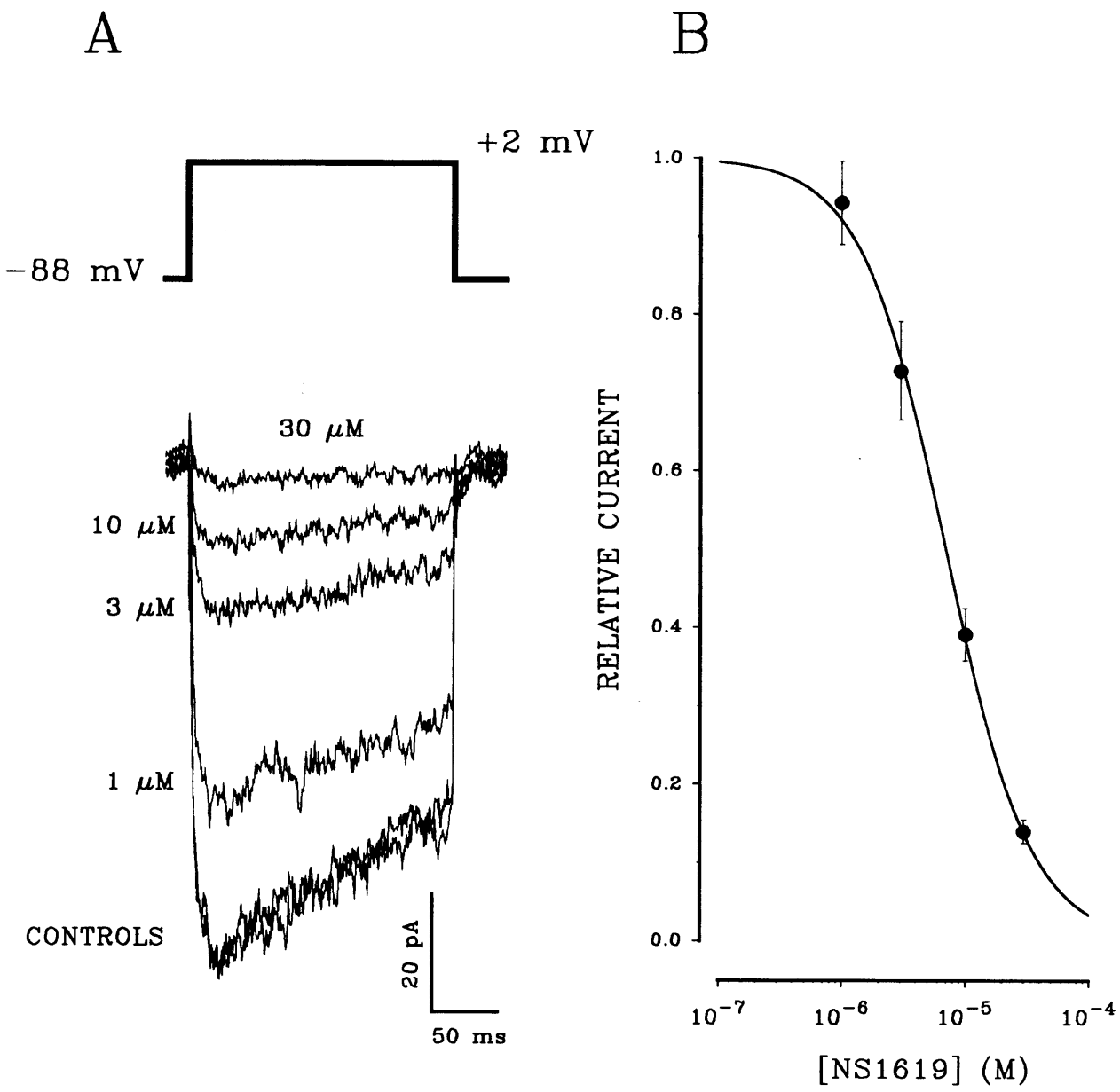


Figure 4.i. Concentration-dependent inhibition of Ca^{2+} channel currents by NS1619 (1-30 μM). Panel A illustrates representative inward currents through Ca^{2+} channels, with 10 mM Ba^{2+} externally, activated following a voltage step from -88 mV to +2 mV illustrating the concentration-dependent inhibition of Ba^{2+} currents by NS1619. Panel B shows the mean concentration-effect relationship for the inhibition of Ba^{2+} currents by NS1619. Each point shows the mean ± s.e. mean from 4 cells, the best fit line has a Hill coefficient of 1.3 giving a K_D of 6.9 μM.

Correcting for leakage currents involved first measuring the leak currents then subtracting a corresponding amount from the 'raw' data during analysis. Leakage currents could be measured and corrected using one of two methods:

1. An average leak current can be constructed using the leak subtraction function built into the patch-clamp software (see methods chapter). This was produced by applying a train of 12 pulses one sixth the amplitude of the command potential. When averaged this data produces the leakage current which can then be subtracted from the raw current traces giving leak subtracted data.

2. Using extracellular Co^{2+} to block inward Ba^{2+} currents, allowing isolation of the leakage current. This is achieved by including 2 mM $CoCl_2$ in the normal 10 mM Ba^{2+} extracellular solution. External Co^{2+} produces a rapid and reversible block of the inward Ba^{2+} currents and so isolates the leakage current. Therefore by subtracting the current measured at a particular command potential in the presence of Co^{2+} from that produced in its absence, the data can be corrected to account for the leakage current giving the true current flowing through Ca^{2+} channels.

A method commonly used to maximise the amplitude of currents through Ca^{2+} channels is to use an ion which permeates the Ca^{2+} channel more readily than Ca^{2+}, such as Ba^{2+} (see chapter 1 section 1.2.3). In a previous study of Ca^{2+} channels using rat basilar arterial smooth muscle cells conducted in this department by Philip Langton, when 10 mM Ba^{2+} was used as the charge carrying ion, the mean peak inward current was ~250 pA. This compared to peak inward currents of 88 and 169 pA when 1.8 mM and 10 mM Ca^{2+} were used as charge carriers (Langton & Standen, 1993). Another reason for using 10 mM Ba^{2+} as the charge carrier is unlike higher concentrations of Ba^{2+} (110 mM), 10 mM Ba^{2+} does not produce a shift in the voltage-dependent activation of Ca^{2+} channels (Smirnov & Aaronson, 1992b), and therefore increases the amplitude of inward currents through Ca^{2+} channels without affecting the I-V relationship. For these reasons 10 mM Ba^{2+} was routinely used as the charge carrier in this study. Ba^{2+} currents were recorded using the conventional whole cell configuration of the patch-clamp technique, which allowed intracellular dialysis with a solution designed to limit the efflux of K^+ through K^+ channels, which would also effectively reduce the amplitude of the inward currents through Ca^{2+} channels.

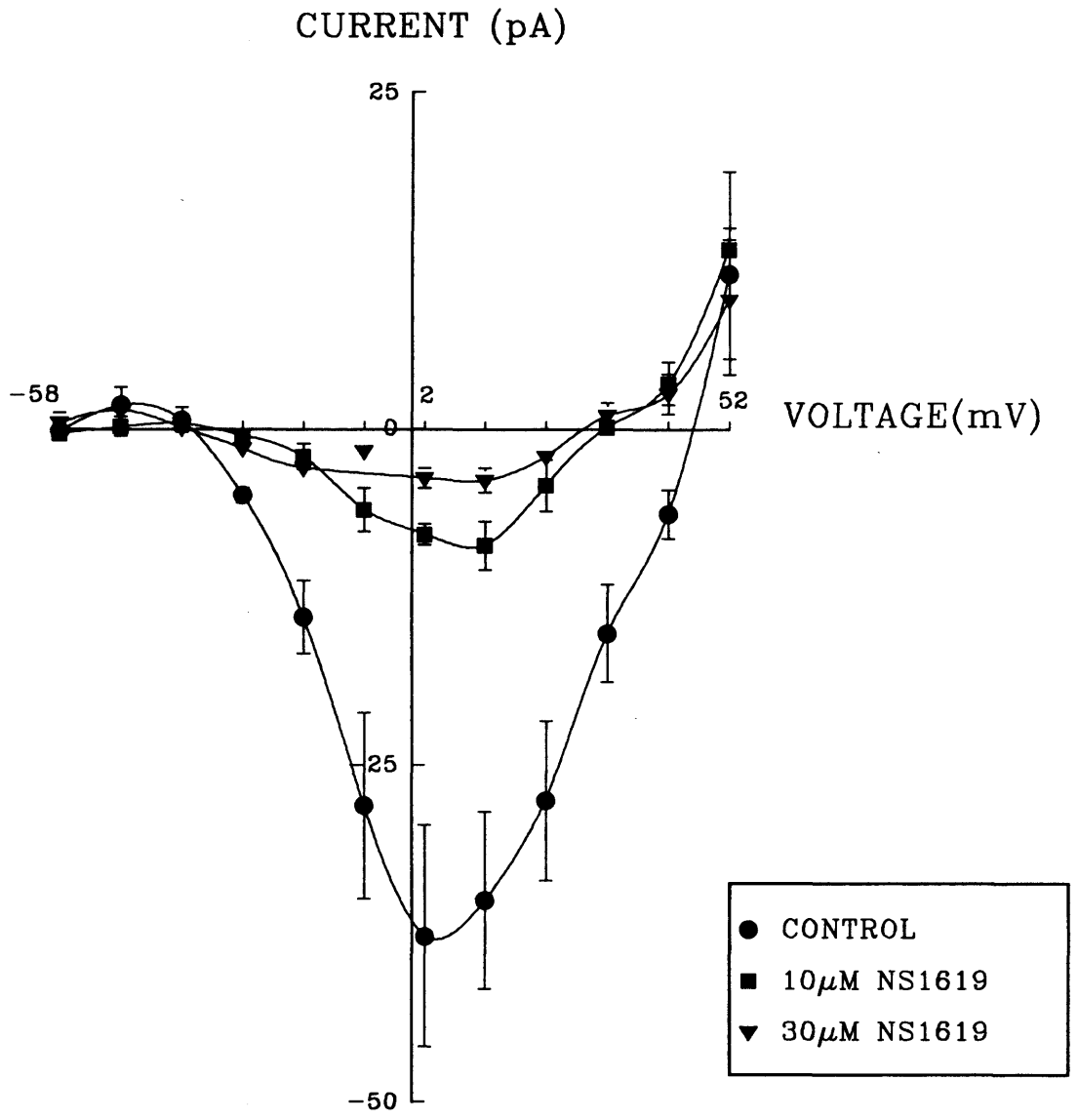


Figure 4.ii. Mean current-voltage relationships illustrating the effects of NS1619 on Ba^{2+} currents. Peak whole cell Ba^{2+} currents through Ca^{2+} channels were measured under control conditions (●), and following the sequential addition of 10 μM (■) and 30 μM (▼) NS1619 (n=4).

This was achieved by substituting K^+ for Cs^+ and using TEA-OH or CS-OH to titre the pH of the intacellular solution to 7.2.

Figure 4.i A shows typical records of inward current measured in response to a voltage step to +2 mV from a holding potential of -88 mV with 10 mM Ba^{2+} externally. Leakage in this example was corrected using 2 mM extracellular Co^{2+} as described above. Following the initial activation of Ba^{2+} currents, the command step was repeated every 15 seconds until a steady state level of current was activated, as illustrated by the 2 control currents in figure 4.i A. This was done because Ca^{2+} currents in these cells have been previously shown to run up with time (Langton 1993), and to measure the effects of NS1619 on voltage-activated Ca^{2+} channels accurately, it was important to assess the effects when the current had reached a stable level. Increasing concentrations of NS1619 (1-30 μM) were then sequentially superfused onto the cell under test and the command potential delivered until the level of inhibition reached a steady state. Figure 4.i A clearly illustrates the concentration-dependent reduction in currents following the addition of NS1619, with 30 μM NS1619 producing almost complete block of the Ba^{2+} current in this particular cell. The reduction in Ba^{2+} current occurred quickly and was apparent after the first voltage step (15 seconds after including NS1619 in the superfusing solution). The level of inhibition reached a steady-state within sixty seconds and was readily reversible, recovering to 85% of control levels after washing with NS1619-free bath solution for two minutes (n=3). Similar effects were obtained from experiments performed on 3 additional cells and the results are summarised in figure 4.i B. This figure illustrates the mean concentration-effect curve for NS1619 (1-30 μM) on inward Ba^{2+} currents. All currents were measured as peak inward current following leak subtraction as described above and normalised by expressing them as a fraction of the maximum current measured in the same cell to compensate for the variation in the absolute amplitude of the controls. The K_D value for the inhibition of Ba^{2+} currents by NS1619 was found to be approximately 7 μM (see figure 3.i B) and this curve was well fit with a Hill coefficient of 1.3, suggesting a 1:1 effect of the drug with the channel.

A characteristic of some inhibitors of Ca^{2+} channels is that they exhibit a phenomenon termed *voltage-dependence*. In essence, this means that the level of inhibition of the current by a particular drug varies with changes in the membrane potential. A previous study describing the inhibitory effect of NS1619 on Ca^{2+} channels did not attempt to determine if the inhibition of voltage-activated Ca^{2+} currents by NS1619 exhibited any voltage-dependence (Edwards *et al.*, 1994).

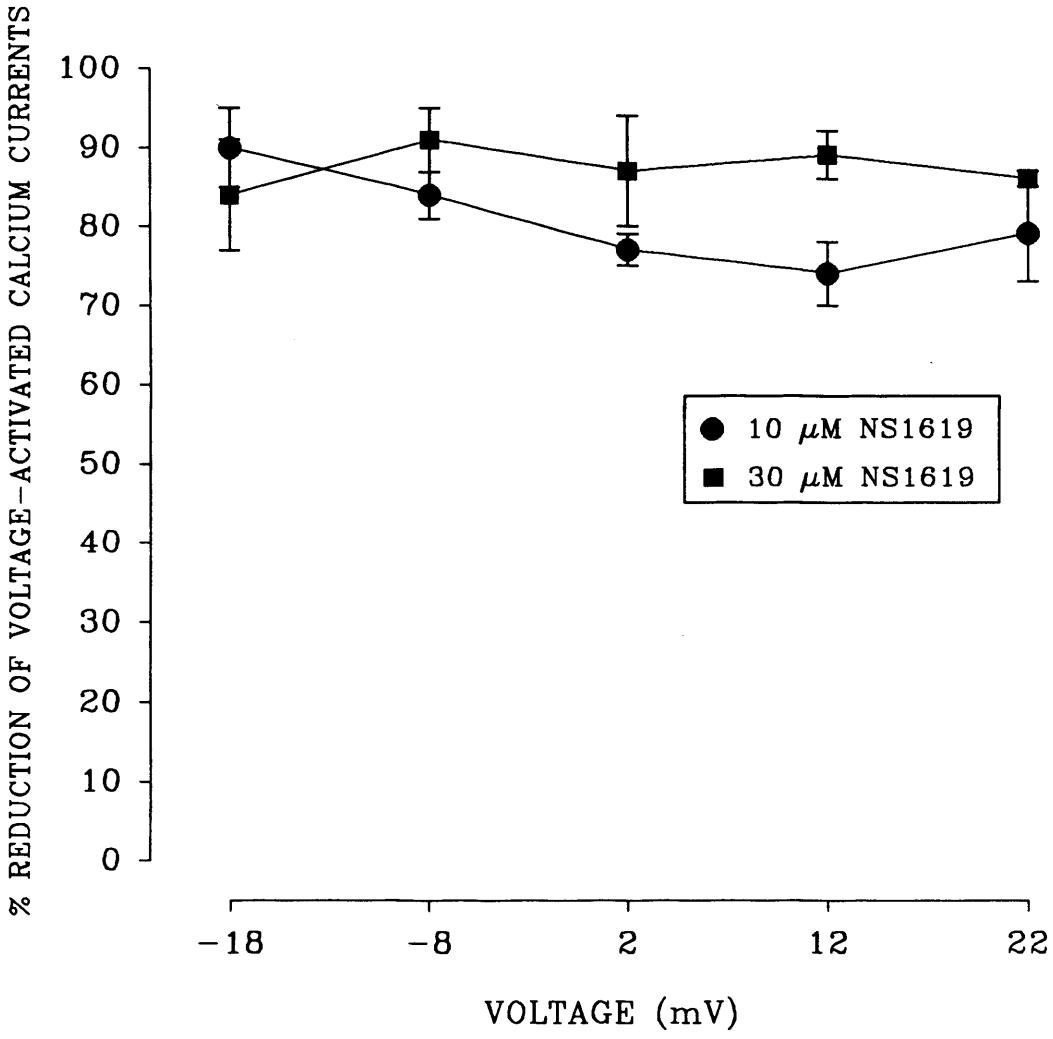


Figure 4.iii. Lack of voltage-dependence of the effects of NS1619 on Ca^{2+} channels . The points show the mean percentage inhibition of Ba^{2+} currents from 4 cells measured over the voltage range -18 to 22 mV in 10 μM NS1619 (●) and 30 μM NS1619
(■).

As this was one of the aims of this study, I investigated the effect of changes in the membrane voltage on the level of Ba^{2+} current inhibition by NS1619. Voltage-dependent inhibition of channels by a drug requires that a range of different membrane potentials are used and the percentage inhibition of the current is measured over that range. Figure 4.ii illustrates I/V relationships for peak inward current recorded in 10 mM Ba^{2+} in the absence, and presence of NS1619 (10 and 30 μM) from 4 cells. Leak subtraction was performed by producing an average leak current using the function built into the patch-clamp software described in more detail in chapter 2. In all experiments, the cell was clamped throughout at -68 mV and Ba^{2+} currents were activated by a sequence of depolarising command potentials from -58 mV rising in 10 mV increments to a final level of +58 mV. The I/V curve possessed the 'bell' shape curve characteristic of Ca^{2+} channel activation in these cells, rising sharply between -35 and 0 mV and peaking at ~5 mV. These results are similar to previous studies of Ba^{2+} currents using cells isolated from this artery (Langton & Standen 1993; Langton, 1993). The addition of 10 and 30 μM NS1619 reduced the inward current in a concentration-dependent manner with 30 μM NS1619 almost completely blocking the current. By measuring the percentage reduction at each potential produced by NS1619 and plotting this across the range of potentials used, the effects of changes in membrane voltage on the level of inhibition were determined. Figure 4.iii illustrates the percentage reduction of the inward Ba^{2+} current by NS1619 (10-30 μM) over -18 to 22 mV. No significant differences in mean percentage reduction of the Ba^{2+} current by NS1619 was apparent, with approximately 75% and 90% inhibition produced by 10 and 30 μM NS1619 respectively (n=4). These data demonstrated that inhibition of Ba^{2+} currents by NS1619 was probably voltage-independent.

4.2.2 Inhibition of K_V channels by NS1619

A recent study using smooth muscle cells isolated from the rat portal vein, a 'phasic' blood vessel, reported that 10 μM NS1619 reduced the whole cell outward current due to inhibition of K_V channels but a further increase to 30 μM NS1619, reduced the level of inhibition of K_V currents (Edwards *et al.*, 1994). This contrasted with the initial description of NS1619 where no auxiliary effects of NS1619 were reported (Olesen *et al.*, 1994a). While this additional effect is probably inconsequential when defining the functional effects of NS1619, it was worthy of investigation to help further define the pharmacology of the drug.

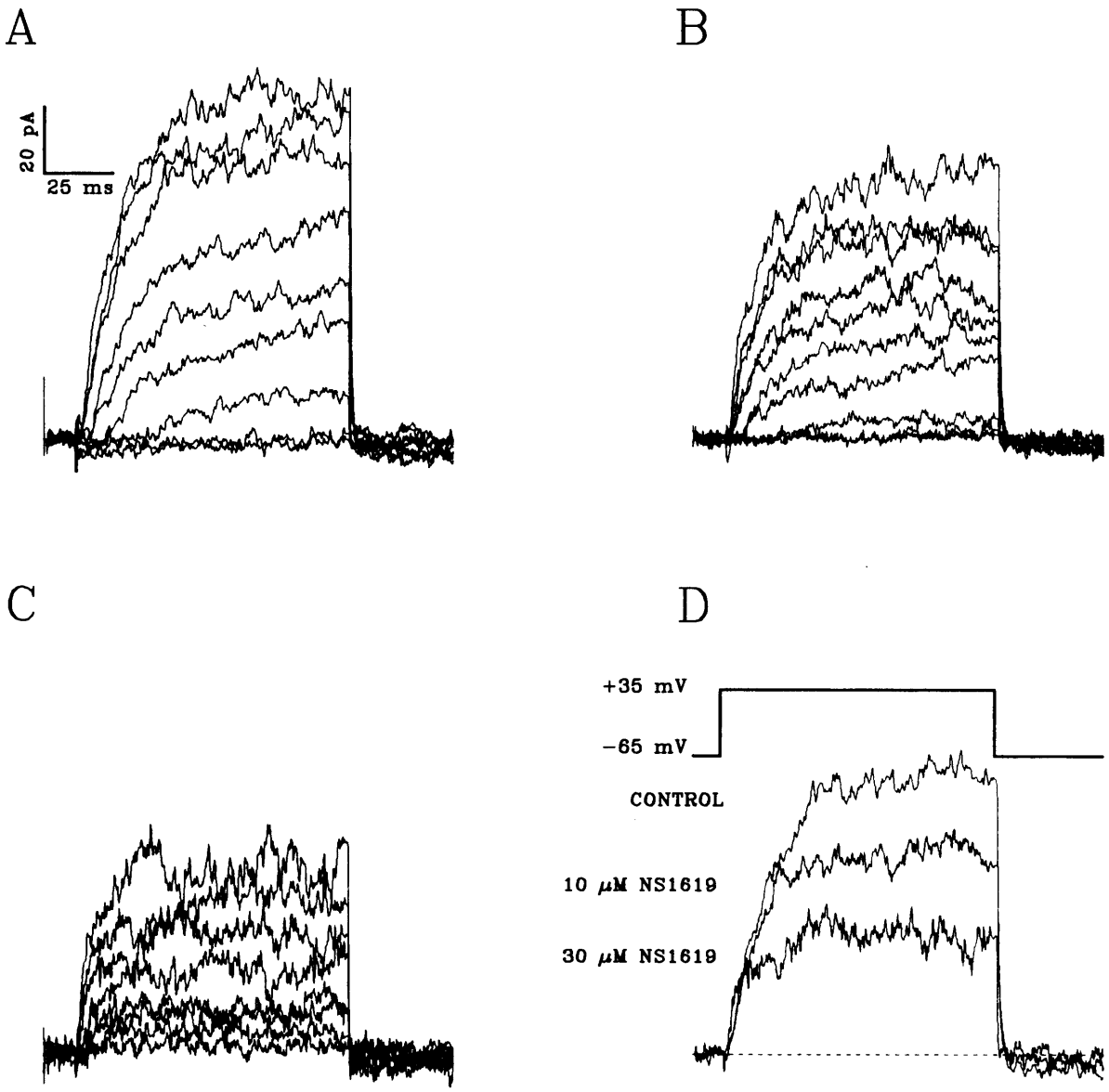


Figure 4.iv. Concentration-dependent inhibition of K_V currents by NS1619. Currents were recorded using the conventional whole cell configuration of the patch-clamp technique with 5 mM EGTA in the pipette solution and 100 nM IbTX in the external solution to minimise BK_{Ca} channel activation. Panel A shows representative families of currents activated under control conditions (holding potential -65 mV). Panels B & C show currents from the same cell following the addition of 10 and 30 μM NS1619 to the cell for 1 minute. Panel D highlights currents activated at +35 mV in the presence of 0, 10 & 30 μM NS1619 extracted from panels A-C for clarity. Peak outward currents at this potential were reduced relative to control by 27 % and 52 % respectively.

I therefore examined the effects of increasing concentrations of NS1619 (10-30 μM) on the amplitude of K_V currents. Obviously assessing the blocking effects of a compound against one population of channels when the same drug also activates other channels present in the same cells posed a potential problem. To accurately measure the blocking effects of NS1619 on K_V currents may have required superfusing NS1619 onto cells until the level of inhibition reached a stable level. This, however may lead to the activation of BK_{Ca} channels, especially at higher concentrations of NS1619, with the resulting outward currents distorting the level of inhibition of K_V currents by NS1619. To counter the activation of BK_{Ca} channels, but still assess the blocking effects of NS1619 on K_V currents, the following steps were taken to reduce the activation of BK_{Ca} channels.

1. The intracellular Ca^{2+} concentration was buffered to a low level by including 10 mM of the Ca^{2+} chelator EGTA in the pipette solution and adding no additional Ca^{2+} to this pipette solution.

2. 100 nM IbTX was included in all superfusing solutions. In previous experiments (see chapter 3 figure 3.iii A), 100 nM of IbTX was found to rapidly and almost completely abolish BK_{Ca} channel activation in response to 20 μM NS1619.

3. As the activation of BK_{Ca} channels in whole cells in response to NS1619 was relatively slow (>1 minute) compared to the blocking effects of NS1619 on Ca^{2+} channels (< 1 minute), the effects of NS1619 on K_V channels were measured after the addition of NS1619 to test cells for 1 minute. This may have resulted in the block of K_V channels by NS1619 being slightly underestimated but I feel this step was necessary to reduce any BK_{Ca} channel activation.

Similar conditions to these have previously been reported to allow the study of K_V currents in isolation (Volk *et al.*, 1991) and should allow the effects of NS1619 to be examined on K_V channels without significant levels BK_{Ca} channel opening also occurring.

Cells under study were continuously superfused with a physiological saline solution containing 6 mM K^+ which could be switched to an identical solution containing increasing concentrations (10-30 μM) of NS1619 as required. The pipette (intracellular) solution contained 140 mM K^+ so an approximately physiological K^+ concentration gradient existed across the membrane.

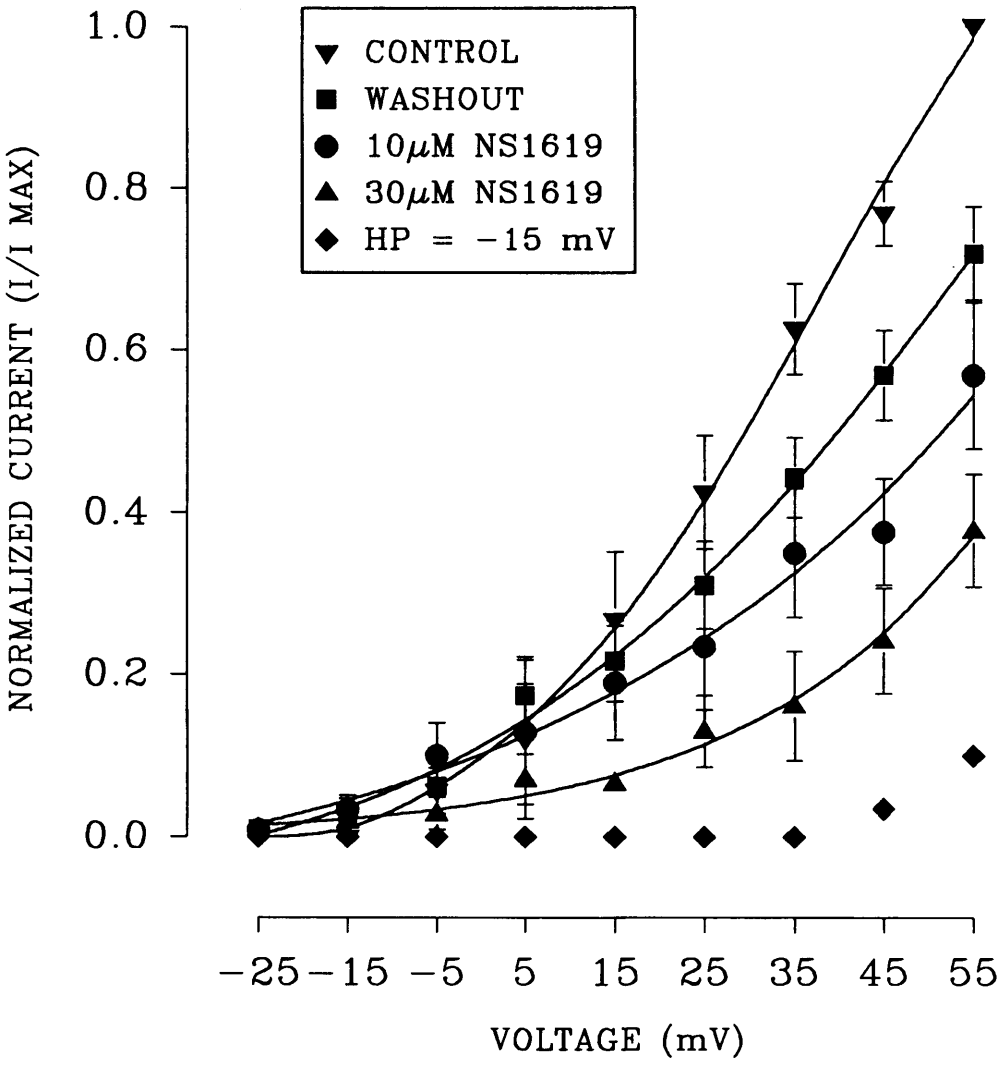


Figure 4.v. Mean current-voltage relationship using the recording conditions described in figure 4.iv for control (▼), 10 μM NS1619 (●), 30 μM NS1619 (▲), washout of NS1619 (■) and currents activated from a holding potential of -15 mV (◆). The points shown are the mean ± s.e. mean from 4 cells each of which was exposed to above conditions. Currents from each cell were normalised to the maximum current measured from that cell

K_V channel currents were activated from cells clamped at -65 mV in response to a sequence of depolarising command potentials from -55 mV, rising in 10 mV increments to a final level of 55 mV. Under these conditions the dominant outward currents were due to the activation of voltage-activated currents as illustrated in figure 4.iv A. This particular figure illustrates a typical record of families of outward voltage-activated currents recorded using the above conditions. Leakage currents were subtracted digitally from these data by producing an average leakage current as described previously, and then subtracting this current from the raw data. Activation of K_V currents occurred at potentials positive to -25 mV (no currents were activated at potentials negative to -25 mV so these traces have been omitted from all figures) and possessed the appearance characteristic of delayed rectifier K^+ currents, with the current taking approximately 50 ms to reach a steady state level and showing no inactivation during the length of the command potential (100 ms shown in figure 4.iv, although pulses 500 ms in length were also used). Figures 4.iv B and C illustrate the currents measured following the sequential addition of 10 and 30 μM NS1619 to the same cell. NS1619 clearly reduced these outward voltage-activated currents in a concentration-dependent manner. To better illustrate this inhibition, figure 4.iv D illustrates currents from the same cell activated by a voltage step to +35 mV, under control conditions and in the presence of 10 and 30 μM NS1619. The peak outward currents illustrated at this potential were 90, 66 and 43 pA, corresponding to reductions in the peak current amplitude relative to the control by 27% and 52% in the presence of 10 and 30 μM NS1619 respectively. Another reason for wanting to inhibit outward currents through BK_{Ca} channels is that the production of an outward current by K_{ATP} channel openers, such as cromakalim has previously been proposed to explain the inhibition of K_V currents by the KCOs (McHugh & Beech, 1995). The proposed mechanism of action underlying this effect involves the increased concentration of K^+ extracellularly following the activation of an outward K^+ current by cromakalim, which reduces the transmembrane concentration gradient for K^+. The reduced driving force for K^+ reduces the electrochemical gradient for K^+ efflux and the amplitude of the K_V currents. To ensure that a similar process could not explain any of the effects on K_V currents observed in this study, BK_{Ca} currents were blocked so that any reduction in K_V was not simply due to an increase in the extracellular K^+ concentration.

Figure 4.v illustrates the mean I/V relationship from similar experiments performed on 4 cells.

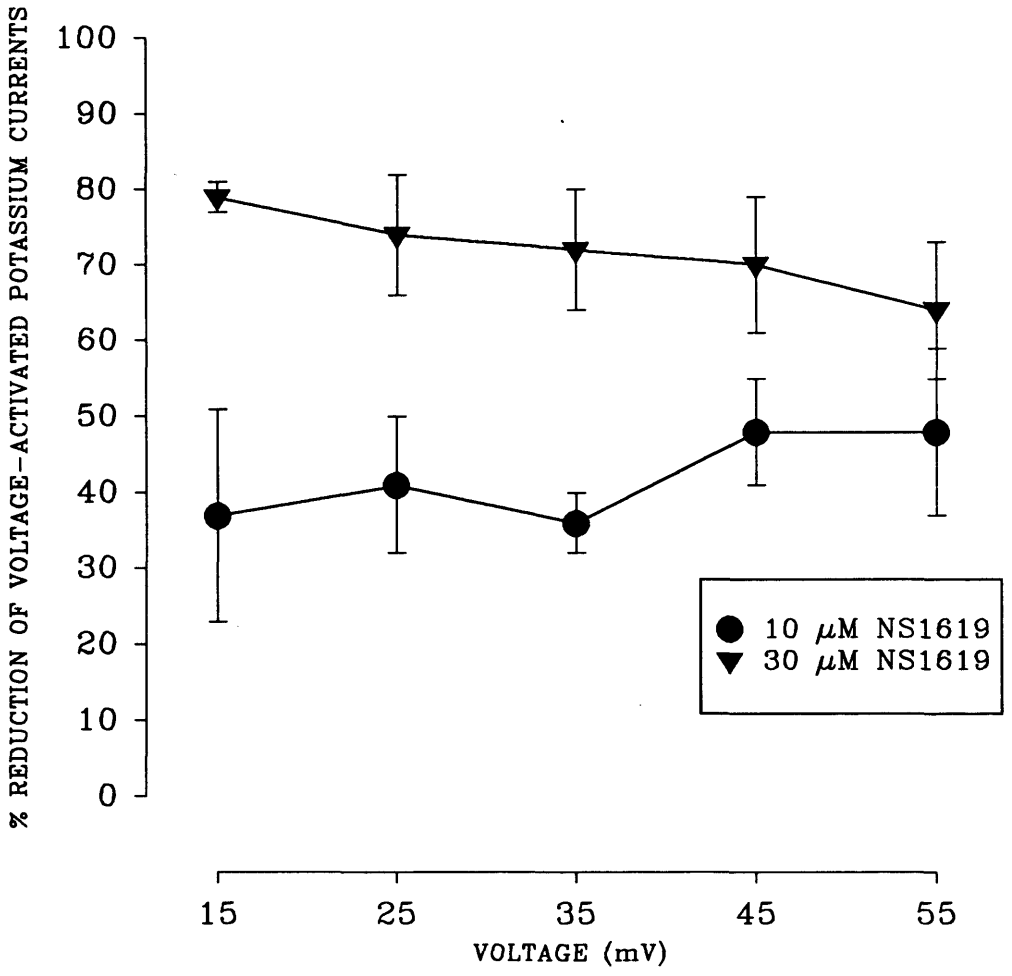


Figure 4.vi. Lack of voltage-dependence of the action of NS1619 on K_V currents. The points show the mean ± s.e. mean inhibition of K_V currents measured over the voltage range 15 to 55 mV from 4 cells in 10 μM (●) and 30 μM NS1619 (▼).

Currents were measured as peak outward current and normalised for each cell by expressing them as a fraction of the maximum control current in that cell, therefore removing the variation in the amplitude of control currents. The control I-V curve clearly illustrates that the channel responsible for the production of this current was voltage-dependent, being activated by depolarising command potentials positive to -25 mV. The lowest curve, produced using the same command sequence but a different (-15 mV) holding potential, also illustrates that this channel underwent considerable voltage-dependent inactivation. Voltage-dependent inactivation is a characteristic of K_V channels and these experiments helped to confirm that the voltage-activated currents described here were due to the activation of K_V channels. Figure 4.v also summarises the effects of NS1619 on the K_V currents and illustrate the concentration-dependent reduction of K_V currents by NS1619. This effect was reversible, with the currents recovering to 60 -90 % of the control level after perfusing with NS1619-free solution for 2 minutes.

Voltage-dependence of this effect of NS1619 was examined by plotting the percentage reduction of the current produced by 10 and 30 μM NS1619 over the range of membrane potentials 15-55 mV. Figure 4.vi summarises these data and illustrates that the reduction in the outward current produced by NS1619 was not significantly different over this range of membrane potentials, indicating that the block of K_V channels was probably voltage-independent. It may appear at first glance as if the level of inhibition of K_V currents by 30 μM NS1619 was showing some voltage-dependence, with the percentage block of K_V currents decreasing as the membrane is being increasingly depolarised. This apparent effect is however probably due to some limited activation of BK_{Ca} channels occurring in the presence of 30 μM NS1619 at more depolarised membrane potentials. This point can be illustrated by examining the records in figure 4.iv C, when despite all the measures used to inhibit BK_{Ca} channels, it is clear that at these more depolarised potentials, +35 to +55 mV, the current traces display the 'noisy' appearance characteristic of BK_{Ca} channel activation. Therefore, at more depolarised potentials 30 μM NS1619 produced limited activation of BK_{Ca} channels, despite employing conditions unfavourable to the activation of the BK_{Ca} channel. This small level of activation probably increases the outward current slightly, giving the false impression that the inhibition of K_V currents by 30 μM NS1619 was showing voltage dependence. This effect might also explain the apparent concentration-dependent reduction in inhibition of K_V currents by 30 μM NS1619 previously reported (Edwards *et al.*, 1994).

4.3 DISCUSSION

The initial report describing the pharmacological profile of NS1619 was conducted using a variety of cells, including cultured bovine aortic smooth muscle cells, and suggested that the effects of NS1619 were specific to BK_{Ca} channels (Olesen *et al.*, 1994a). A more recent study conducted using cells acutely isolated from rat portal vein reported that NS1619, in addition to activating BK_{Ca} channels, blocked DHP-sensitive Ca^{2+} channels, K_V channels and K_{ATP} channels over a similar range of concentrations to those that opened BK_{Ca} channels (Edwards *et al.*, 1994). Functional results from the study by Edwards and co-workers established that the vasorelaxant effects of NS1619 were unaffected by specific inhibitors of BK_{Ca} channels, with the authors concluding that the vasorelaxant effects of NS1619 could be entirely explained by Ca^{2+} channel block rather than BK_{Ca} channel opening. Results from the present study confirm that NS1619 has pronounced effects on other ion channels over a similar range of concentrations to those that opened BK_{Ca} channels. These additional effects, especially the Ca^{2+} channel blocking effects, are important when defining how NS1619 ultimately produces vasorelaxation.

4.3.1 Inhibition of Ba^{2+} currents by NS1619

NS1619 inhibited DHP-sensitive Ca^{2+} channels in rat basilar artery smooth muscle cells. This effect was fast in onset (maximum inhibition < 1 minute) and reversible. This result was unsurprising as NS004, a compound structurally related to NS1619, and also described as an opener of BK_{Ca} channels (Olesen *et al.*, 1994b), has also been reported to inhibit Ca^{2+} currents (Sargent *et al.*, 1993; McKay *et al.*, 1994) and that these Ca^{2+}-channel blocking properties formed the basis of NS004 vasorelaxant and cardioprotective effects (Sargent *et al.*, 1993). In the present study, NS1619 blocked Ca^{2+} channels with a K_D value of 7 μM. This effect was independent of changes in the membrane potential and was due to a 1:1 interaction of drug with channel. It is unclear why the initial description of NS1619 found this compound to have no effect on voltage-activated Ca^{2+} channels. However Olesen and his co-workers assessed the effects of NS1619 on Ca^{2+} channels by measuring changes in the intracellular global Ca^{2+} concentration using FLUO-3 following stimulation of mouse cerebellar granule cells with 60 mM K^+, rather than direct measurements of I_{Ca} with patch-clamp techniques (Olesen *et al.*, 1994a).

Cerebellar granule cells have been reported to contain at least three types of voltage-activated Ca^{2+} channels in addition to DHP-sensitive Ca^{2+} channels, including N-, P- and Q-type channels (Huston *et al.*, 1995; Warming *et al.*, 1997). It is therefore possible that Ca^{2+} influx through voltage-activated Ca^{2+} channels unaffected by NS1619 may have 'disguised' the effects of this compound on DHP-sensitive Ca^{2+} channels in these cells.

4.3.2 Inhibition of delayed rectifier K^+ channels by NS1619

NS1619 was found to reduce K_V currents in a concentration-dependent manner. This effect was fast in onset (≤ 1 minute), readily reversible and independent of changes in the membrane potential. This observation contrasts with a previous report which claimed that NS1619 did not affect K_V channels in mouse cerebellar granule cells (Olesen *et al.*, 1994a), but partially agrees with a subsequent study which reported that NS1619 reduced K_V currents in rat portal vein smooth muscle cells (Edwards *et al.*, 1994). However in contrast to Edwards *et al.*, (1994), who reported that increasing the concentration of NS1619 above 10 μM to 30 μM resulted in a reduction in the level of inhibition, I report that 30 μM NS1619 further depressed K_V currents. The reasons for this discrepancy can probably be explained by differences in the experimental design and how the inhibition of K_V currents by NS1619 were assessed in the two studies. In the present study for example, as explained in some detail in section 4.2.2, the effects of NS1619 on K_V currents were examined using conditions designed to limit BK_{Ca} channel activation and the effects of NS1619 on K_V currents were measured before NS1619 should produce substantial activation of BK_{Ca} channels. These measures I feel allowed a more accurate assessment of the blocking effects of NS1619 on K_V currents, as additional contaminating currents would either be abolished or would be relatively small. Despite these measures, I still observed that at more depolarised potentials in the presence of 30 μM NS1619 some BK_{Ca} channel activation still occurred (see figure 4.iv C). If, as seems likely, this also occurred in the experiments performed by Edwards and her co-workers then this may have appeared in the whole cell record as an apparent reduction in the level of inhibition of K_V produced by 30 μM NS1619, when in fact it simply reflected an increased level of BK_{Ca} channel activation by the higher concentration of NS1619.

Why the initial report describing the effects of NS1619 did not find that NS1619 had additional effects on K_V channels is unclear.

The most obvious explanation is that the sub-unit composition of K_V channels expressed in mouse cerebellar granule cells is different to those expressed in smooth muscle cells, which appear to be composed entirely of Kv 1.5 subunits (Overturf *et al.*, 1994; Clement-Chomienne *et al.*, 1997). A large number of native K_V channels and Kv channel subunits has been identified using molecular biological and electrophysiological techniques. Different Kv subunits have been shown to possess different sensitivities to blocking agents. CTX, for example, a frequently used BK_{Ca} channel blocker, also blocks some Kv 1.3 channels with higher affinity than it blocks BK_{Ca} (Lewis & Cahalan, 1988; Price *et al.*, 1989; Swanson *et al.*, 1990; Deutsch *et al.*, 1991). IbTX, which has a similar sequence and three-dimensional structure to CTX does not block Kv 1.3 channels in T lymphocytes (Deutsch *et al.*, 1991; Leonard *et al.*, 1991). The channels in mouse cerebellar granule cells may therefore be composed of Kv sub-units that are insensitive to NS1619, while those that comprise the K_V channel in smooth muscle were markedly affected by NS1619.

The effects of NS1619 on K_{ATP} channels were not assessed, primarily because appreciable currents to the K_{ATP} channel opening drugs pinacidil and cromakalim could not be produced using cells isolated from the rat basilar artery. This observed inability to activate appreciable K_{ATP} currents using K^+ channel opening drugs agrees well with functional and sharp electrode electrophysiological studies performed on rat cerebral arteries, where these drugs have been shown to be poor vasorelaxant agents in this particular vascular bed (McCarron *et al.*, 1991; McPherson & Stork, 1992).

Despite NS1619 being a fairly potent blocker of K_V channels, the studies performed investigating the effects of NS1619 on cell membrane potential (see chapter 3) demonstrated that blocking these channels, as NS1619 would have done, did not depolarise the membrane potential, with the hyperpolarisation due to BK_{Ca} channel activation being the dominant effect. This suggests that K_V channels may not be particularly important in determining the resting membrane potential in this particular vascular bed.

4.4 CONCLUSION

These experiments have helped to further define the pharmacological profile of NS1619 by assessing the effects of the drug on tonic smooth muscle cells, as a previous study of the effects of NS1619 was performed on phasic smooth muscle.

The effects on NS1619 in phasic and tonic smooth muscle appears to be very similar but what has become increasingly clear is that NS1619 is pharmacologically a 'dirty' agent, with a whole range of effects on channels other than BK_{Ca} channels. NS1619 blocks DHP-sensitive Ca^{2+} channels over a similar concentration range to that which activates BK_{Ca} channels. It would appear that this effect most probably explains NS1619s vasorelaxant properties. NS1619 also reduced K_V currents, an effect however that did not prevent 30 μM NS1619, a concentration of NS1619 that produced substantial block of K_V currents, from producing membrane hyperpolarisation, perhaps indicating that K_V channels may not be as important in setting the membrane potential in these cells as they are in other vascular beds. These additional effects of NS1619 should be taken into account when using this compound especially when attempting to attribute any functional effects of this drug to a specific channel type. NS1619 is however a useful experimental compound and can used to demonstrate the presence of BK_{Ca} channels in a preparation.

CHAPTER FIVE

Activation of BK_{Ca} channels in rat cerebral artery smooth muscle cells by the nitric oxide donor SIN-1A and by authentic nitric oxide

5.1 INTRODUCTION

5.1.1 Synthesis of nitric oxide

In the early 1980s a series of pioneering experiments determined that the presence of an intact endothelium was required to elicit relaxation of isolated arteries to agonists such as acetylcholine (Furchott & Zawadski, 1980). This led to the idea that acetylcholine did not produce vasorelaxation by directly interacting with receptors located in the membrane of vascular smooth muscle cells but via the endothelium cells. The resulting agonist-receptor interaction initiated the release of a labile factor which then diffused into the adjacent smooth muscle cells producing vasorelaxation. This *endothelium-derived relaxing factor* (EDRF), as it was initially termed, was later identified as nitric oxide (NO) or a labile compound capable of releasing NO (Ignarro *et al.*, 1987; Palmer *et al.*, 1987).

The discovery of NO represents one of the most important discoveries in recent years in the field of vascular biology and cell signalling as a whole and led to an explosion of interest in the field (for reviews see Snyder & Bredt, 1992; Schmidt *et al.*, 1993; Garthwaite & Boulton, 1995; Rand & Li, 1995).

NO can exist in three distinct forms. These are: NO^+ the nitrosium ion, NO* the neutral free radical and NO^- the nitroxyl ion. The exact form of NO synthesised by nitric oxide synthase (NOS) in the endothelium and which produces vasorelaxation has yet to be identified, although NO* is generally assumed to be form of NO produced by endothelial nitric oxide synthase. The NO^- ion is definitely not the correct form of NO produced by the endothelium as it possesses only one-thousandth the relaxant activity of NO* and NO^+ (Feelish *et al.*, 1994).

NO is highly reactive and diffusible and therefore readily crosses cell membranes. These characteristics, coupled with its short half-life *in vitro* (~5 s), although *in vivo* its half-life may be longer, make NO an ideal regulator of local blood flow.

The production of NO in vascular endothelial cells is catalysed by the enzyme *nitric oxide synthase* (NOS). Three distinct isoforms of this enzyme have been well characterised and classified according to the tissues from where they were originally isolated or the chronological order in which they were isolated and cloned (for review see Griffith & Stuehr, 1995):

1. Endothelial NOS (eNOS), sometimes called NOS III. The activity of eNOS is stimulated following an increase in the endothelial Ca^{2+} concentration (see figure 5.i). Ca^{2+} binds to calmodulin, forming the versatile Ca^{2+}-calmodulin complex, which activates many Ca^{2+}-dependent enzymes, including eNOS, and activates eNOS. Activated eNOS catalyses the production of NO which produces vasorelaxation. eNOS, unlike the other members of the NOS family, is thought to be associated with the endothelial cell membrane, a characteristic which may explain the activation of eNOS by shear stress (Buga *et al.*, 1991)

2. Neuronal NOS (nNOS), sometimes called NOS I. The activity of nNOS is also controlled by agonist-induced increases in Ca^{2+} concentration, an event which occurs following glutamate binding to N-methyl D-aspartate (NMDA) receptors (see Garthwaite & Boulton, 1995).

3. Inducible NOS (iNOS), sometimes called NOS II. iNOS is widely distributed and has been identified in virtually all mammalian cells. iNOS is fully active at normal intracellular Ca^{2+} concentrations as it has a very high affinity for calmodulin and they remain bound even during low basal Ca^{2+} concentrations found in resting cells. The expression of iNOS is regulated by the production of mRNA which can be rapidly induced by endogenous inflammatory mediators such as tumour necrosis factor, γ interferon and interleukins as well as some products of gram-positive and gram-negative bacteria such as endotoxin and lipopolysaccharide.

The widely accepted hypothesis for the production of NO by eNOS is the five-electron oxidation of one of the two guanidino nitrogens of L-arginine, yielding NO and L-citrulline (for reviews see Schmidt *et al.*, 1993; Dinerman *et al.*, 1993; Griffith & Stuehr, 1995) as illustrated in figure 5.i. This cartoon is greatly simplified and the generation of NO requires molecular oxygen and a number of additional co-factors, including NADPH, tetrahydrobiopterin, flavin adenine dinucleotide and flavin mononucleotide. NO synthesis by eNOS is a Ca^{2+}-dependent process which is triggered following the binding of an agonist such as acetylcholine to a muscarinic receptor of the M_3-subtype.

Binding of acetylcholine activates, via a heterotrimeric G-protein, phospholipase C (PLC), which catalyses the hydrolysis of phosphoinositides, such as phosphatidylinositol 4,5,-biphosphate (PIP_2), a minor component (3-5%) of the plasma membrane of eukaryotic cells, yielding inositol 1,2,5- triphosphate (IP_3). IP_3 then binds to IP_3 receptors located on the sarcoplasmic reticulum which produces Ca^{2+} release. Physical stimuli such as shear stress also results in NO production. Though poorly understood, this effect may be related to deformation of the endothelial plasma membrane by pulsatile blood flow which subsequently activates membrane associated eNOS by a mechanism currently not understood.

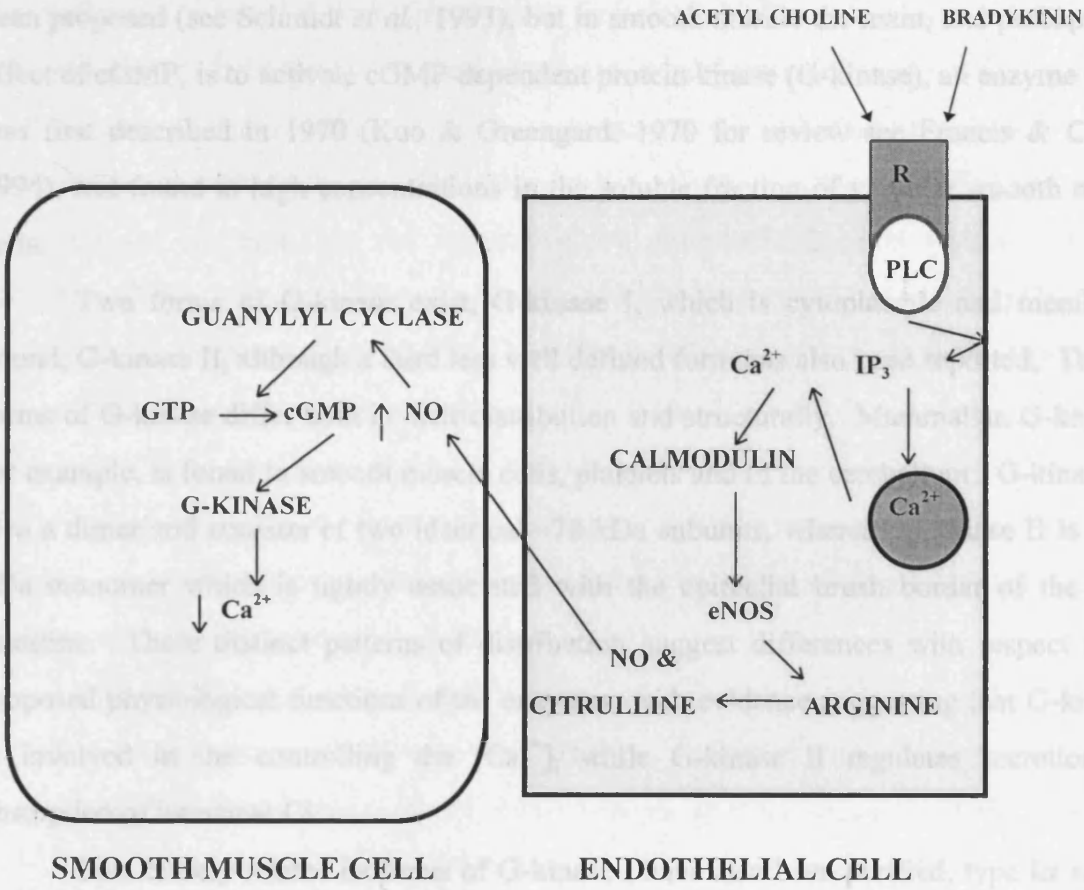


Figure 5.i. Synthesis and effects of Nitric Oxide

5.1.2 Effects of NO

The main effect of NO in vascular smooth muscle is the activation of the enzyme soluble guanylyl cyclase (GC-S), although additional effects of NO have been described which may occur as a result of over-production of NO which may be related to the pathophysiological and toxic effects of NO (see Gross & Wolin, 1995). NO binds to iron in the haem moiety at the active site of GC-S leading to a large increase in the V_{max} of the enzyme, presumably as a result of a conformational change in GC-S (Traylor & Sharma, 1992). The activation of GC-S leads to the formation of guanosine cyclic 3', 5'-monophosphate (cGMP) from guanosine triphosphate (GTP), which is present in excess intracellularly, leading to an increase in the intracellular concentration of cGMP. A number of effects of cGMP have been proposed (see Schmidt *et al.*, 1993), but in smooth muscle the main, and perhaps only effect of cGMP, is to activate cGMP-dependent protein kinase (G-kinase), an enzyme which was first described in 1970 (Kuo & Greengard, 1970 for review see Francis & Corbin, 1994), and found in high concentrations in the soluble fraction of vascular smooth muscle cells.

Two forms of G-kinase exist, G-kinase I, which is cytoplasmic and membrane-bound, G-kinase II, although a third less well defined form has also been reported. The two forms of G-kinase differ both in their distribution and structurally. Mammalian G-kinase I, for example, is found in smooth muscle cells, platelets and in the cerebellum. G-kinase I is also a dimer and consists of two identical ~78 kDa subunits, whereas G-kinase II is an 86 kDa monomer which is tightly associated with the epithelial brush border of the small intestine. These distinct patterns of distribution suggest differences with respect to the proposed physiological functions of the enzymes, with evidence suggesting that G-kinase I is involved in the controlling the $[Ca^{2+}]_i$ while G-kinase II regulates secretion and absorption of intestinal Cl.

Two closely related isoforms of G-kinase I have also been purified, type Iα and Iβ, which are present in approximately equal proportions in porcine, bovine and human vascular smooth muscle (Francis & Corbin, 1994). No difference in the catalytic rate appears to exist between the two isoforms, which are also both fully activated by similar concentrations of cGMP (100-300 nM) at 20 °C (Francis & Corbin, 1994). However, studies using substrate specific cGMP analogues reported a strong correlation between the activation of G-kinase Iα and the ability to relax vascular smooth muscle (Francis *et al.*, 1988).

G-kinase I has many diverse effects but the exact mechanism(s) by which it ultimately produces relaxation of smooth muscle are still unknown (for reviews see Schmidt *et al.*, 1993; Lincoln & Cornwell 1993; Lincoln *et al.*, 1996; Rembold, 1996). What is clear is that NO, nitrovasodilators and other cGMP-elevating agents relax various vascular and non-vascular smooth muscle preparations contracted with either receptor-occupying agonists or with depolarising concentrations of K^+. Because contractile agonists and K^+-induced depolarisation produce constriction of smooth muscle by different mechanisms, G-kinase has been proposed to have a number of effects on various intracellular systems which account for the decrease in the intracellular Ca^{2+} and relaxation produced following the activation of G-kinase (for reviews see Felbel *et al.*, 1988; Cornwell & Lincoln 1991; Francis & Corbin, 1994; Lincoln *et al.*, 1996; Rembold, 1996). These effects are summarised in figure 5.ii and table 5.i. and include: inhibition of voltage-activated Ca^{2+} channels (Clapp & Gurney, 1991); stimulation of a sarcoplasmic reticulum Ca^{2+}-pumping ATPase via phosphorylation of phospholamban, a Ca^{2+}-ATPase regulatory protein, (Sarcevic *et al.*, 1989; Lincoln, 1989; Cornwell *et al.*, 1991); activation of a sarcoplasmic reticulum Ca^{2+}-ATPase via the stimulation of phosphatidylinositol-4-phosphate (PIP) formation (Vrolix *et al.*, 1988); stimulation via phosphorylation of a 240 kDa protein regulator of a membrane bound Ca^{2+}-ATPase (Yoshida *et al.*, 1991); phosphorylation of the IP_3 receptor reducing agonist-induced Ca^{2+} release (Komalavilas & Lincoln, 1996); reducing the Ca^{2+}-sensitivity of MLCK, therefore reducing the phosphorylation of MLC_{20} and generation of contractile force (McDaniel *et al.*, 1992) and activating myosin light chain phosphatase which dephophorylates phosphorylated MLC_{20}, therefore inhibiting crossbridge cycling and force generation (Lee & Kitazawa, 1997).

Surprisingly the vasorelaxant effects of NO,nitrovasodilators and membrane permeable activators of cGMP were less effective against K^+-induced contractions than against agonist-induced tone (Lincoln, 1983; Lincoln & Fisher-Simpson, 1983; Francis *et al.*, 1988; Jones *et al.*, 1990). This suggests that one or more of the vasorelaxant mechanisms of NO and the cGMP signalling system was inhibited in high external K^+. This raised the possibility that one effect of NO may involve opening K^+ channels leading to membrane hyperpolarisation.

A number of subsequent studies reported that NO, nitrovasodilators and atrial (A) and cerebral (C)-type natriuretic peptides, produce opening of plasmalemal K^+ channels, and the resulting hyperpolarisation may in part contribute to vascular smooth muscle relaxation and reduced slow wave activity in visceral smooth muscle preparations (Williams *et al.*, 1989; Thornbury *et al.*, 1991; Tare *et al.*, 1991; Taniguichi *et al.*, 1992; Robertson *et al.*, 1993; Bolotina *et al.*, 1994; Koh *et al.*, 1995; Chen & Rembold, 1996; Banks *et al.*, 1996; Plane *et al.*, 1996; Wellman *et al.*, 1996). Of the many mechanisms which have been proposed to account for the effects of NO, I am going to concentrate solely on this latter mechanism, namely that NO is a hyperpolarising factor and this hyperpolarisation may be involved in the relaxation of vascular smooth muscle in response to nitrovasodilators.

EFFECTS OF cGMP/G-KINASE	EFFECT ON INTRACELLULAR $[Ca^{2+}]$
Activation of membrane-bound Ca^{2+}-ATPase via the phosphorylation of a regulator protein.	↓ Increased Ca^{2+} extrusion from the smooth muscle cell, resulting in a reduction in the free $[Ca^{2+}]_i$.
Activation of SR Ca^{2+}-ATPase via the phosphorylation of the regulatory protein phospholamban.	↓ Increased Ca^{2+} sequestration by the SR therefore reducing the free $[Ca^{2+}]_i$.
Inhibition via phosphorylation of agonist-evoked PLC activity	↓ Inhibition of IP_3 production therefore reducing agonist-induced Ca^{2+} release from the SR
Phosphorylation and inhibition of the IP_3 receptor	↓ Inhibition of IP_3-induced Ca^{2+}-release from the SR and therefore reduced agonist-evoked Ca^{2+} release
Inhibition of L-type Ca^{2+} channels	↓ Inhibition of voltage dependent Ca^{2+} influx, therefore reducing high K^+ and agonist-induced Ca^{2+} influx leading to a reduction in the $[Ca^{2+}]_i$
Activation of K^+ channels	↓ Membrane hyperpolarisation leading to a decrease in Ca^{2+} influx through DHP-sensitive Ca^{2+} channels.
cGMP-dependent reduction in the Ca^{2+}-sensitivity of MLCK	No effect but a reduction in force production for a given $[Ca^{2+}]_i$.

Table 5.i. Summary of the effects of elevating cGMP and activating G-kinase in smooth muscle cells. Adapted from Lincoln *et al.*, (1996) and Rembold, (1996).

5.1.3 NO as a hyperpolarising factor?

While the role of nitric oxide (NO) as an endothelium-derived relaxing factor (EDRF) is well established, its proposed role as a hyperpolarising factor in smooth muscle is more controversial, a fact illustrated by the results from electrophysiological studies where the effects of NO on membrane potential and K^+ channels varies considerably.

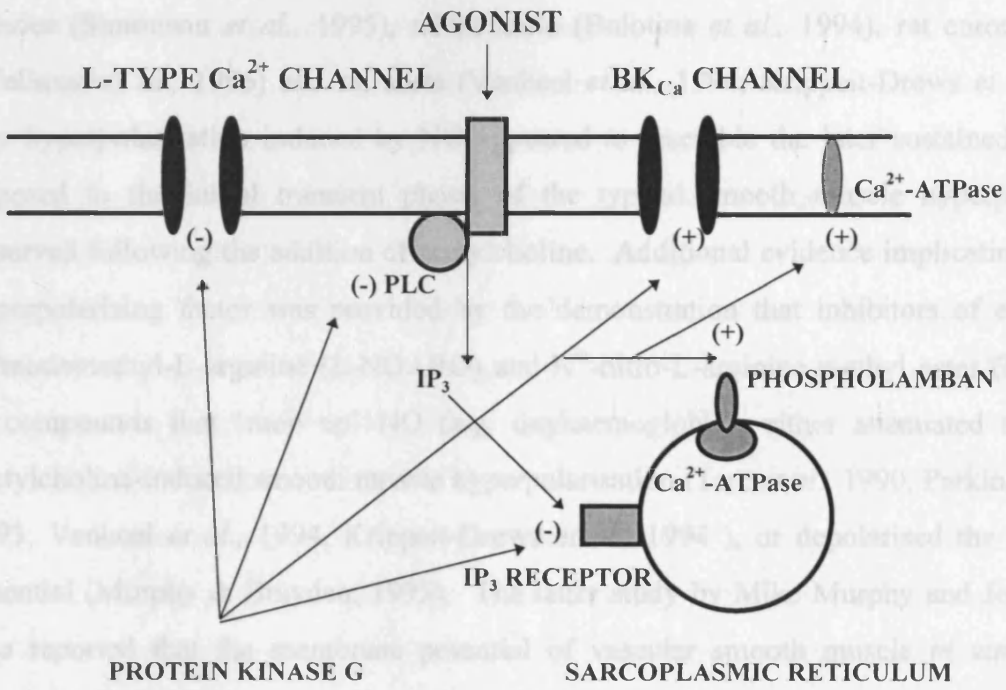


Figure 5.ii. Illustration of the proposed effects of G-kinase reported in various smooth muscle cell preparations. Adapted from Lincoln *et al.*, 1996 in Biochemistry of Smooth Muscle Contraction Academic Press.

A number of studies for example, have reported that authentic NO, NO donors such as sodium nitroprusside (SNP), glyceryl trinitrate (GTN), 3-morpholinosydnonimine (SIN-1A) and A and C-type natriuretic peptides hyperpolarised a variety of vascular smooth muscle preparations including guinea pig , rat, sheep, and rabbit coronary arteries (Parkington *et al.*, 1993; Parkington *et al.*, 1995), rabbit basilar artery (Rand & Garland, 1992), canine and porcine coronary arteries (Taniguchi *et al.*, 1992; Banks *et al.*, 1996), small rat mesenteric arteries (Garland & McPherson 1992; Plane *et al.*, 1996), rabbit mesenteric arteries (Murphy & Brayden, 1995), guinea pig mesenteric and uterine artery (Tare *et al.*, 1990), rat cerebellar arteries (McPherson & Stork, 1992), rat tail artery (Chen & Rembold, 1996), horse penile arteries (Simonson *et al.*, 1995), rabbit aorta (Bolotina *et al.*, 1994), rat coronary artery (Wellman *et al.*, 1996) and rat aorta (Vanheel *et al.*, 1994; Krippeit-Drews *et al.*, 1994). The hyperpolarisation induced by NO appeared to resemble the later sustained phase, as opposed to the initial transient phase, of the typical smooth muscle hyperpolarisation observed following the addition of acetylcholine. Additional evidence implicating NO as a hyperpolarising factor was provided by the demonstration that inhibitors of eNOS (e.g. N^{G}monomethyl-L-arginine (L-NOARG) and N^{ω}-nitro-L-arginine methyl ester (L-NAME), or compounds that 'mop up' NO (e.g. oxyhaemoglobin), either attenuated the typical acetylcholine-induced smooth muscle hyperpolarisation (Tare *et al.*, 1990, Parkington *et al.*, 1993, Vanheel *et al.*, 1994, Krippeit-Drews *et al.*, 1994), or depolarised the membrane potential (Murphy & Brayden, 1995). The latter study by Mike Murphy and Joe Brayden also reported that the membrane potential of vascular smooth muscle *in vitro* became depolarised following removal of the endothelium when compared to the membrane potential of endothelium-intact vessels, (Murphy & Brayden, 1995), perhaps indicating that continuous basal release of NO occurs, producing smooth muscle hyperpolarisation.

Conversely other studies have demonstrated that even saturated solutions of NO had little or no effect on membrane potential (Komroi *et al.*, 1988; Brayden 1990; Plane & Garland, 1993; Waldron & Garland, 1994; Frieden & Beny, 1995) and that acetylcholine-induced hyperpolarisation persisted even when vessels were pre-treated with inhibitors of eNOS. This led to the suggestion that part of the acetylcholine-induced membrane hyperpolarisation was due to the release of a currently unidentified humoral factor termed endothelium derived hyperpolarising factor (EDHF) (for reviews see Taylor & Weston, 1988; Komori & Vanhoutte, 1990).

Acetylcholine has also been reported to directly hyperpolarise vascular endothelial cells via the activation of an apamin-sensitive K^+ channel and a CTX-sensitive K^+ channel other than the BK_{Ca} channel, possibly the intermediate conductance Ca^{2+}-activated K^+ channel (Busse *et al.*, 1988; Marchenko & Sage 1996). These authors proposed that acetylcholine-induced endothelial hyperpolarisation may then hyperpolarise the underlying smooth muscle cells, raising the possibility that the direct hyperpolarisation of the endothelium may partly explain the hyperpolarisation of smooth muscle by acetylcholine.

Where smooth muscle hyperpolarisation due to the activation of a K^+ conductance has been demonstrated, contrasting results as to the identity of the K^+ channel affected have been reported. Several studies have reported that the BK_{Ca} channel was activated (Williams *et al.*, 1989; Taniguchi *et al.*, 1992; Robertson *et al.*, 1993; Bolotina *et al.*, 1994; Archer *et al.*, 1994; Hampl *et al.*, 1995; Banks *et al.*, 1996; Wellman *et al.*, 1996), while others have claimed that ATP-dependent (K_{ATP}) channels were the target (Garland & McPherson 1992; Miyoshi *et al.*, 1994; Murphy & Brayden 1995; Parkington *et al.*, 1995) and one study even reported that NO activated K_V channels (Yuan *et al.*, 1996). Also a direct G-kinase-independent mechanism was proposed by Valerie Bolotina and her co-workers in Boston to account for the activation of BK_{Ca} channels in excised membrane patches by NO solutions (0.5-5 μM) (Bolotina *et al.*, 1994).

In summary, where NO has been reported to act as a hyperpolarising factor, the activation of BK_{Ca}, K_{ATP} or even K_V channels in smooth muscle have been reported to explain how this hyperpolarisation may be produced which may then contribute to part of the relaxation of smooth muscle produced by NO.

In the present study I investigated the effects of NO on single smooth muscle cells isolated from the rat basilar artery using 3-morpholinosydnonimine (SIN-1A), a thermolabile source of NO with a half life at room temperature of several hours (Feelisch *et al.*, 1989), and authentic NO in the form of a saturated solution (Feelisch, 1991). I aimed to determine if NO affected the activity of K^+ channels in basilar artery smooth muscle cells and if so to unequivocally identify the channels involved. Unlike most of the other patch clamp studies of this nature, I recorded steady-state whole cell currents at membrane potentials approximating to the physiological RMP of arterial smooth muscle cells (-60 to -40 mV) rather than at more depolarised potentials.

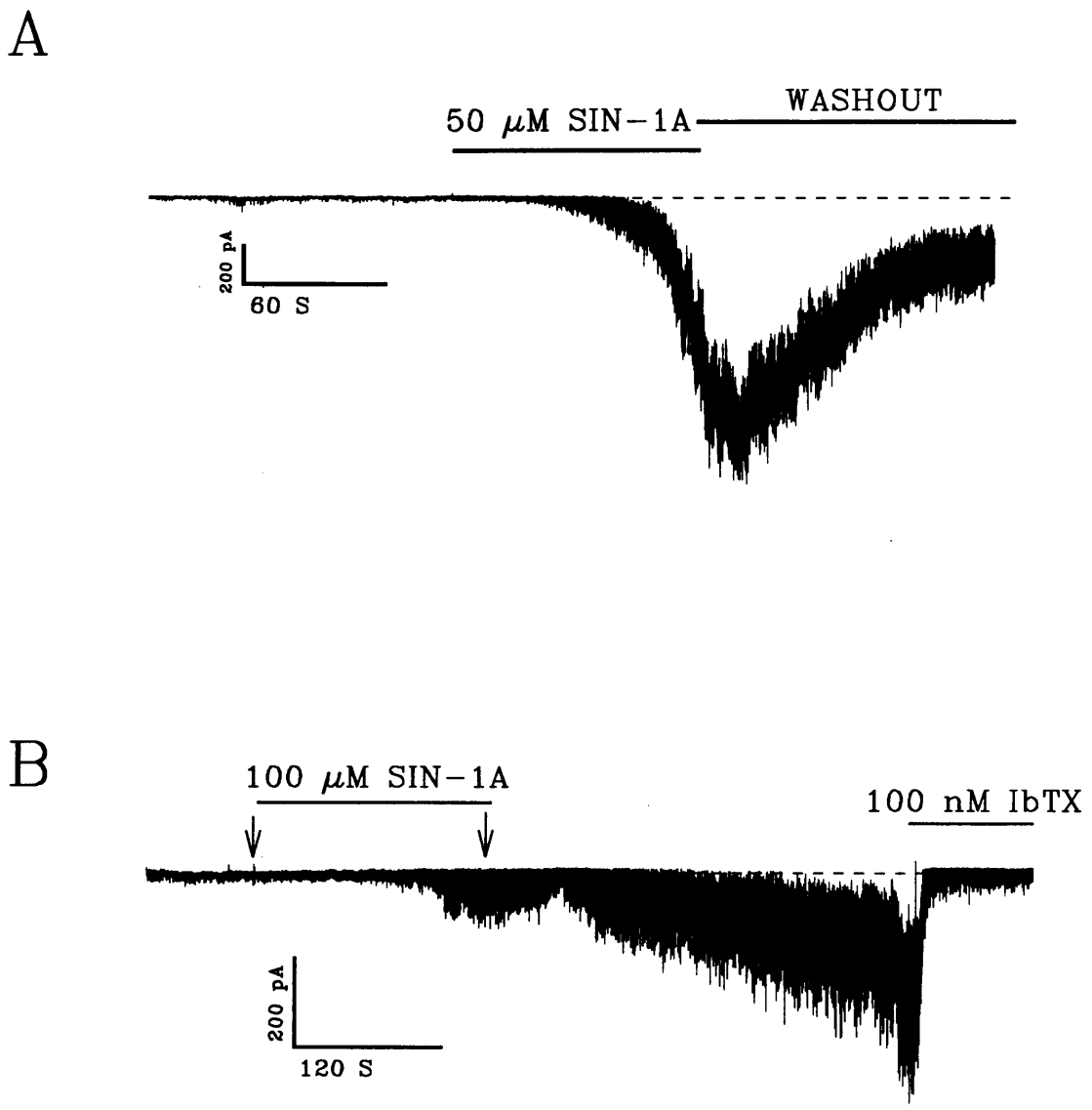


Figure 5.iii. Activation of whole cell currents by the nitric oxide donor SIN-1A. Access was gained in both examples using the conventional whole-cell configuration of the patch-clamp technique and the cells were held at -60 mV (panel A) and -40 mV (panel B) respectively and bathed throughout in a solution containing 140 mM K^+ and 50 units ml^{-1} of super oxide dismutase. Both records were filtered at 1.0 kHz and sampled at 5.0 kHz and the continuous dotted line indicates the zero current level. SIN-1A was added where indicated and the arrows indicate points where the recording was paused and whole cell currents were activated using voltage ramps.

5.2 RESULTS

5.2.1 Induction of whole cell currents by the nitric oxide donor SIN-1A was due to the activation of K^+ channels

If NO is a hyperpolarising factor then it should have effects on the activity of K^+ channels leading to the development of a K^+ current. To preclude the involvement of any reactive breakdown products of NO being responsible for the effects observed in this study all the experiments described were performed in the presence of superoxide dismutase (50 units ml^{-1}), an enzyme which catalyses the conversion of the superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen. This inhibited the accumulation of O_2^- anions, therefore ensuring that NO did not react with O_2^- to form the peroxynitrite radical ($ONOO^-$), as a study recently reported that O_2^- and $ONOO^-$ activated BK_{Ca} channels (Wei *et al.*, 1996).

Figure 5.iii illustrates the effects of SIN-1A (50 and 100 μM) on steady state currents recorded from two rat basilar artery smooth muscle cells. Whole cell access was gained using the conventional whole cell configuration of the patch clamp technique and the cells were clamped at -60 mV (panel A) and -40 mV (panel B) respectively. Both these recordings were made in symmetrical 140 mM K^+, so that the opening of any K^+ channels would result in the production of inward currents. Symmetrical high K^+ was used to maximise the unitary current amplitude of any K^+ channels, so even if only small numbers of channels were opened the resulting currents should be large enough to measure (see solutions appendix at the end of the methods chapter for the composition of experimental solutions).

Before SIN-1A was added the basal level of channel activity in both these cells was low. This was expected because of the combination of using only relaxed smooth muscle cells for patch-clamp experiments, the negative holding potentials used and the low Ca^{2+} intracellular solution used in all experiments. This point is perhaps better illustrated by the control whole cell currents activated by voltage ramps illustrated in figures 5.iv A, 5.v B, 5.vii A and B and summarised in figures 5.viii A and B. Clearly the small control currents reflect the low levels of channel activity in unstimulated cells under these conditions. In the recordings illustrated in figure 5.iii 'noisy' inward currents began to develop approximately 30 s to 2 minutes after adding SIN-1A to the superfusing solution. The addition of SIN-1A was then ended leaving the cells 'washing' in the 140 mM K^+ solution to try and reverse the SIN-1A-activated current.

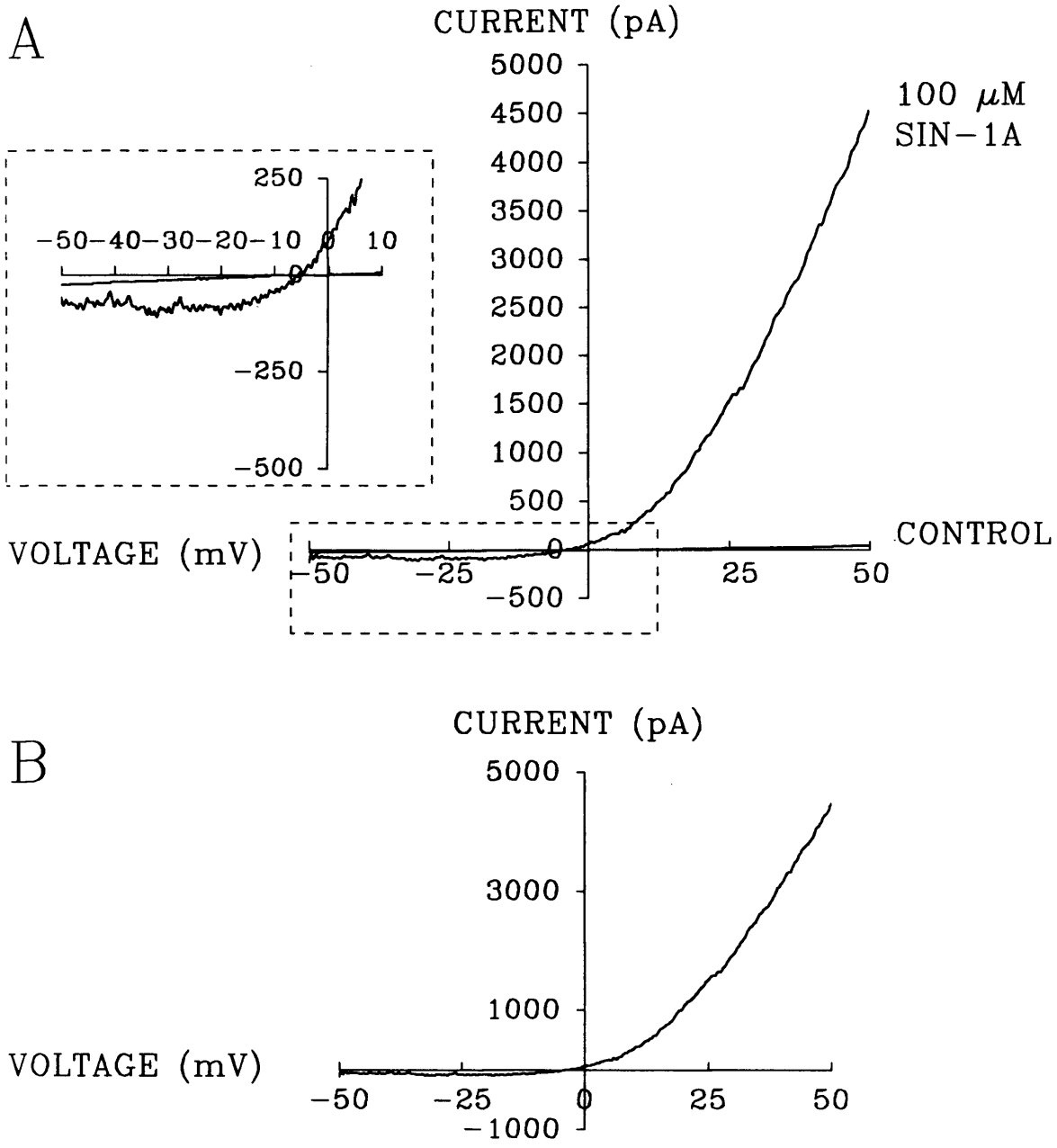


Figure 5.iv. Whole cell and difference currents activated by voltage ramps from a rat basilar smooth muscle cell. Figure 5.iv A shows whole cell currents activated by 50 ms voltage ramps run from -50 mV to 50 mV at the times illustrated in figure 5.iii B. Each ramp is the average from a minimum of 20 individual ramps The expanded section highlights the increased current at the negative potentials as well as at more depolarised potentials and shows that the SIN-1A activated current reversed close to 0 mV, the E_K in this experiment. Figure 5.iv B illustrates the difference between the SIN-1A-activated current and the control current and reflects the increase in current above the control level produced following the addition of SIN-1A.

In the example illustrated in panel A the inward current reached an amplitude of ~1200 pA before the addition of SIN-1A was halted with the current then clearly falling to ~400 pA after washing for ~three minutes, a level still many times higher than the level of activity recorded before SIN-1A was added. In the example illustrated in panel B an inward current of ~100 pA developed after exposure to SIN-1A for ~two minutes. In this example the vertical arrows indicate times when the recording was paused and voltage ramps were used to activate whole cell currents, which are illustrated in figure 5.iv A. When the recording was resumed the current did not decline after washing for approximately six minutes with 140 mM K^+ but continued to slowly increase, reaching ~250 pA. In similar experiments performed on four other cells a reduction in the SIN-1A-activated current of 30-60 % was observed with prolonged washing whereas two cells showed little or no reduction. What was evident was the effects of SIN-1A on whole cell currents were slow to reverse at room temperature, an observation which was not entirely unexpected as previous patch-clamp studies of the activation of K^+ channels by authentic NO and NO donors in rat pulmonary artery and bovine aortic smooth muscle cells reported that the NO-induced current required prolonged (<10 minutes) washing at room temperature to partially reverse the activation (Archer *et al.*, 1994; Williams *et al.*, 1989). The mean inward current measured when an approximately steady-state had been reached following the addition of SIN-1A was 700 ± 450 pA (n=10), and in all cases the currents developed within five minutes.

Figure 5.iv A shows the whole cell currents activated by 50 ms voltage ramps from -50 to 50 mV from the cell illustrated in figure 5.iii B. Series resistance and capacitance compensation were used when required. A minimum of 20 individual ramps were run, and the resulting currents were averaged, producing a mean whole cell current. Clearly inward and outward currents were increased at all membrane potentials with respect to control currents following the addition of 100 μM SIN-1A, especially at more positive potentials. An inward current of ~100 pA for example was produced at -50 mV, whereas ~4500 pA of outward current was activated at +50 mV. This provided strong evidence that the channel being activated following the addition of SIN-1A exhibited voltage-dependence or was an outwardly rectifying channel.

Figure 5.iv B illustrates the difference between the current activated following the addition of SIN-1A and the control current, which highlights the increase in current above controls following the addition of SIN-1A.

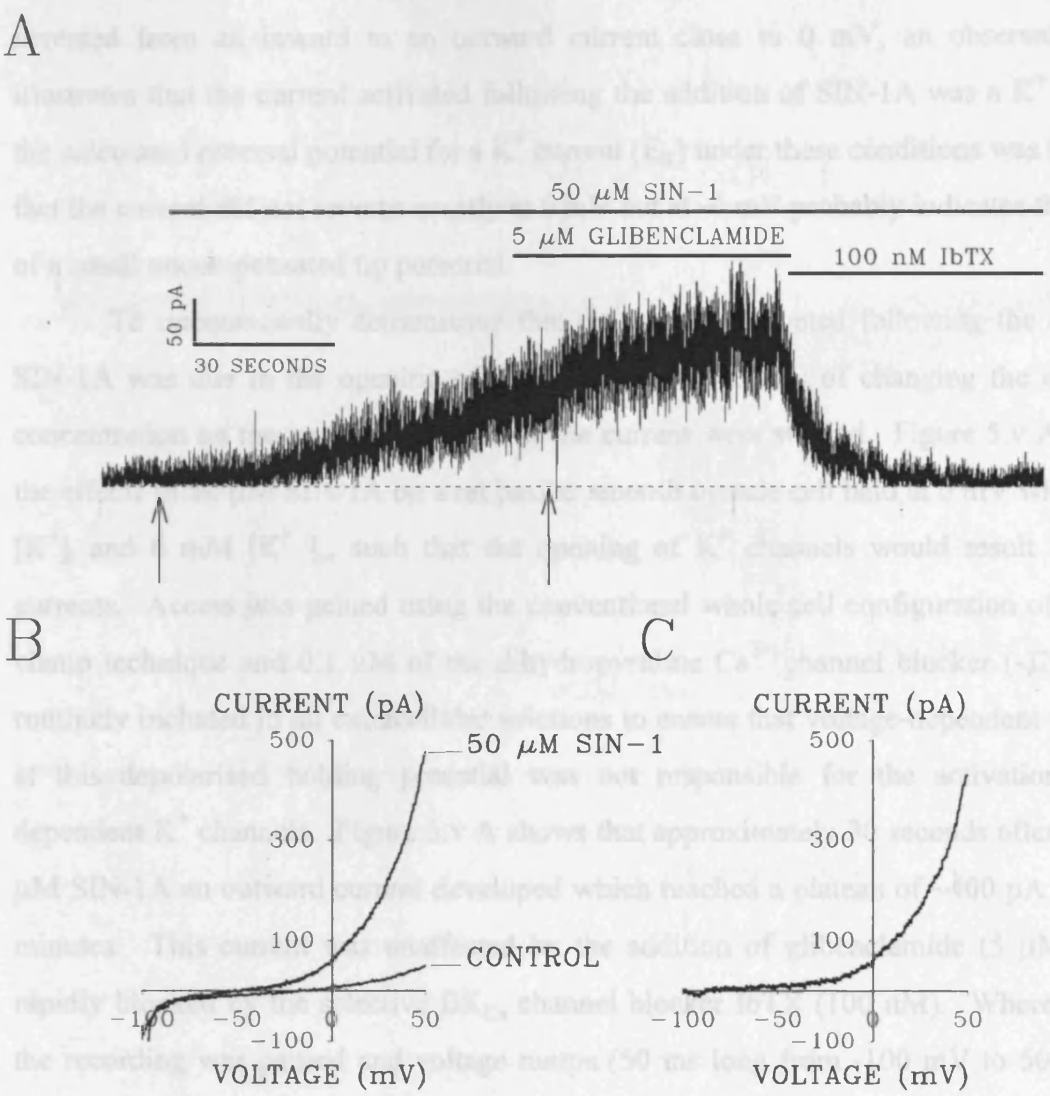


Figure 5.v. Activation of whole cell current from a rat basilar artery smooth muscle cell in a physiological K^+ gradient. Figure 5.v A shows the activation of a whole cell current following the addition of 50 μM SIN-1A to a rat basilar artery myocyte. Access was gained using the conventional whole cell configuration of the patch-clamp technique. The cell was bathed throughout in a solution containing 6 mM K^+ and 50 units ml^{-1} of superoxide dismutase. Drugs were added where indicated and the arrows indicate points where the recording was paused and voltage-ramps were run to activate whole cell currents. Figures 5.v B illustrates the whole cell currents activated by the voltage ramps 50 ms in length from -100 mV to 50 mV. The whole cell current was increased from 50 pA at 50 mV under control conditions to approximately 500 pA at the same potential following the activation of a whole cell current by 50 μM SIN-1A. Figure 5.v C shows that the SIN-1A-activated current reversed close to E_K, -79.5 mV under these conditions, and exhibited voltage-dependence.

This difference current also shows that the direction of the current activated by SIN-1A reversed from an inward to an outward current close to 0 mV, an observation which illustrates that the current activated following the addition of SIN-1A was a K^+ current, as the calculated reversal potential for a K^+ current (E_K) under these conditions was 0 mV. The fact the current did not reverse exactly at 0 mV but at -4 mV probably indicates the presence of a small uncompensated tip potential.

To unequivocally demonstrate that the current activated following the addition of SIN-1A was due to the opening of K^+ channels, the effects of changing the external K^+ concentration on the reversal potential of the current were studied. Figure 5.v A illustrates the effects of 50 μM SIN-1A on a rat basilar smooth muscle cell held at 0 mV with 140 mM $[K^+]_i$ and 6 mM $[K^+]_o$, such that the opening of K^+ channels would result in outward currents. Access was gained using the conventional whole cell configuration of the patch-clamp technique and 0.1 μM of the dihydropyridine Ca^{2+} channel blocker (-)202791 was routinely included in all extracellular solutions to ensure that voltage-dependent Ca^{2+} influx at this depolarised holding potential was not responsible for the activation of Ca^{2+}-dependent K^+ channels. Figure 5.v A shows that approximately 30 seconds after adding 50 μM SIN-1A an outward current developed which reached a plateau of ~400 pA after ~four minutes. This current was unaffected by the addition of glibenclamide (5 μM) but was rapidly blocked by the selective BK_{Ca} channel blocker IbTX (100 nM). Where illustrated the recording was paused and voltage ramps (50 ms long from -100 mV to 50 mV) were used to activate whole cell currents. Figure 5.v B illustrates the resulting average whole cell current, which clearly show that currents activated following the addition of 50 μM SIN-1A were increased at all potentials with respect to control currents, especially at positive membrane potentials. Figure 5.v C illustrates the difference between the SIN-1A activated current and control currents. It is fairly clear from the difference current that the outward current is being activated relatively close to the calculated reversal potential (~-80 mV under these conditions), confirming that following the addition of SIN-1A a K^+ conductance developed.

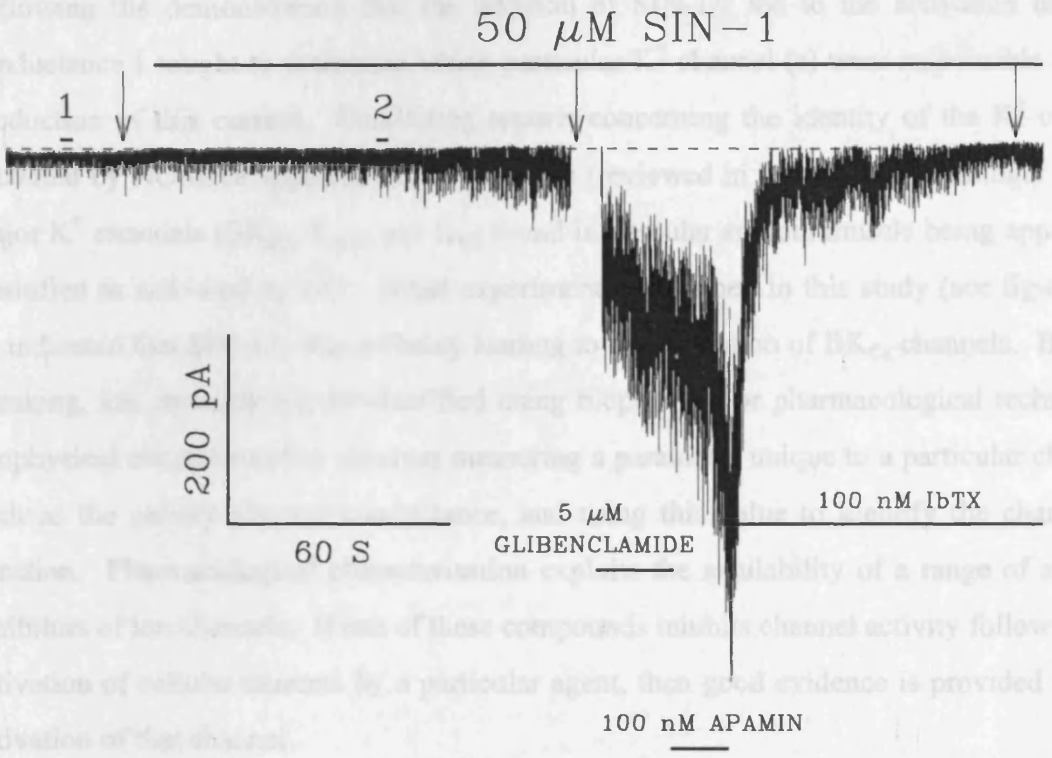


Figure 5.vi. Selective activation of BK_{Ca} channels underlay the whole cell current activated following the addition of SIN-1A. Access was gained using the conventional whole-cell configuration of the patch-clamp technique and the cell was held at -40 mV in symmetrical 140 mM K^+ and 50 units ml^{-1} of super oxide dismutase. The record illustrated was filtered at 1.0 kHz and sampled at 5.0 kHz and the continuous dotted line indicates the zero current level. Following the addition of 50 μM SIN-1A and the activation of an inward current, glibenclamide, apamin and IbTX were added where illustrated at the concentrations shown. IbTX selectively inhibited the SIN-1A-activated current. The arrows indicate the times where the recording was paused and voltage ramps were run to activate whole cell currents.

5.2.2 Effects of selective potassium channel inhibitors on the whole cell current activated by SIN-1A

Following the demonstration that the addition of SIN-1A led to the activation of a K^+ conductance I sought to determine which particular K^+ channel (s) were responsible for the production of this current. Conflicting reports concerning the identity of the K^+ channel activated by NO have appeared in the literature (reviewed in 5.1.3), with seemingly all the major K^+ channels (BK_{Ca}, K_{ATP} and K_V) found in vascular smooth muscle being apparently identified as activated by NO. Initial experiments performed in this study (see figure 5.iii B) indicated that SIN-1A was probably leading to the activation of BK_{Ca} channels. Broadly speaking, ion channels can be classified using biophysical or pharmacological techniques. Biophysical characterisation involves measuring a parameter unique to a particular channel, such as the unitary channel conductance, and using this value to identify the channel in question. Pharmacological characterisation exploits the availability of a range of specific inhibitors of ion channels. If one of these compounds inhibits channel activity following the activation of cellular currents by a particular agent, then good evidence is provided for the activation of that channel.

The channel activated following the addition of SIN-1A was identified as a K^+ channel on the basis of shifts in E_K with changes in the external K^+ concentration. To further identify which particular K^+ channel was being activated by SIN-1A the following K^+ channel blockers were used. Glibenclamide, a relatively specific inhibitor of K_{ATP} channels, apamin, a selective inhibitor of SK_{Ca} channels and iberiotoxin (IbTX) or charybdotoxin (CTX) specific inhibitors of BK_{Ca} channels. The effects of these agents on the current activated by SIN-1A are shown in figure 5. vi. This figure illustrates a recording made from a cell held at -40 mV using the conventional whole-cell configuration of the patch-clamp technique. The cell was bathed in symmetrical 140 mM K^+, such that the opening of K^+ channels would produce inward currents. Prior to the addition of SIN-1A the recording was paused where illustrated and voltage ramps (-50 to 50 mV) were used to activate whole cell currents. These data were then averaged as described previously and the resulting control whole cell currents are illustrated in figure 5.vii. SIN-1A (50 μM) was then added to the superfusing solution which led to the development of an inward current over approximately a two minute period.

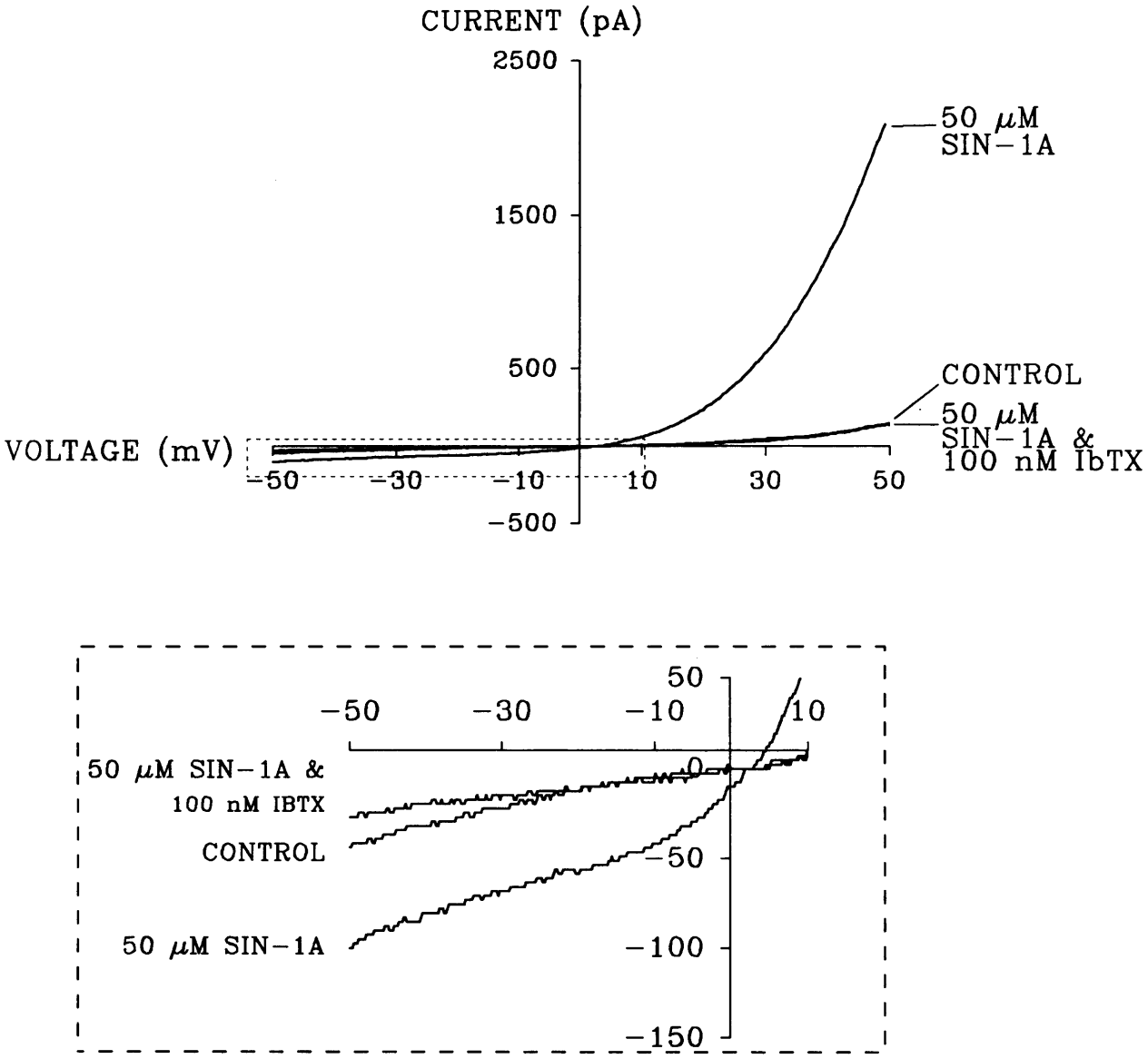


Figure 5.vii. Demonstration that the voltage-dependent whole cell current produced following the addition of SIN-1A was due to the activation of BK_{Ca} channel. The currents are the averaged data from a minimum of 20 individual ramps which were run at the points illustrated in figure 5. vi. Following the addition of 50 μM SIN-1A the whole cell currents were increased at all potentials relative to controls and reversed close to 0 mV, the E_K for a K^+ channel under these conditions. The increase in current was reduced below control levels following the addition of IbTX.

Voltage ramps (-50 to 50 mV) were used where illustrated (note the recording was not paused during this experiment due to an oversight on the part of the investigator, consequently this section was simply deleted for aesthetic reasons), and the average whole cell currents activated by the voltage ramps are illustrated in figure 5.vii. Inward and outward currents were increased at all membrane potentials in the presence of 50 μM SIN-1A with respect to the control currents, especially at the more positive ramp potentials and the current reversed at +3 mV, close to the calculated E_K (0 mV). When the recording was resumed the level of inward current continued to increase even when glibenclamide (5 μM) and apamin (100 nM), were included in the superfusing solution. IbTX (100nM) however, rapidly (<30 seconds) inhibited the SIN-1A-activated inward current. The rapid block by IbTX dramatically contrasts with the slow reversal over a period of minutes of the current following the removal of SIN-1A and prolonged washing (see figures 5.iii A and B). The demonstration that IbTX selectively inhibited the SIN-1A-activated current was confirmed in a further six cells, an effect also illustrated in figures 5.iii B and 5.v A.

5.2.3 Whole cell currents activated by ramps are increased by SIN-1A and inhibited by IbTX

When voltage ramps were used to activate whole cell currents, it was obvious that the channel underlying the current activated by SIN-1A was either voltage-dependent or was an outwardly rectifying channel. However, IbTX blocked the effects of SIN-1A thus confirming that the channel activated by SIN-1A was the BK_{Ca} channel, which is a voltage-dependent channel whose P_{open} increases with depolarisation (Benham *et al.*, 1986 and also see chapter 6 figure 6.iii). Figure 5.vii illustrates the voltage-dependent nature of the channel activated following the addition of SIN-1A. At -50 mV for example, ~100 pA of inward current was produced, an effect which is perhaps more clearly illustrated by the expanded section in this figure, whereas at +50 mV (where an identical driving force for K^+ existed) the current activated was ~2000 pA, a 20 fold increase. Data from four similar experiments are summarised in the histograms in figures 5.viii A and B which illustrate the mean currents (n=4) measured at -50 and +50 mV under control conditions and after the addition of SIN-1A (50-100 μM) and SIN-1A (50-100 μM) and 100 nM IbTX, with significance ($p<0.05$ Student's paired t-test) illustrated by *. Clearly mean whole cell currents measured at either -50 and 50 mV following the addition of SIN-1A were significantly increased with respect to controls.

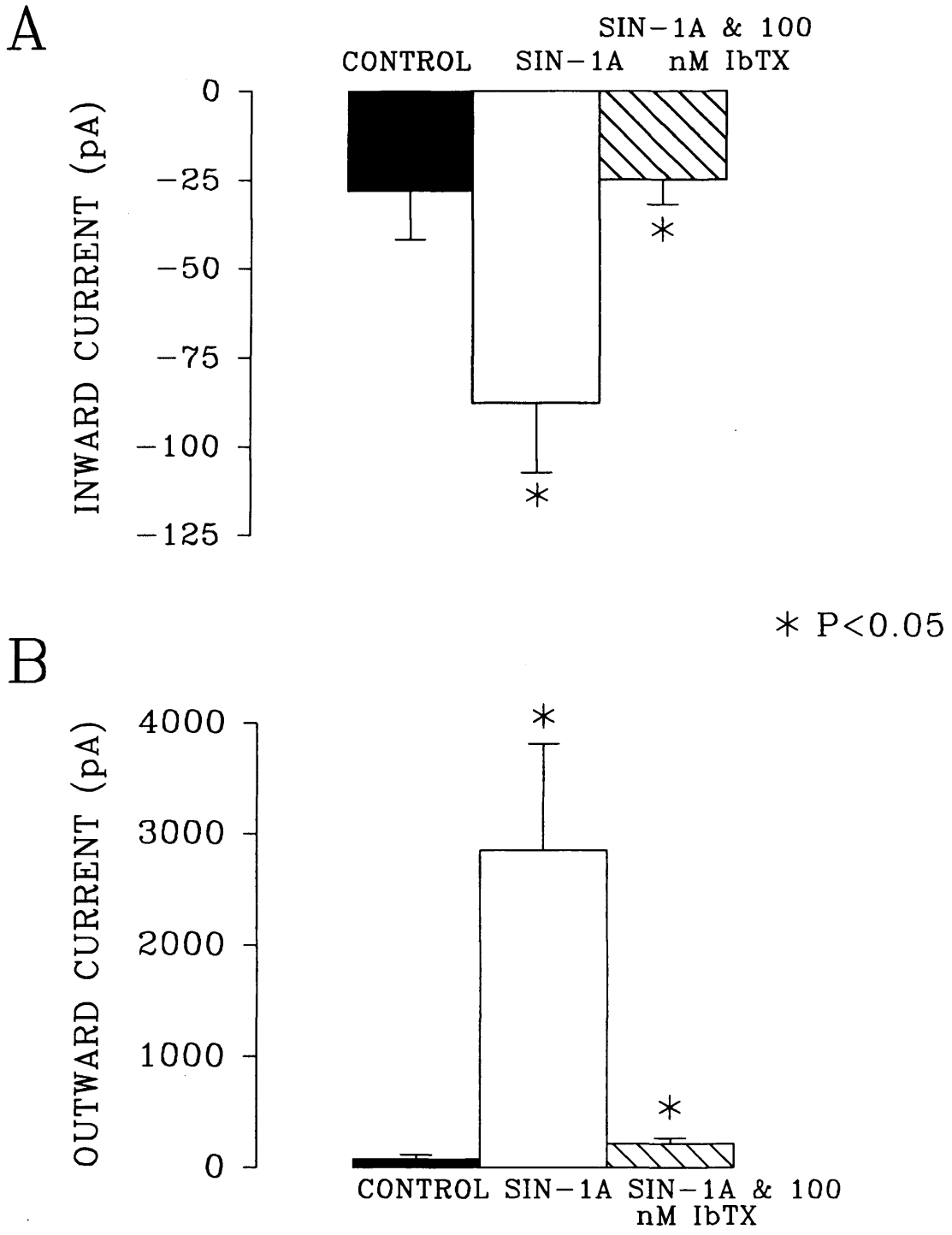


Figure 5.viii. Histograms summarising the effects of SIN-1A and SIN-1A and IbTX on whole cell currents activated by voltage ramps. Panel A shows the mean current activated at -50 mV (n=4). Addition of SIN-1A significantly increased the whole cell current at this potential while IbTX significantly reduced the SIN-1A-activated current. Panel B shows the effects at 50 mV again showing the significant increase in current following the addition of SIN-1A, which again was significantly reduced by IbTX. By comparing the 2 histograms it is clear the channel being activated by SIN-1A was exhibiting a considerable degree of voltage-dependence.

The mean inward control current at -50 mV, for example, was 28 ± 13.9 pA (n=4), increasing to 87.5 ± 19.8 pA (n=4), approximately a 3-fold increase. However at +50 mV the mean control outward current was 80.3 ± 34.4 pA (n=4) whereas the current activated following the addition of SIN-1A was 2849.2 ± 963.9 pA (n=4), an approximately 36-fold increase with respect to the control current. The increases in current at both membrane potentials were blocked by the addition of 100 nM IbTX. These experiments provided additional evidence that the channel activated by SIN-1A was the BK_{Ca} channel as the channels were both voltage-dependent and were blocked by IbTX, a specific blocker of BK_{Ca} channels.

5.2.4 Activation of a large-conductance channel following the addition of SIN-1A

Obviously pharmacological methods provided sufficient evidence to characterise the channels opened following the addition of SIN-1A as BK_{Ca} channels. However, these channels could also be characterised by measuring the unitary conductance of the channel and comparing this values to previously reports and also to data illustrated in chapter 6. The unitary conductance can be specific for particular channels and is therefore used to identify particular channels. In the early part of some recordings, before large levels of channel activation occurred, I was able to measure the unitary current amplitude of the channels being increasingly opened following the addition of SIN-1A by producing histograms of channel open and closed states. This process is possible because of the high input resistance of arterial smooth muscle cells, the large unitary current amplitude of BK_{Ca} channels and low noise recordings made in the laboratory. Figures 5.ix A and B illustrate consecutive 50 ms sections of recording expanded from the sections highlighted in figure 5. vi, with the continuous dotted lines indicating channel open and closed levels. Figure 5 .ix A illustrates recordings prior to the addition of 50 μM SIN-1A and show the low basal level of channel activity. The only event which occurred with any regularity was the opening of a channel with a unitary current amplitude of ~10 pA. This relatively low level of channel activity sharply contrasts with the recordings illustrated in figure 5.ix B which show increasing opening of channels with large unitary current amplitudes. The current amplitude of this channel, measured from the histogram illustrated in figure 7C, was 10.74 ± 1.5 pA at -40 mV corresponding to a unitary conductance of 268.5 pS in symmetrical 140 mM K^+. The mean unitary conductance from six different cells in symmetrical 140 mM K^+ was 257 pS.

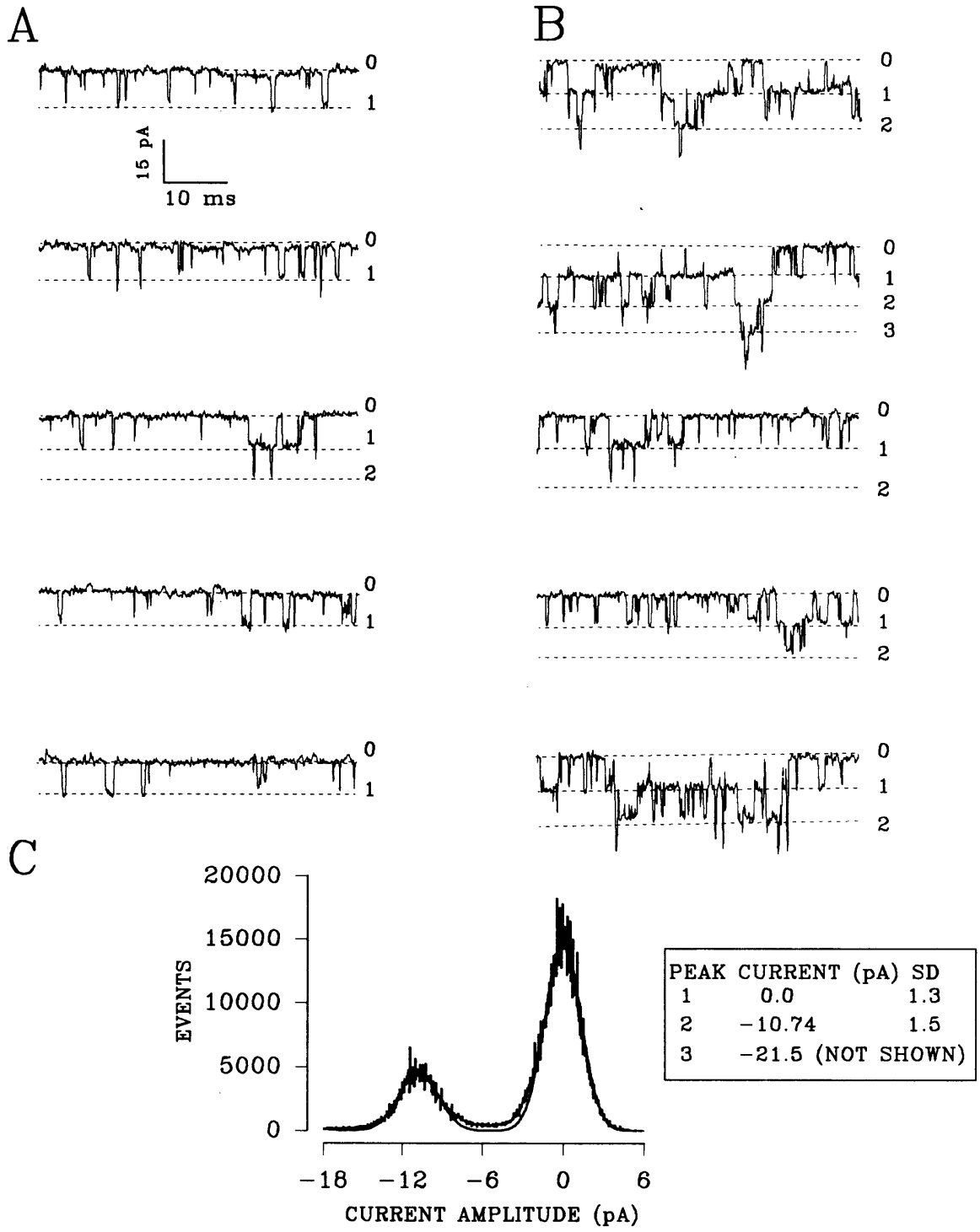


Figure 5.ix. Activation of large-conductance channels by SIN-1A. These records are expanded from the sections marked 1 and 2 in figure 5.vi. Panel A shows 50 ms sections before SIN-1A was added while panel B shows sections taken after 50 μM SIN-1A was added. Clearly the activity of a channel with a large unitary current amplitude was increased following the addition of SIN-1A. Panel C illustrates the amplitude histogram of the channel activated by SIN-1A.

These measurements of the unitary current amplitude provided further proof that SIN-1A was leading to the opening of BK_{Ca} channel, as previous studies of BK_{Ca} channels in smooth muscle have reported a similar unitary conductance (Benham *et al.*, 1986; Taniguchi et al., 1992). In summary, the channel activated following the addition of SIN-1A was identified as the BK_{Ca} channel on the basis of its voltage-dependence, sensitivity to IbTX and large unitary channel conductance.

5.2.5 Activation of a steady state charybdotoxin-sensitive current by authentic NO

Having activated whole cell K^+ currents with the NO donor SIN-1A, I sought to try and reproduce this effect using authentic NO solution, as it was possible that additional breakdown products of SIN-1A with reactivities not shared by NO could be responsible for activating the whole cell currents described previously. The effects of authentic NO were also assessed using cells clamped at membrane potentials equating to the physiological RMP of arterial smooth muscle cells. Figure 5. x illustrates the effects of authentic NO on a single smooth muscle cell held at -60 mV in symmetrical 140 mM K^+ solution. Before infusing NO into the recording chamber the basal level of channel activity was low. Again this was demonstrated by the small whole cell currents activated by voltage ramps (-50 mV to 50 mV) illustrated in figure 5.xi A and the expanded sections of recording shown in figure 5.xii A, which are taken from the section highlighted in figure 5.x. 2 ml of saturated (~1.0 mM) solution of NO, dissolved in degassed (see methods) 140 mM K^+ solution, was slowly infused into the bath over a period of ~two minutes using a gas-tight syringe and gas-impermeable tubing (the volume of the bath measured at the end of the experiment was 7 ml). During the infusion of NO the frequency of opening of a channel with a large unitary current amplitude appeared to increase. Where indicated the recording was paused and voltage ramps (-50 to 50 mV) were again used to activate whole cell currents. 5 μM of the K_{ATP} channel inhibitor glibenclamide was then superfused onto to the cell, using the pressure superfusion system described in the chapter 2, it was then replaced, having had no effect, with 100 nM charybdotoxin (CTX), a relatively selective inhibitor of BK_{Ca} channels (Miller *et al.*, 1985; Gimenez-Gallego *et al.*, 1988), which rapidly and reversibly blocked the developing current. The recording was paused again and voltage ramps were used to activate whole cell currents. CTX was then removed and the inward current slowly returned over the course of ~three minutes.

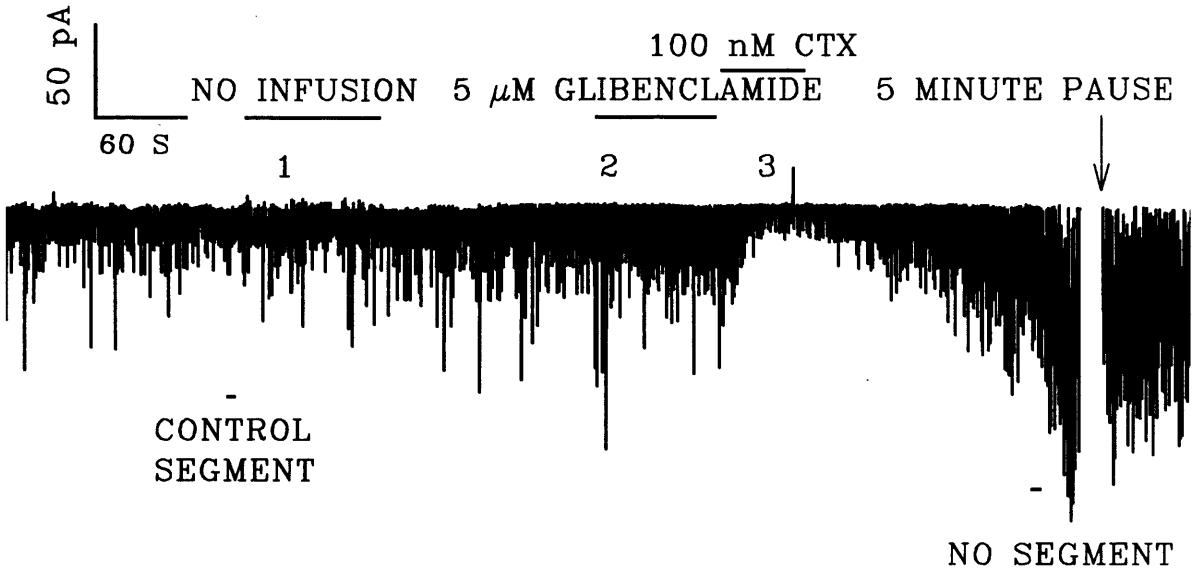
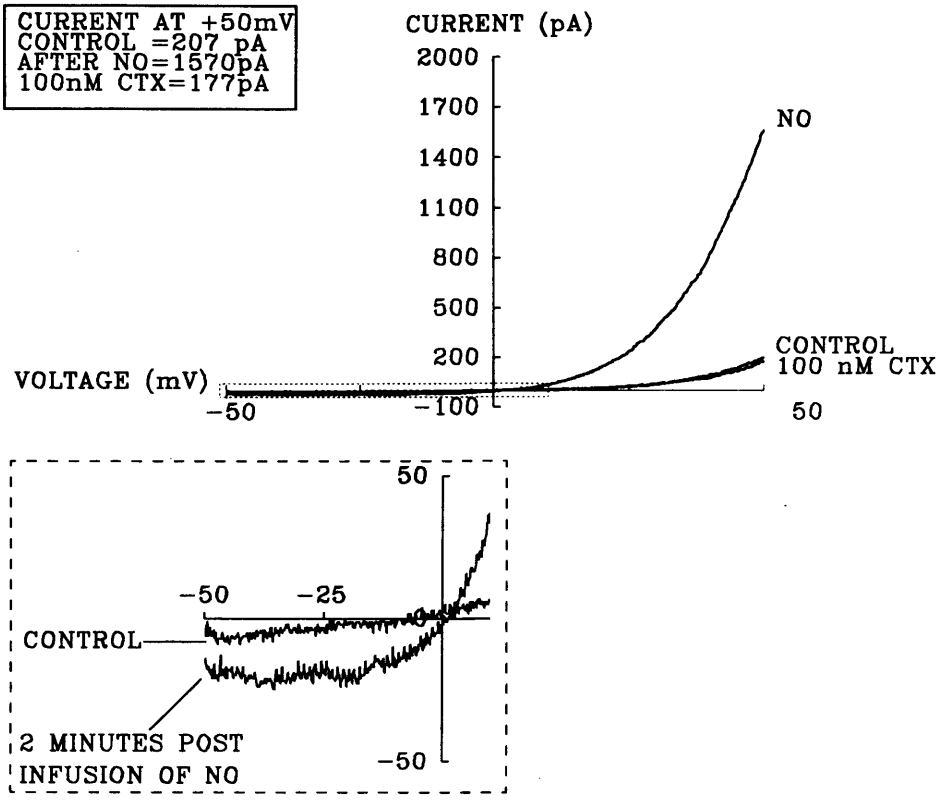


Figure 5.x. Activation of a CTX-sensitive current by authentic NO. Access was gained using the conventional whole cell configuration of the patch-clamp technique. The cell was held at -60 mV and bathed in 140 mM K^+. The record illustrated was filtered at 1.0 kHz and sampled at 5.0 kHz. At the time shown a saturated solution of NO was infused into the recording chamber and led to the development of an inward current. The amplitude of this current was unaffected by glibenclamide but was reversibly reduced by CTX. Prolonged washing had little effect on the NO-induced current. At the times illustrated the recording was paused and voltage ramps were run to activate whole cell currents.

A



B

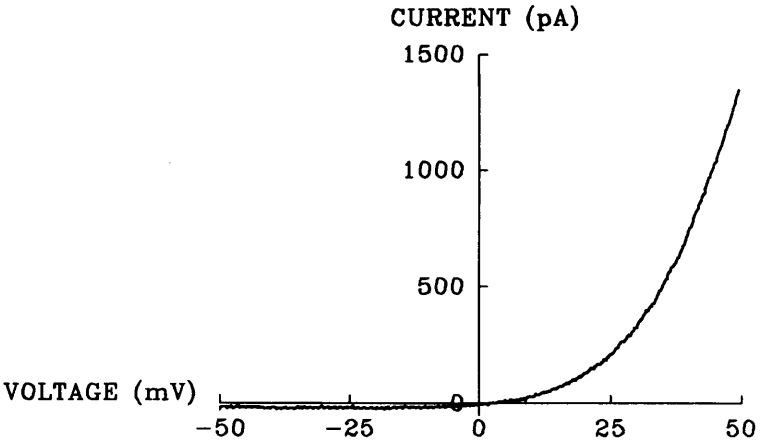


Figure 5.xi. Voltage-dependent whole cell currents were increased following the addition of NO. Panel A illustrates the whole currents activated following voltage ramps. These ramps were run at the points illustrated in figure 5 x and illustrate that NO increased whole cell currents at all potentials with respect to controls, especially at positive potentials. The addition of CTX reduced the NO-induced current to control levels. Panel B illustrates the difference current which shows that the NO-induced current reversed at 0 mV, the E_K for a K^+ current under these conditions.

The effects of NO, like those of SIN-1A, were slow to reverse at room temperature as illustrated by the elevated current level after washing with 140 mM K^+ solution for ~ten minutes.

5.2.6 Whole cell currents activated by voltage ramps were increased after the infusion of NO

Figure 5.xi A illustrates the mean whole cell currents activated by voltage ramps before the infusion of NO, which were <10 pA and 207 pA at -50 and 50 mV respectively. Following the activation of a whole cell current by NO the currents activated at -50 mV and 50 mV were increased to -25 pA and to 1570 pA respectively, increases of 2.5 and 7.6 times with respect to control values. The whole cell currents activated by NO, like the currents previously activated by SIN-1A were therefore voltage-dependent with a greater degree of channel activation occurring at depolarised potentials. Figure 5.xi B illustrates the difference current, which is simply the increase in current above the control level following the addition of NO, which reversed close to 0 mV, as expected for a K^+ current under these conditions. After the addition of the BK_{Ca} channel blocker, CTX, the current activated following the ramp was reduced to 177 pA at +50 mV. The currents activated by NO, like the currents activated by SIN-1A, exhibited profound voltage-dependence and were blocked by a BK_{Ca} blocker, suggesting that these currents also resulted from the activation of BK_{Ca} channels by NO.

5.2.7 The channel activated by NO had a large unitary conductance and single channel amplitude

The unitary conductance of the channels activated after the infusion of NO was also measured to confirm their identity. Figure 5.xii A illustrates consecutive 1500 ms section records expanded from figure 5.x and highlights the low level of activity of a channel with a unitary conductance of 252 pS. Following the addition of NO and the subsequent activation of the whole cell current illustrated in figure 5.x, the openings of this channel became more frequent, as illustrated by the consecutive sections shown in figure 5.xii B. The unitary conductance of these channels was 249.5 pS, indicating that NO was opening BK_{Ca} channels, as these unitary conductance values in symmetrical 140 mM K^+ are typical of smooth muscle BK_{Ca} channels (Benham *et al.*, 1986).

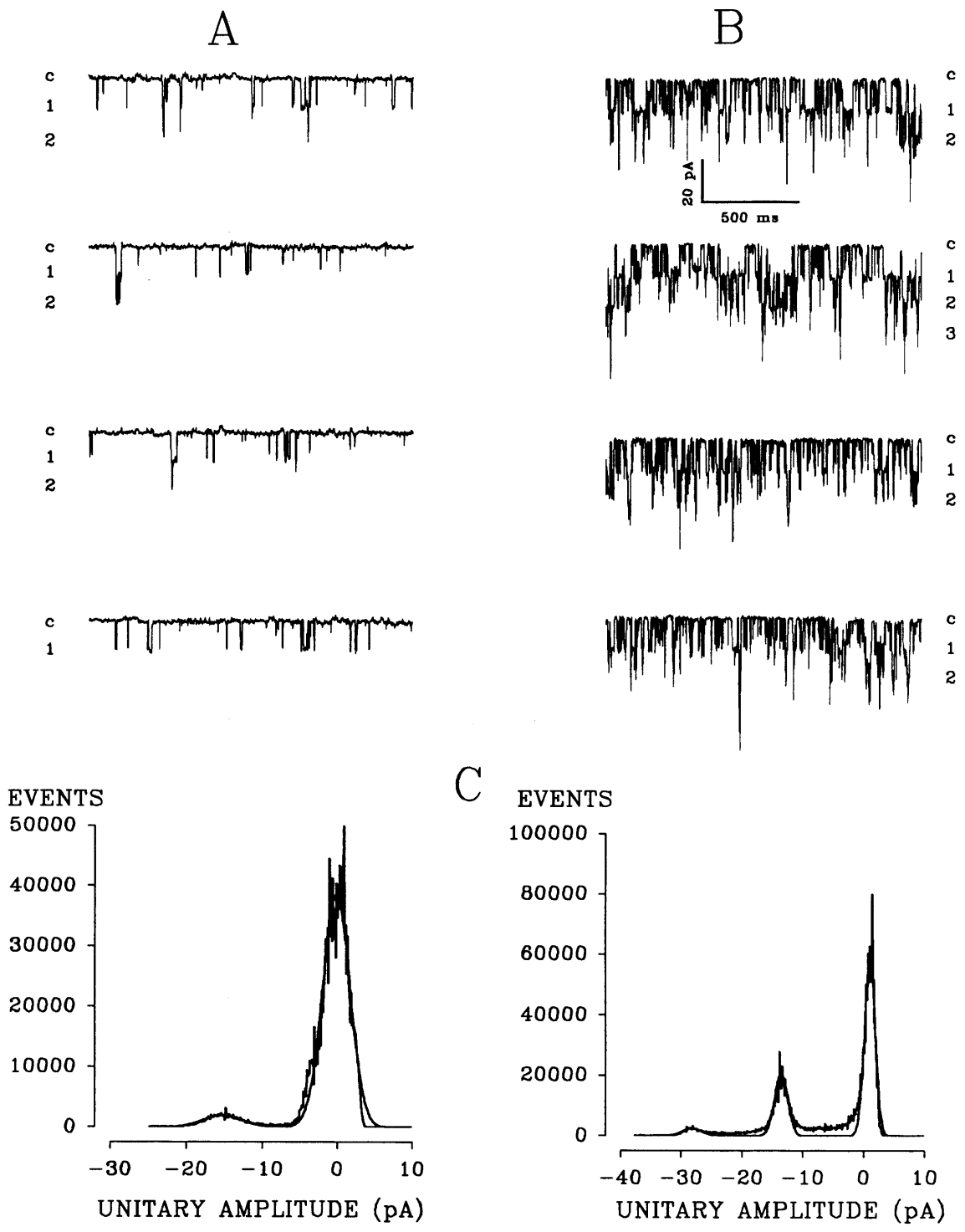


Figure 5.xii. Activation of large-conductance channels by authentic NO. These records are expanded from the sections marked in figure 5.x. Panel A shows 50 ms sections before NO was added, while panel B shows sections taken following the infusion of NO into the recording chamber. Clearly the activity of a channel with a large unitary current amplitude was increased following the addition of NO. Panel C illustrates histograms of the large amplitude channel before and after the addition of NO. It is clear that NO did not affect the unitary current amplitude of the channel

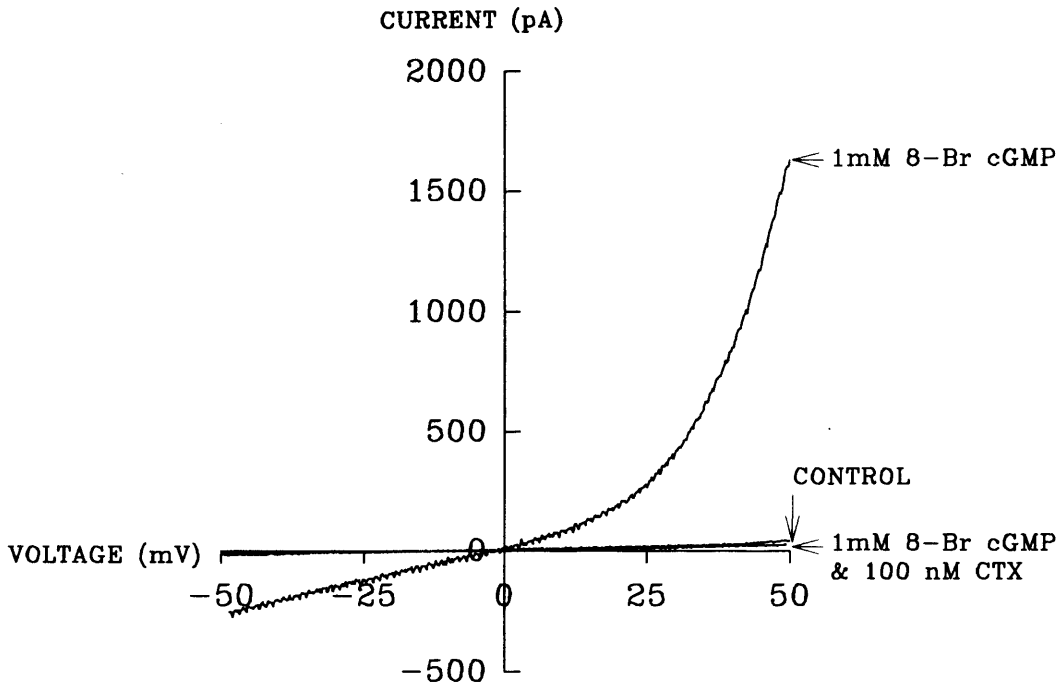


Figure 5.xiii. Activation of a CTX-sensitive current by a membrane-permeable activator of G-kinase. This figure illustrates whole cell currents activated by voltage ramps from a rat basilar myocyte. Access was gained in this example using the conventional whole cell configuration of the patch-clamp technique and the cell was held at 0 mV and bathed in 140 mM K^+. The lower trace shows the averaged currents activated under control conditions. 1 mM 8-bromo cyclic GMP was then perfused onto the cell and the upper trace shows the currents activated in the presence of this compound. Clearly the currents were increased with respect to controls at all potentials and the current reversed at 0 mV, E_K under these conditions. 100 nM CTX was then perfused onto the cell which reduced the current activated in the presence of 8-bromo cyclic GMP to control levels.

5.2.8 Whole cell currents were increased following the addition of a membrane-permeant cGMP analogue

Having activated whole cell currents using both a NO donor and authentic NO and further identified that the activation of BK_{Ca} channels was responsible for the development of these currents in these cells, the question remained as to how are the effects of NO being transduced? Were these effects due to a direct effect of NO on the channel or was NO leading to the activation of G-kinase which then produced channel opening presumably via a mechanism involving a phosphorylation reaction. To try and answer this important question I assessed the effects on whole cell currents of a membrane-permeant activator of G-kinase, 8-bromo cyclic GMP. Using this compound ensured that any increase in the level of channel activity occurred was due to activation of G-kinase rather than direct channel activation by NO. Figure 5.xiii illustrates the effects of 1 mM 8-bromo cyclic GMP on whole cell currents activated by voltage ramps (-50 mV to 50 mV). Access was gained using the conventional whole cell configuration of the patch-clamp technique and the currents illustrated were averaged from a minimum of 20 individual ramps. The control currents illustrate the low level of channel activity in an unstimulated cell, however following the addition of 8-bromo cyclic GMP and the development of a steady state whole cell current (data not shown), the currents activated were increased at all potentials relative to control. These currents reversed at 0 mV, E_K under these conditions, and were voltage-dependent with larger currents activated as the membrane potential was depolarised. The third ramp shows the reduced level of current when 100 nM CTX was added in addition to 8-bromo cyclic GMP. The voltage-dependence and the sensitivity of the current to CTX confirmed that opening of BK_{Ca} channels occurred following the addition of a membrane permeant activator of G-kinase.

5.2.9 Approximately 40 % of cells studied responded to SIN-1A

Out of a total of 39 cells 17 cells (44%) responded to SIN-1A, with the activation of current within five minutes. Three cells out of a total of seven responded to authentic NO solutions and one cell out of six responded to 8-bromo cyclic GMP. Control cells, which were simply cells where access was achieved using the conventional whole cell configuration but no NO or SIN-1A was added to the superfusing solution, never developed the currents seen in response to NO or SIN-1A (n=4).

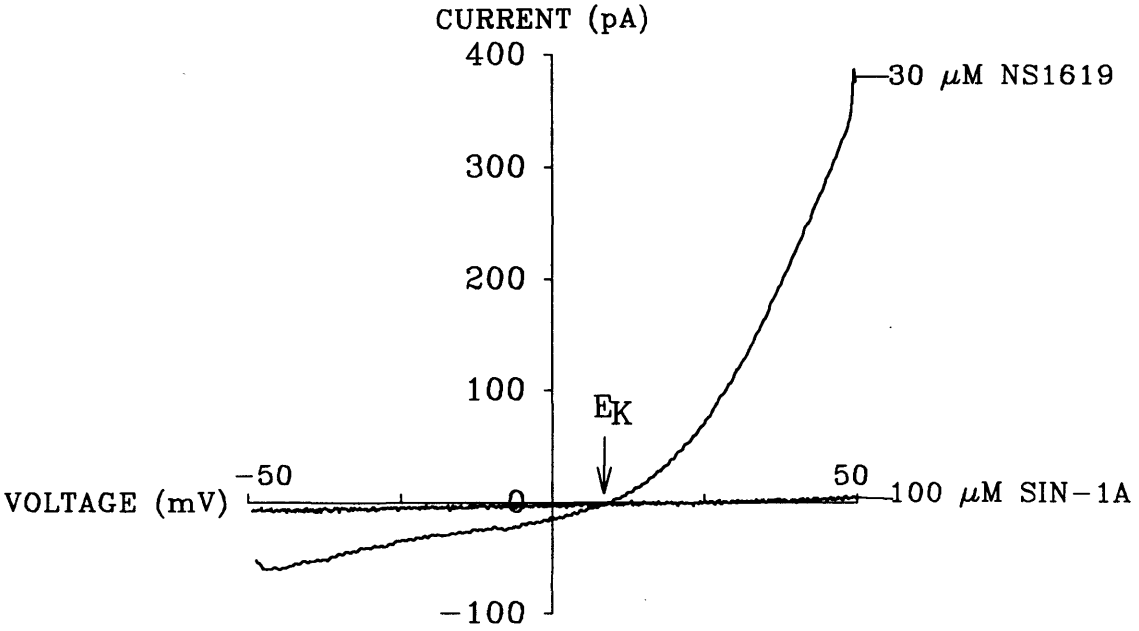


Figure 5.xiv. Whole cell currents illustrating the presence of BK_{Ca} channels in a cell that did not respond to SIN-1A. Whole cell currents were activated using voltage ramps run from -50 mV to 50 mV from cells held at 0 mV in symmetrical 140 mM K^+. Access was gained using the conventional whole cell configuration of the patch-clamp technique. The lower ramp shows the whole cell currents activated following the addition of 100 μM SIN-1A to the cell for >10 minutes. While the upper ramp shows currents from the same cell activated following the addition of 30 μM of the BK_{Ca} channel activator NS1619. The current activated by NS1619 showed voltage-dependence and reversed close to E_K, 0 mV under these conditions.

The cells which developed currents in response to NO were classified as 'responders' and the remainder 'non-responders'. To ensure that the non-responders actually contained BK_{Ca} channels, the BK_{Ca} channel activator, NS1619, was used as a positive control. As described and illustrated in chapter 3, NS1619 directly activates BK_{Ca} channels in number of tissues (Olesen *et al.*, 1994a; Sellers & Ashford, 1994) including rat basilar arterial smooth muscle cells (Holland *et al.*, 1996 and also see chapter 3). Figure 5.xiv, lower trace, illustrates average whole cell currents activated by voltage ramps (-50 to 50 mV) from an unresponsive cell. Clearly when compared to whole cell currents recorded from responsive cells (see figures 5.iii A, 5.v A, 5.vi and 5. ix) no currents were activated. The addition of 30 μM NS1619 led to the activation, after a characteristic delay, of an inward current (data not shown). The upper ramp illustrates the average whole cell currents activated by 30 μM NS1619, which showed voltage-dependence and reversed at 8 mV, close to the E_K (0 mV in this experiment) and which were blocked by 100 nM CTX (data not shown). The unitary conductance of the NS1619-activated channel in symmetrical 140 mM K^+ was ~260 pS, a value similar to previous measurements of the conductance of BK_{Ca} channels described earlier in this chapter (see also chapter 6) and by previous studies of BK_{Ca} channels in vascular smooth muscle cells (Benham *et al.*, 1986). These experiments confirmed that BK_{Ca} channels were present in smooth muscle cells that did not respond to SIN-1A and NO.

5.3 DISCUSSION

5.3.1 The nitric oxide-induced current resulted from the activation of BK_{Ca} channels

Application of both the NO donor, SIN-1A, and authentic NO to single rat basilar artery smooth muscle cells led to the development of whole cell currents. The channel responsible for these currents was identified as a K^+ channel, on the basis of the current reversal potential shifting with E_K. These channels were further identified as BK_{Ca} channels because of their voltage-dependence, their large (~250 pS) unitary conductance in symmetrical 140 mM K^+ and their sensitivity to IbTX and CTX.

These observations demonstrated that the addition of NO leads to the activation of BK_{Ca} channels in rat basilar artery smooth muscle cells and are in agreement with previous electrophysiological studies where authentic NO and SIN-1A activated BK_{Ca} channels in smooth muscle cells isolated from rat pulmonary arteries (Archer *et al.*, 1994), rabbit aorta (Bolotina *et al.*, 1994) and rabbit cerebral arteries (Robertson *et al.*, 1993). What the present study further reports is that the addition of SIN-1A or NO resulted in the activation of BK_{Ca} channels at negative potentials, which more accurately represent the RMP of these cells, as well as at more depolarised potentials. The observation that NO could activate BK_{Ca} channels at negative, physiologically-relevant membrane potentials is of pivotal importance, as it illustrates that NO may hyperpolarise arterial smooth muscle via the opening of BK_{Ca} channels.

Of the many *in vitro* electrophysiological studies where it has been that NO hyperpolarises smooth muscle (Tare *et al.*, 1990; McPherson & Stork 1992; Rand & Garland 1992; Garland & McPherson 1992; Parkington *et al.*, 1993; Bolotina *et al.*, 1994; Vanheel *et al.*, 1994; Krippeit-Drews *et al.*, 1994; Parkington *et al.*, 1995; Murphy & Brayden 1995) some studies further reported that the NO-induced hyperpolarisation was reversed by glibenclamide, an inhibitor of K_{ATP} channels (Rand & Garland 1992; Garland & McPherson 1992; Murphy & Brayden 1995; Parkington *et al.*, 1995), thus raising the possibility that K_{ATP} channel activation underlay the NO-induced hyperpolarisation in some blood vessels. The results of the present study however indicate that NO activates BK_{Ca} channels in rat cerebral smooth muscle cells. I do not feel however that these results necessarily contradict these many previous studies but may simply be explained by well documented differences in the ion channel populations in different arterial beds of different species. K_{ATP} channels for example, have previously been reported to be present at a very low density or indeed may be absent in the rat cerebral circulation, as demonstrated by the poor relaxant properties of the K_{ATP} channel openers, cromakalim and pinacidil, at concentrations that fully relaxed mesenteric arteries (McCarron *et al.*, 1991; McPherson & Stork, 1992). A conclusion supported by my own studies using rat basilar arterial myocytes and the K_{ATP} channel openers levcromakalim and pinacidil (Holland unpublished observations). The study by McPherson & Stork (1992) also reported that SNP hyperpolarised rat cerebral arteries, including basilar arteries by a mechanism that did not involve K_{ATP} channels, as demonstrated by the lack of effect of the K_{ATP} channel inhibitor, glibenclamide, on this effect.

In the study performed by McPherson & Stork (1992), K_{ATP} channel openers such as cromakalim (30 μM) produced only a modest increase in membrane potential (2±1 mV) and did not relax rat anterior cerebral arteries with established myogenic tone. SNP however consistently produced larger glibenclamide-resistant membrane hyperpolarisations (14±5 mV) and relaxed vessels with myogenic tone. The activation of BK_{Ca} channels by NO reported by the present study could therefore explain the glibenclamide-resistant hyperpolarisation produced by SNP in the rat cerebral circulation, a conclusion supported by Grant McPherson (McPherson, personal communication).

5.3.2 How does NO lead to BK_{Ca} channel activation in rat basilar smooth muscle cells?

Where BK_{Ca} channel activation has been reported to produce hyperpolarisation of vascular smooth muscle two mechanisms have been proposed to explain this effect:

1. NO activates GC-S located in the cytoplasm of the smooth muscle cell, which leads to the opening of BK_{Ca} channels via the activation of G-kinase and a subsequent phosphorylation of either the BK_{Ca} channel or a closely associated regulatory site group. Support for this mechanism was provided by the demonstration that G-kinase Iα catalytic subunits, the G-kinase I isoform currently thought to be the isoform of G-kinase which produces vasorelaxation (Francis & Corbin, 1994), activated BK_{Ca} channels when applied to the cytoplasmic surface of inside-out membrane patches from dog coronary, rabbit cerebral arteries and tracheal smooth muscle (Taniguichi *et al.*, 1992; Robertson *et al.*, 1993; Zhou *et al.*, 1996).

2. More controversially one study has claimed that NO directly activates BK_{Ca} channels as a result of nitrosylation of structural -SH groups (Bolotina *et al.*, 1994).

This latter mechanism was proposed following the demonstration that authentic NO produced activation of BK_{Ca} channels in excised membrane patches in the presence of methylene blue, a non-selective inhibitor of GC-S (Bolotina *et al.*, 1994).

Which of the above mechanisms best describes the results obtained in this study? The second mechanism (direct activation) probably does not account for the results presented here.

The main reason for this assertion is the observation that ~40% of cells tested responded to SIN-1A or NO, a value similar to a previous study which reported that ~30% of rat pulmonary smooth muscle cells responded to NO with the development of whole cell CTX-sensitive currents (Archer *et al.*, 1994). If NO did directly activate BK_{Ca} channels like NS1619, which I found to activate whole cell currents in close to 100 % of cells studied, surely a higher proportion of cells would have responded to NO with the activation of a current. Additional evidence was produced when I assessed the effects of SIN-1A and NO on excised inside-out membrane patches where I found no direct activation of BK_{Ca} channels in isolated patches (see chapter 6). These results directly contrast with those published by Bolotina *et al.*, (1994), where direct activation of BK_{Ca} by NO was reported. Also in the present study the application of a membrane permeant activator of G-kinase led to the development of a whole cell current which resembled the currents produced following the addition of NO and SIN-1A. Therefore in summary:

1. In smooth muscle cells isolated from the rat basilar artery and rat pulmonary artery a similar percentage of cells responded to NO with the production of a K^+ current, which in both cases was found to be due to the activation of BK_{Ca} channels.

2. No evidence was found in the present study to imply that NO directly activated BK_{Ca} channels perhaps indicating that the activation of G-kinase via NO-induced increases in the concentration of intracellular cGMP was a more likely explanation of the results presented in this study.

3. Preliminary experiments using a membrane permeant activator of G-kinase appeared to supported the idea that the G-kinase pathway was involved in the activation of BK_{Ca} channels.

This leads to the questions why did only approximately half of all the cells studied respond to NO and would the activation of BK_{Ca} currents in 40 % of cells produce vasorelaxation via membrane hyperpolarisation?

A recent study claimed that morphological differences existed in isolated rat pulmonary artery smooth muscle cells which explained apparent electrophysiological differences in the cell population (Archer *et al.*, 1996).

Stephen Archer and his co-workers proposed that pulmonary artery smooth muscle cells could be broadly divided into three distinct groups on the basis of apparent morphological, electrophysiological and pharmacological differences. These groups were:

1. K_{DR} cells which expressed predominantly K_V channels and did not respond to NO

2. K_{Ca} cells which expressed predominately BK_{Ca} channels and responded to NO with the development of whole cell currents, due to the opening of BK_{Ca} channels via the cyclic GMP signal transduction pathway.

3. 'Mixed cells' which expressed both K_V and BK_{Ca} channels and which also responded to NO with the activation of a whole cell current due to BK_{Ca} channel opening.

If similar morphological and electrophysiological differences existed in basilar arterial smooth muscle cells they could explain why 40 % of cells responded to NO. Even to my untrained eye, once dissociated basilar arterial smooth muscle cells displayed a considerable degree of structural variation, possibly representing a heterogeneous, rather than a homogeneous population. The morphological differences however could simply result from the dissociation process, which obviously subjects the artery to a cocktail of enzymes (see chapter 2) and a more detailed study by a trained microscopist would be required to determine if real morphological differences did exist. If consistent morphological differences could be identified and related to electrophysiological differences then some form of classification would be useful. However, I found no evidence to indicate that any obvious differential channel distribution existed which would allow these cells to be classified in a similar way to pulmonary artery smooth muscle cells. Whenever I recorded K_V or BK_{Ca} channels, for example, all the cells studied contained both these channels, which made the use of channel blockers essential when studying the individual channels in isolation (Holland, unpublished observations). I therefore found no evidence to classify rat basilar arterial smooth muscle cells according to the categories proposed by Stephen Archer and his colleagues and therefore no evidence to use this classification to explain why 40 % of cells responded to SIN-1A.

An alternative explanation as to why 40 % of cells studied responded to SIN-1A could be that only those smooth muscle cells lining the lumen of the artery and therefore in close proximity to the endogenous source of NO, i.e. the endothelium, responded to NO. Although clearly a hypothesis, this could plausibly explain why this percentage of cells responded to NO. Alternatively the necessary biochemical elements required to produce BK_{Ca} channel activation (GC-S and G-kinase) may not survive the cell isolation procedure whereas the BK_{Ca} channels, which have been shown by many studies to be very robust, fair much better.

The additional observation that NO produced prolonged activation of BK_{Ca} channels, reported in the present study and in previous reports (Williams *et al.*, 1988; Archer *et al.*, 1994), may potentially explain the smaller, extended hyperpolarisation produced by nitrovasodilators and acetylcholine previously described in some functional studies (McPherson & Stork, 1992; Vanheel *et al.*, 1994; Krippeit-Drews *et al.*, 1994). The latter two studies reported that following the addition of acetylcholine or nitrovasodilators, washing for up to 10 minutes produced only a partial recovery of the NO- or acetylcholine-induced hyperpolarisation, with the membrane still between 6-8 mV more hyperpolarised than before pre-addition levels.

Vascular smooth muscle is *single unit* smooth muscle (see section 1.5.1) and acts as a *single functional unit* because of the gap junctions which electrically and chemically link the individual cells. If the membrane potential of one cell becomes hyperpolarised, this hyperpolarisation should 'spread' to surrounding cells electrically linked to this cell. Activation of 40 % of the cells in a syncitium may explain the hyperpolarisation of vascular smooth muscle due to the opening of BK_{Ca} channels by NO, acetylcholine and nitrovasodilators reported previously (Rand & Garland, 1992; Garland & McPherson 1992; Taniguchi *et al.*, 1992; Bolotina *et al.*, 1994; Vanheel *et al.*, 1994; Krippeit-Drews *et al.*, 1994; Simonson *et al.*, 1995; Banks *et al.*, 1996; Chen & Rembold, 1996; Plane *et al.*, 1996; Wellman *et al.*, 1996).

In conclusion, following the addition of SIN-1A or NO whole cell currents developed in approximately 40 % of cells. These currents were due to the opening of BK_{Ca} channels, most probably due to the activation of G-kinase by NO, rather than a direct effect of NO on the BK_{Ca} channel. This effect occurred at holding potentials equating to the RMP of vascular smooth muscle and may help partially explain the results from a number of previous functional studies where NO and nitrovasodilators have been reported to produce hyperpolarisation by opening BK_{Ca} channels.

No evidence was found for classifying dissociated rat basilar artery smooth muscle cells on the basis of a previously proposed classification used to distinguish between rat pulmonary artery smooth muscle cells.

CHAPTER SIX

Effects of NO and cGMP-dependent protein kinase G catalytic subunits on BK_{Ca} channel activity

6.1 INTRODUCTION

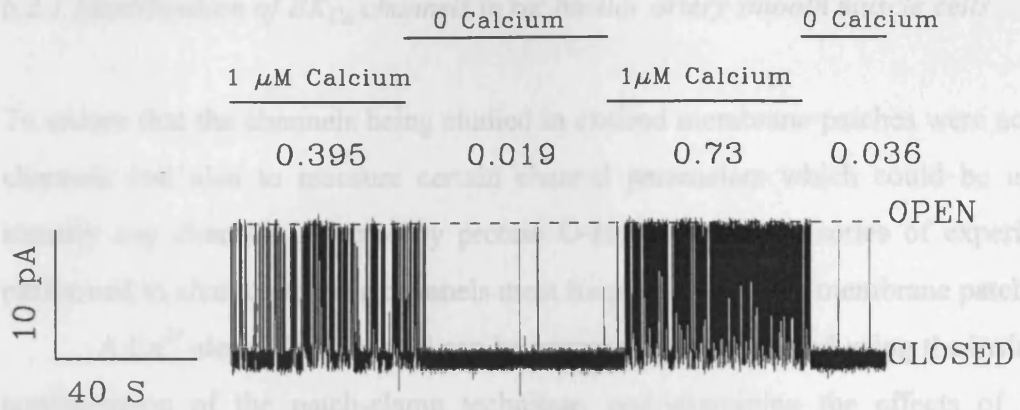
Results presented in chapter 5 illustrated that a K^+ conductance developed following the addition of NO to single rat basilar smooth muscle cells. This K^+ current was due to the opening of BK_{Ca} channels, therefore posing the question how was this effect transduced? The following mechanisms have previously been proposed to account for this activation of BK_{Ca} channels by NO and the G-kinase signalling pathway in smooth muscle cells:

1. NO activates soluble guanylyl cyclase (GC-S) located within smooth muscle cells, resulting in an increase in the level of intracellular cGMP. This increase in cGMP activates cGMP-dependent protein kinase (G-kinase), which, presumably via a reaction involving phosphorylation of the BK_{Ca} channel or a closely associated site, leads to an increase in the P_{open} of the BK_{Ca} channel. This increase in channel P_{open} underlies the subsequent development of a K^+ current (Taniguchi *et al.*, 1992; Robertson *et al.*, 1993).

2. NO is a free radical and is therefore highly reactive and combines with sulphhydryl (-SH) groups within the BK_{Ca} channel. Nitrosylation of -SH groups in the channel or associated regulatory subunits chemically modifies the BK_{Ca} channel, leading to an increase in the channel P_{open} independently of the activation of GC-S and G-kinase (Bolotina *et al.*, 1994).

Initial experiments suggested that the currents produced following the addition of NO to single smooth muscle cells in the present study involved G-kinase. The basis of this assumption was that following the addition of a membrane permeant activator of G-kinase, 8 bromo-cGMP, CTX-sensitive currents developed, a result similar to a previous study using a pituitary tumour cell line (White *et al.*, 1993). Also initial experiments performed using an inhibitor of G-kinase, KT5823, suggested that KT5823 reversed SIN-1A-induced whole cell currents (Holland unpublished observations). To unequivocally determine which one, if any, of the above mechanisms could account for the activation of a whole cell current in rat basilar artery myocytes, inside-out membrane patches were used. This configuration was chosen so the effects of activated protein kinase G catalytic subunits and NO when applied directly to the cytoplasmic surface of the membrane could be examined.

A



B

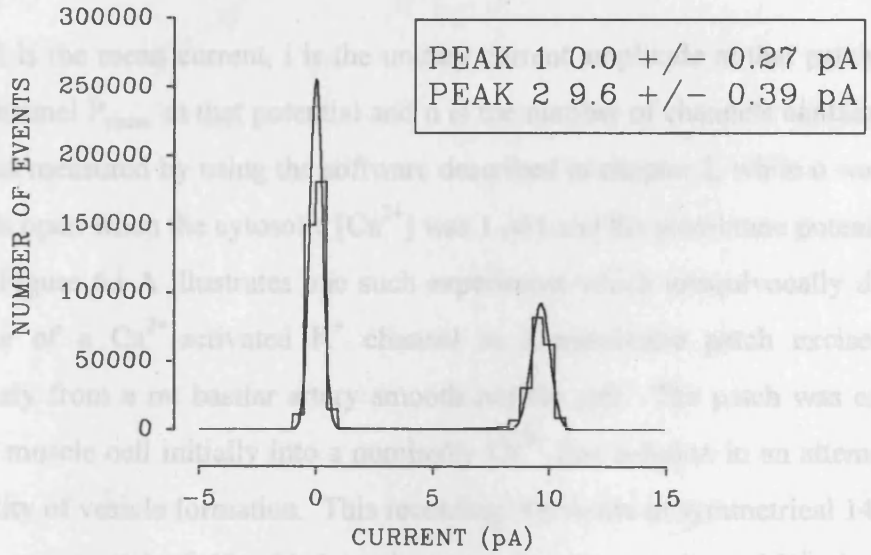


Figure 6.i. Identification of a Ca^{2+}-activated channel in an excised *inside-out* membrane patch from a rat basilar artery smooth muscle cell. Figure 6.i A illustrates a recording made at 40 mV in symmetrical 140 mM K^+. Channel openings are upward deflections with the continuous dotted line indicating the channel open level. This record was filtered at 1 kHz and sampled at 5 kHz. Where indicated the $[Ca^{2+}]$ at the cytoplasmic surface of the patch was either 1 μM or nominally zero. The value below the bar indicates the channel P_{open} measured following exposure of the patch to that particular concentration of Ca^{2+}. Figure 6.i B illustrates a histogram of the open and closed events with 1 μM free-Ca^{2+} at the cytoplasmic surface of the patch allowing a measurement of the unitary current amplitude of the channel to be made.

6.2 RESULTS

6.2.1 Identification of BK_{Ca} channels in rat basilar artery smooth muscle cells

To ensure that the channels being studied in excised membrane patches were actually BK_{Ca} channels and also to measure certain channel parameters which could be used to help identify any channels affected by protein G-kinase or NO, a series of experiments were performed to characterise the channels most frequently found in membrane patches.

A Ca^{2+}-dependent channel can be unequivocally identified using the inside-out patch configuration of the patch-clamp technique, and examining the effects of varying the cytoplasmic Ca^{2+} concentration on the P_{open} of the channels contained in the membrane patch using equation 12.

$$\mathbf{I=iP_{open}\,n} \qquad [12]$$

Where I is the mean current, i is the unitary current amplitude at that patch potential, P_{open} is the channel P_{open} at that potential and n is the number of channels contained in the patch. P_{open} was measured by using the software described in chapter 2, while n was the number of channels open when the cytosolic [Ca^{2+}] was 1 μM and the membrane potential was 50 mV.

Figure 6.i A illustrates one such experiment which unequivocally demonstrates the presence of a Ca^{2+}-activated K^+ channel in a membrane patch excised as described previously from a rat basilar artery smooth muscle cell. The patch was excised from the smooth muscle cell initially into a nominally Ca^{2+}-free solution in an attempt to reduce the possibility of vesicle formation. This recording was made in symmetrical 140 mM K^+ using a holding potential of 40 mV throughout, so that the opening of K^+ channels would be visualised as upward deflections. When the cytosolic solution was nominally free from Ca^{2+}, the P_{open} of a channel with large unitary amplitude was the only identifiable event, although the opening of this channel occurred infrequently (P_{open} 0.01). Increasing the free Ca^{2+} concentration from nominally zero to 1 μM produced rapid activation of the channel with the large unitary amplitude. With its P_{open} increasing to 0.395 when measured over a period of approximately 40 seconds. The Ca^{2+} concentration at the cytosolic surface was then reduced to nominally Ca^{2+}-free, which produced a dramatic, rapid reduction in the activity of this channel, which fell to 0.02 when measured over ~40 seconds.

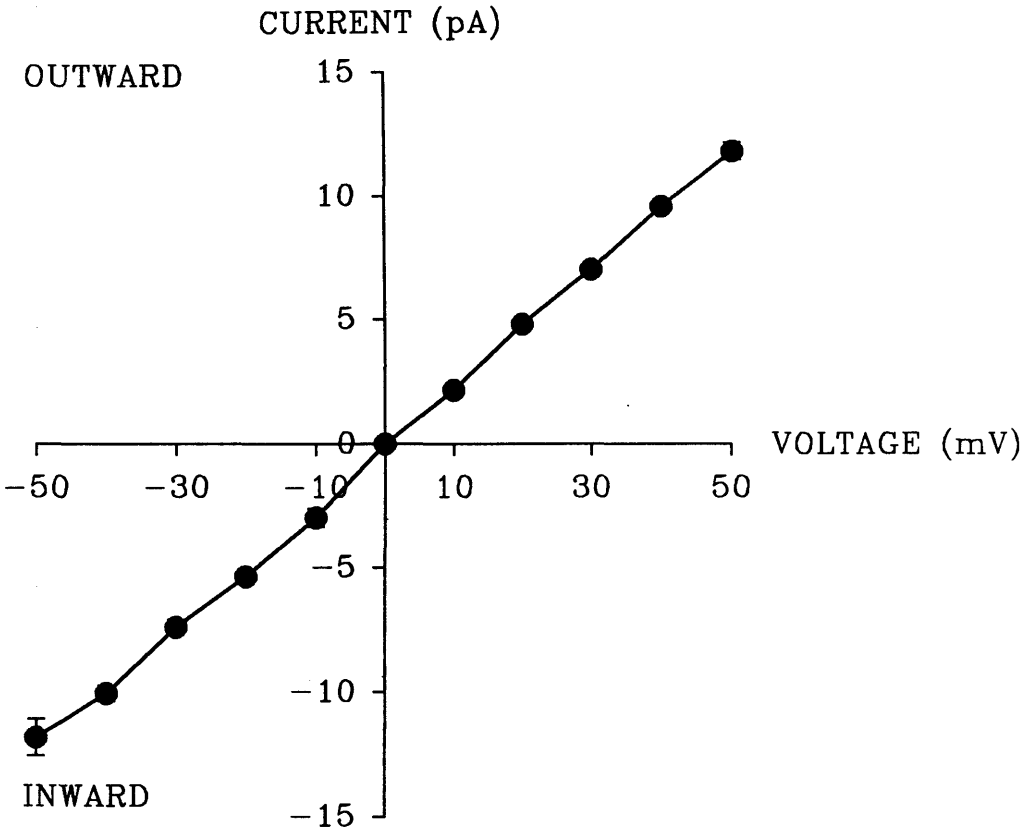


Figure 6.ii. Single channel current / voltage relationship for the Ca^{2+}-dependent channel. This figure illustrates I / V relationship of single Ca^{2+}-dependent channel currents measured in symmetrical 140 mM K^{+}. The data points represent means ± S.E. of 4 inside-out patches exposed to a solution containing 1 μM free-Ca^{2+}. The channel slope conductance calculated between 0 and +50 mV was 236 pS.

When the free Ca^{2+} concentration was increased to 1 μM the level of channel activity again rapidly increased (P_{open} 0.73 over approximately 40 seconds). Experiments performed on three separate patches yielded similar results, confirming that the channel contained in the patch was a Ca^{2+}-dependent channel. These channels were further identified as K^+ channels by substituting the cytosolic K^+ for Na^+ with the Cl^- concentration constant (140 mM). This modification abolished current flowing through the patches (data not shown). Figure 6.i B illustrates a histogram of open and closed events produced using the data illustrated in figure 6.i A. From this histogram the unitary current amplitude and conductance of the Ca^{2+}-dependent K^+ channel were measured, parameters which can help to identify unknown channels. The unitary current amplitude of the Ca^{2+}-dependent K^+ channel in the experiment illustrated was 9.6 ± 0.4 pA at 40 mV, which corresponds to a single channel conductance of 240 pS in symmetrical 140 mM K^+ using Ohms law (see Equation 10). This process was then repeated so that the unitary current amplitude and P_{open} could be measured at 30, 20, 10.....-50 mV, with the resulting data summarised in the I-V relationship shown in figure 6.ii. These experiments provided additional information concerning the identity of the channel by identifying its reversal potential and also examining the effects of membrane depolarisation on its P_{open}. Figure 6.ii clearly illustrates that the direction of current flow reversed at, or close to, 0 mV, confirming the channel was a K^+ channel. This I-V relationship is also linear indicating that this Ca^{2+}-dependent K^+ channel did not rectify, or if it did rectify in an intact cell whatever was responsible for producing rectification was rapidly lost following patch excision. Having identified a Ca^{2+}-dependent K^+ channel in inside-out membrane patches from a rat basilar artery smooth muscle, the channel required further characterisation, as three classes of Ca^{2+}-activated K^+ channels have previously been described (Latorre *et al.*, 1989; McManus, 1991), each possessing unique biophysical and pharmacological properties.

The mean unitary channel conductance of this Ca^{2+}-dependent K^+ channel in symmetrical 140 mM K^+, measured between 0 and +50 mV from the current-voltage relationship, was 236 pS (n=4). This large single channel conductance characterised this channel as the BK_{Ca} channel, an assessment that was further supported when the effects of voltage on channel P_{open} and current passing through excised inside-out membrane patches were examined (see equation 12). These data, illustrated in figure 6.iii A and B respectively, clearly show that depolarisation increased both the P_{open} of the Ca^{2+}-dependent channel and the amount of current passing through the channel, characteristics of BK_{Ca} channels (Barrett *et al.*, 1982; Benham *et al.*, 1986).

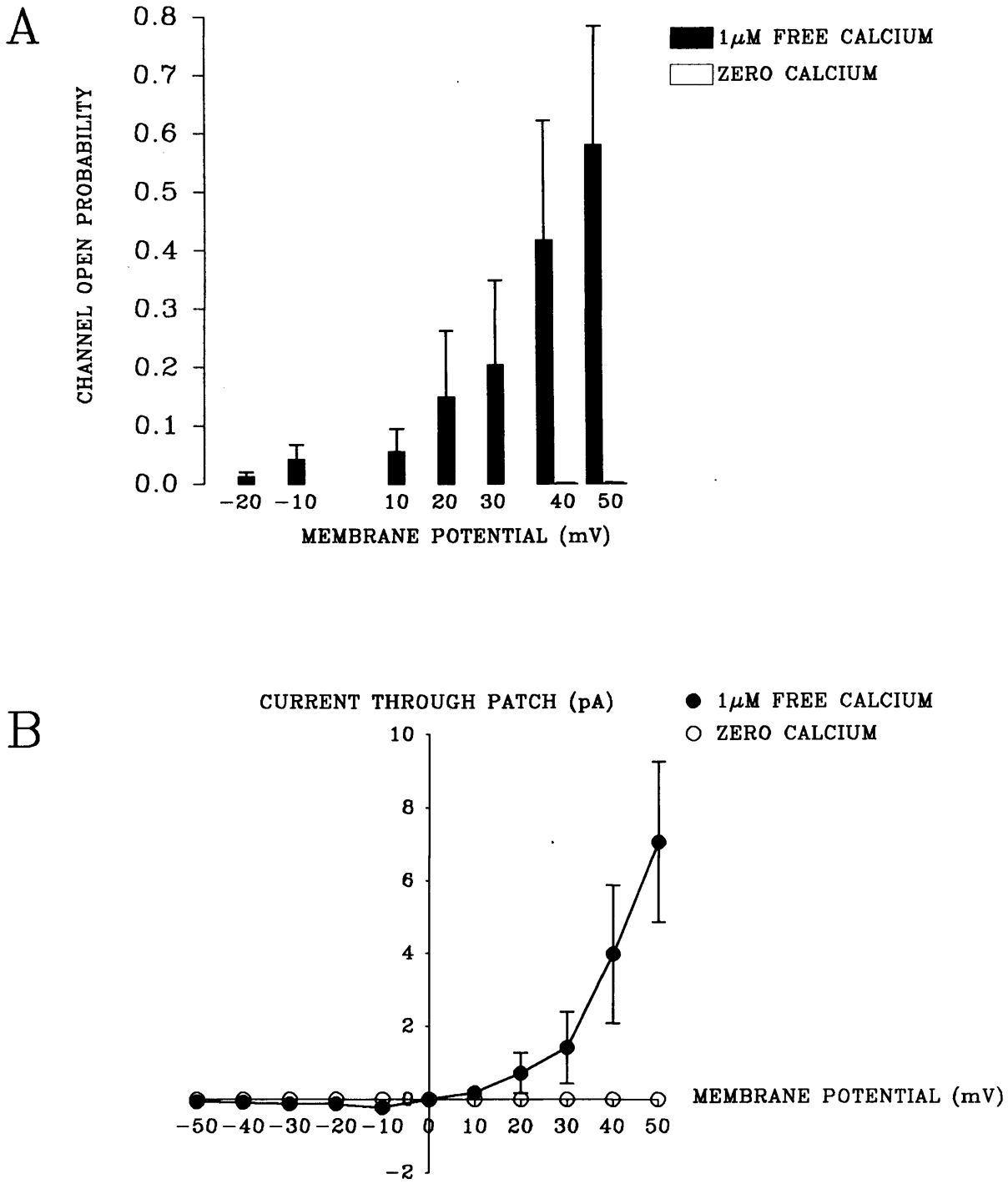


Figure 6.iii. Effects of changes in the cytoplasmic [Ca^{2+}] and voltage on channel P_{open} and the amount of current passing through the patch. Figure 6.iii A illustrates the effects of membrane depolarisation on the open probability of the Ca^{2+}-dependent channel. The histogram represents mean data from 4 inside-out patches exposed to solutions containing 1 μM free-Ca^{2+} and nominally Ca^{2+}-free. Channel P_{open} was measured under each condition for a minimum of 40 seconds. Figure 6.iii B illustrates the effects of different membrane potentials (-50 to 50 mV) on the mean current passing through inside-out patches excised from rat basilar artery smooth muscle cells (n=4). All experiments were conducted in 140 mM symmetrical K^+ and the data illustrated were obtained from patches exposed to solutions that contained 1 μM free-Ca^{2+} or were Ca^{2+}-free.

For example, by comparing the mean P_{open} and current passing through the patch at -20mV and 20 mV, where the electrochemical driving force through the channel was identical, it is clear that both parameters were greater at 20mV. This could not be explained by the channel preferentially passing outward current, as BK_{Ca} channels in these excised patches did not rectify (see figure 6.ii). This increase in P_{open} therefore occurs as a result of the channel being increasingly open as the patch potential is depolarised.

In summary, a channel with the characteristics of the BK_{Ca} channel was identified in excised patches from rat basilar smooth muscle cells. This channel was identified on the basis of its unitary conductance and its Ca^{2+}-and voltage-dependence. No other Ca^{2+}-activated channels were observed in any of the patches studied although sub-states of the BK_{Ca} channel were occasionally observed but were not further investigated. This result was unsurprising as most studies of Ca^{2+}-activated K^+ channels, using excised patches from vascular visceral smooth muscle preparations, failed to report the presence of Ca^{2+}-activated K^+ channels other than BK_{Ca} channels (Benham *et al.*,1986; Langton *et al.*,1991; Taniguchi *et al.*, 1992; Robertson *et al.*, 1993; Holland *et al.*,1996), although a recent report claimed to have identified a 68 pS apamin-sensitive Ca^{2+}-activated K^+ channel in membrane patches excised from renal arteriolar smooth muscle (Gebremedhin *et al.*, 1996).

6.2.2 Effects of ATP, cGMP and protein kinase G on the activity of BK_{Ca} channels

The opening of BK_{Ca} channels in whole cell experiments by both the NO donor, SIN-1A and native NO described in the previous chapter suggested that the activation of G-kinase, via the increase in cGMP, may be involved in the modulation of BK_{Ca} channels. To assess the effects of G-kinase on BK_{Ca} channel activity the inside-out patch configuration was used. One of the advantages of using excised inside-out membrane patches is that the cytoplasmic surface of the membrane is exposed, hence *inside-out*, which allows the effects of membrane impermeant proteins, such as G-kinase, to be investigated by simply perfusing them directly onto this normally inaccessible surface of the membrane, while simultaneously monitoring channel activity. Inside-out patches were excised as described previously and the presence of BK_{Ca} channels was confirmed as detailed in section 6.2.1. Following successful excision the patch was moved away from the bottom of the bath and therefore the smooth muscle cell from which the patch was derived.

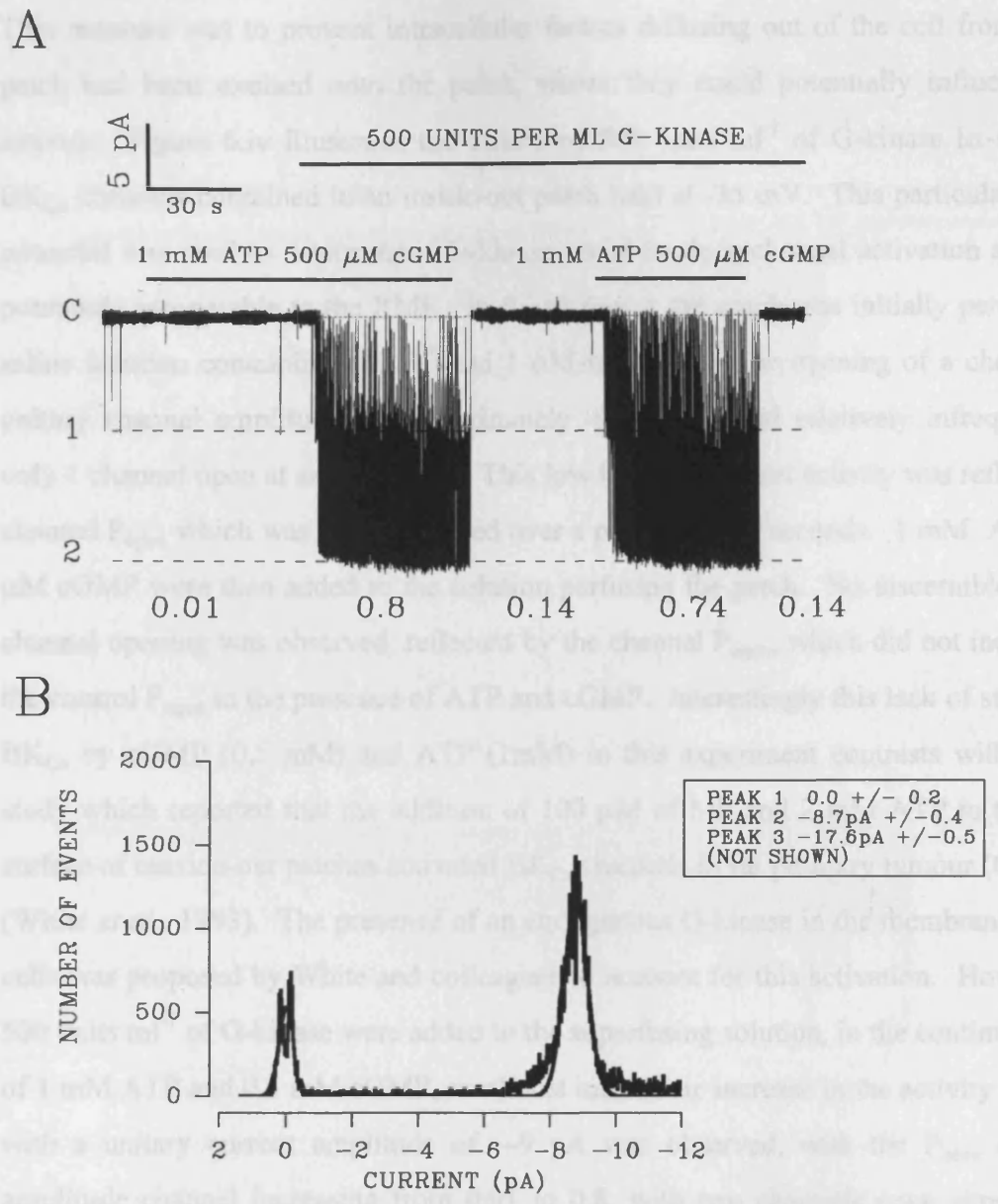


Figure 6.iv. G-kinase increases single channel activity in inside-out membrane patches from rat basilar artery smooth muscle cells. Panel A illustrates a representative recording showing the effects of 500 units of G-kinase catalytic subunits on single channel activity in an inside-out patch excised from a rat basilar artery smooth muscle cell. The membrane potential was -35 mV and the recording was made in symmetrical 140 mM K^+ and 1 μM Ca^{2+} at the cytoplasmic surface of the patch. For display this record was filtered at 1 kHz and sampled at 5 kHz. Test compounds were added where indicated and the values below the bars indicate the P_{open} of any channels under those experimental conditions. Figure 6.iv B illustrates a histogram of the channel open and closed events allowing a measurement of the unitary current amplitude of the channel activated by G-kinase to be made.

This measure was to prevent intracellular factors diffusing out of the cell from which the patch had been excised onto the patch, where they could potentially influence channel activity. Figure 6.iv illustrates the effects of 500 units ml^{-1} of G-kinase Iα-sub-units on BK_{Ca} channels contained in an inside-out patch held at -35 mV. This particular membrane potential was used to determine if G-kinase could produce channel activation at membrane potentials comparable to the RMP. In figure 6.iv A the patch was initially perfused with a saline solution containing 140 K^+ and 1 μM-free Ca^{2+}. The opening of a channel with a unitary channel amplitude of approximately -9 pA occurred relatively infrequently, with only 1 channel open at any one time. This low level of channel activity was reflected by the channel P_{open} which was 0.01 measured over a period of ~40 seconds. 1 mM ATP and 500 μM cGMP were then added to the solution perfusing the patch. No discernible increase in channel opening was observed, reflected by the channel P_{open}, which did not increase above the control P_{open} in the presence of ATP and cGMP. Interestingly this lack of stimulation of BK_{Ca} by cGMP (0.5 mM) and ATP (1mM) in this experiment contrasts with a previous study which reported that the addition of 100 μM cGMP and 2 mM ATP to the cytosolic surface of outside-out patches activated BK_{Ca} channels in rat pituitary tumour (GH_4C_1) cells (White *et al.*, 1993). The presence of an endogenous G-kinase in the membranes of GH_4C_1 cells was proposed by White and colleagues to account for this activation. However, when 500 units ml^{-1} of G-kinase were added to the superfusing solution, in the continued presence of 1 mM ATP and 0.5 mM cGMP, an almost immediate increase in the activity of a channel with a unitary current amplitude of ~-9 pA was observed, with the P_{open} of the large amplitude channel increasing from 0.01 to 0.8, with two channels open simultaneously.. When cytosolic ATP and cGMP were removed, leaving the G-kinase catalytic subunit alone, channel activity rapidly fell to control levels, as illustrated by the channel P_{open}, which fell to 0.014. When ATP and cGMP were then re-added, almost identical results were obtained with 2 channels again open simultaneously and the channel P_{open} increasing to 0.74. The requirement for cytosolic ATP and cGMP for channel opening by G-kinase was re-confirmed, as when they were removed the channel P_{open} again rapidly fell to control levels. To identify the channel whose P_{open} dramatically increased in the presence of cytoplasmic ATP, cGMP and G-kinase, I measured the amplitude of this channel using the standard method of producing a histogram of channel open and closed events as described in chapter 2.

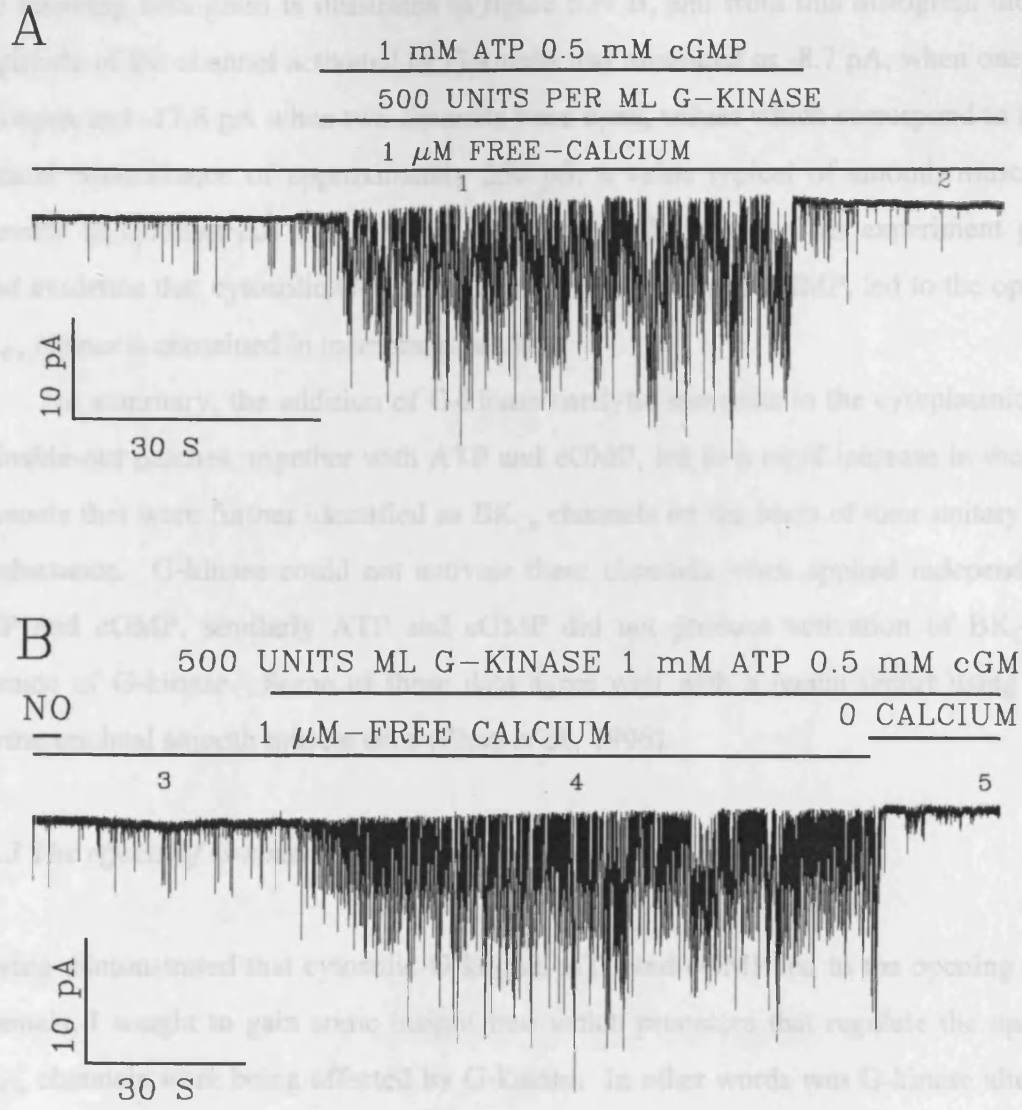


Figure 6.v. Evidence that the activation of BK_{Ca} channels by G-kinase is Ca^{2+}-dependent and NO does not directly activate BK_{Ca} channels in rat basilar artery smooth muscle cells. Panels A and B illustrate single channel recordings from an inside-out patch held at -30 mV in symmetrical 140 mM K^+. These recordings were filtered at 1 kHz and sampled at 5 kHz. The bars indicate when the addition of test compounds to the cytosolic solution took place and the values below the addition bars indicate when the recording was paused and the voltage ramps illustrated in figure 6.vi were run.

The resulting histogram is illustrated in figure 6.iv B, and from this histogram the current amplitude of the channel activated by G-kinase was measured as -8.7 pA, when one channel was open and -17.6 pA when two channels were open, values which correspond to a unitary channel conductance of approximately 250 pS, a value typical of smooth muscle BK_{Ca} channels in symmetrical 140 mM K^+ (Benham *et al.*, 1986). This experiment provided good evidence that cytosolic G-kinase, together with ATP and cGMP, led to the opening of BK_{Ca} channels contained in membrane patches.

In summary, the addition of G-kinase catalytic sub-units to the cytoplasmic surface of inside-out patches, together with ATP and cGMP, led to a rapid increase in the P_{open} of channels that were further identified as BK_{Ca} channels on the basis of their unitary channel conductance. G-kinase could not activate these channels when applied independently of ATP and cGMP, similarly ATP and cGMP did not produce activation of BK_{Ca} in the absence of G-kinase. Some of these data agree well with a recent report using isolated bovine tracheal smooth muscle cells (Zhou *et al.*, 1996).

6.2.3 The effects of G-kinase were Ca^{2+}- dependent

Having demonstrated that cytosolic G-kinase, ATP and cGMP led to the opening of BK_{Ca} channels, I sought to gain some insight into which processes that regulate the opening of BK_{Ca} channels were being affected by G-kinase. In other words was G-kinase altering the Ca^{2+}- or voltage-dependence of the BK_{Ca} channel? Figure 6.v illustrates a recording made from an inside-out patch. The patch potential was held at -30 mV in symmetrical 140 mM K^+ and the free-Ca^{2+} concentration at the cytoplasmic surface was 1 μM. Under these conditions a channel with a unitary channel conductance of 240 pS opened relatively infrequently. Following the addition of 500 units ml^{-1} of G-kinase, together with 1 mM ATP and 500 μM cGMP, a rapid increase in the activity of the large conductance channel occurred, with up to 2 channels open simultaneously. As mentioned in section 6.2.1 in this chapter, sub-states of BK_{Ca} channels occasionally occur. In this particular experiment for example, a BK_{Ca} channel sub-state with a unitary current amplitude of -5.7 pA was identified, corresponding to a unitary conductance of 190 pS. When the ATP and cGMP were removed the level of channel activity rapidly returned to control levels confirming that for the activation of BK_{Ca} channels to occur cytosolic ATP and cGMP were also required. G-kinase, ATP and cGMP were then re-added and channel activation rapidly occurred.

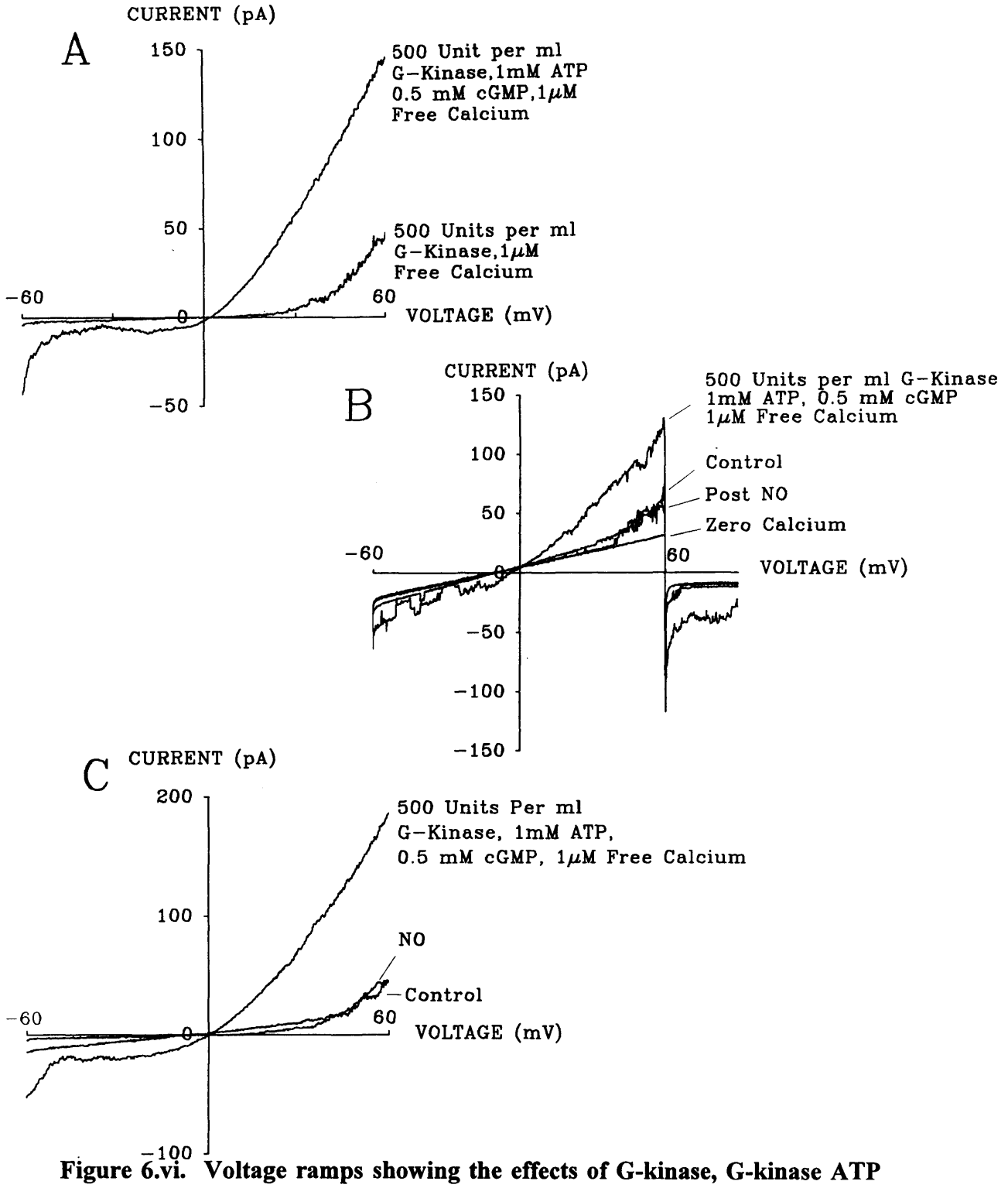


Figure 6.vi. Voltage ramps showing the effects of G-kinase, G-kinase ATP and cGMP and authentic NO on currents activated from an inside-out patch. The currents illustrated in panels 6 A, B and C were activated from the recording illustrated in panels 6.v A and B. In all case the x-axis is the patch potential and the y-axis the amount of current at that potential. Panels 6 A and 6 C illustrate data averaged from a minimum of 20 individual ramps following leak subtraction, while panel 6 B illustrates raw data. All ramps were 50 ms in length and filtered at 1 kHz.

To then try and determine which particular pathway involved in the modulation of BK_{Ca} channels was being affected by G-kinase, I reduced the free-Ca^{2+} concentration at the cytoplasmic surface to nominally zero in the continued presence of G-kinase, ATP and cGMP. This led to a rapid reduction in channel P_{open}, demonstrating that the increase in activity of BK_{Ca} channels by G-kinase, ATP and cGMP also required cytoplasmic Ca^{2+}, perhaps indicating that G-kinase was increasing BK_{Ca} channel P_{open} by an effect on the Ca^{2+}-dependence rather than the voltage-dependence of the BK_{Ca} channel. This observation contrasts with a previous study, where it was reported that the activation of BK_{Ca} channels in cultured hippocampal neurones by β-amyloid-precursor protein (β-APP), via the activation of the cGMP signalling pathway was Ca^{2+}-independent (Furaukawa *et al.*, 1996).

At the time points illustrated in figure 6.v voltage ramps were used to examine the effects of voltage on the channels activated by G-kinase. This was to further verify that the large-conductance channels being activated by G-kinase were BK_{Ca}, channels by confirming that the P_{open} of these channels increased with membrane depolarisation. These data are illustrated in figure 6.vi. A and were corrected for leak by subtracting the average currents activated when the cytosolic [Ca^{2+}] was nominally zero (see figure 6.vi B). This ensured the P_{open} of any BK_{Ca} channels was extremely low and therefore most of the current through the patch in response to the voltage ramp was leak current. This procedure was routinely used to correct for leak currents when analysing patch data. The lower trace in figure 6. vi A illustrates currents activated with 1 μM Ca^{2+} and 500 units per ml^{-1} of G-kinase at the cytosolic face of the patch. Little or no current was activated until the patch potential reached approximately +50 mV, then the activation of outward currents was evident. Following the addition of cGMP and ATP, together with G-kinase and 1 μM Ca^{2+}, the upper currents were activated. These currents were increased at all potentials with respect to controls, especially at positive potentials, indicating that the channel opened by G-kinase displayed voltage-dependence. This information, coupled with previous data, such as the 250 pS slope conductance of this channel in symmetrical 140 mM K^{+}, confirmed that cytosolic G-kinase catalytic sub-units led to the opening of BK_{Ca} channels. Figure 6.vi B illustrates representative individual ramps used to produce the ensemble average ramps. The ramp obtained with nominally zero Ca^{2+} and G-kinase present at the cytosolic surface of the patch illustrates the almost complete absence of BK_{Ca} channel activity under these conditions, justifying using such data to correct for leak currents.

These currents additionally illustrate that the presence of cytosolic Ca^{2+} was necessary for G-kinase to produce BK_{Ca} channel opening, perhaps indicating that the stimulation of BK_{Ca} channels by G-kinase in arterial smooth muscle may involve increasing the Ca^{2+}-sensitivity of the BK_{Ca} channel, possibly by somehow facilitating binding of Ca^{2+} to Ca^{2+}-binding sites located on the channel. An effect like this would be equivalent to increasing the concentration of Ca^{2+} at the cytosolic surface, enabling the BK_{Ca} channel to open at a less depolarised membrane potentials. The currents for example activated by voltage ramps in the presence of G-kinase illustrated in figure 6.vi B upper trace show individual BK_{Ca} channels being activated at membrane potentials as negative as -60 mV.

Therefore, in summary, the addition of G-kinase catalytic subunits together with cGMP and ATP led to an increase in the P_{open} of a channel with characteristics of BK_{Ca} channels i.e. Ca^{2+}- and voltage-dependence, unitary channel conductance. The effects produced by G-kinase were rapid in onset and offset and required cytosolic Ca^{2+}, perhaps indicating that the action of G-kinase ultimately involves altering the Ca^{2+}-dependence of the BK_{Ca} channel.

6.2.4 Authentic NO did not directly open BK_{Ca} channels when applied to inside-out patches

The effects of applying NO, in the form of a saturated solution or the donor compound SIN-1A, directly to the cytoplasmic surface of the patch were also examined. This was because a previous study reported that a methylene blue-resistant part of the vasodilation produced by NO in the rabbit aorta was blocked by CTX (Bolotina *et al.*, 1994). An observation which led Bolotina and her co-workers to propose that this GC-S-resistant portion of the NO-induced vasodilation could be explained by NO reacting with, and chemically modifying, -SH groups that form part of the BK_{Ca} channel itself or an associated regulatory site. The resulting nitrosylation of the BK_{Ca} channel by NO resulted in an increase in the P_{open} of the BK_{Ca} channel (Bolotina *et al.*, 1994). In figures 6.v B and 6.vii, 1 ml of saturated solution (~1 mM NO) of NO, a dose that far exceeded the dose required to produce full relaxation of rat basilar rings performed *in vitro* (Holland unpublished observations), was injected, using a gas-tight syringe, into the recording chamber close to the patch, so minimal oxidative breakdown of NO would occur prior to the NO reaching the patch.

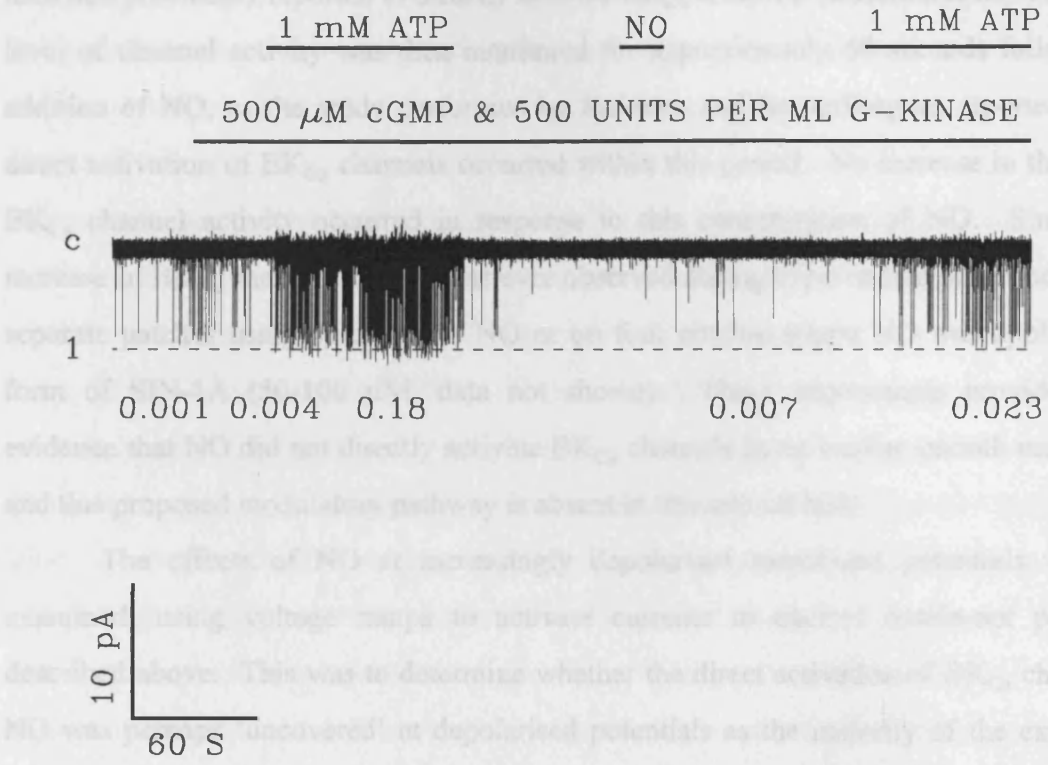


Figure 6.vii. Evidence that the activation of BK_{Ca} channels by G-kinase in inside-out patches from rat basilar artery smooth muscle cells was dependent on cytosolic ATP. This figure illustrates a recording of single channels, made at -30 mV in 140 mM symmetrical K^+ and 1 μM free cytosolic Ca^{2+}. This recording was filtered at 1 kHz and sampled at 5 kHz. The continuous dotted line indicates the channel open level and the solid lines indicate when test compounds were added and removed. The continuous dotted line denotes the channel open level and the values below this line indicates the channel P_{open} under those particular conditions.

At the end of the experiments illustrated in figure 6.v B and 6.vii the bath volumes were found to be approximately 5 ml, corresponding to an equilibrium bath concentration of NO, if no oxidative breakdown occurred, of ~160 μM, a concentration of NO ~30 times higher than that previously reported to directly activate BK_{Ca} channels (Bolotina *et al.*, 1994). The level of channel activity was then monitored for approximately 60 seconds following the addition of NO, as the study performed by Bolotina and her colleagues reported that the direct activation of BK_{Ca} channels occurred within this period. No increase in the level of BK_{Ca} channel activity occurred in response to this concentration of NO. Similarly no increase in BK_{Ca} channel activity was ever observed during experiments performed on four separate patches using solutions of NO or on four patches where NO was applied in the form of SIN-1A (50-100 μM, data not shown). These experiments provided strong evidence that NO did not directly activate BK_{Ca} channels in rat basilar smooth muscle cells and this proposed modulatory pathway is absent in this arterial bed.

The effects of NO at increasingly depolarised membrane potentials were also examined, using voltage ramps to activate currents in excised inside-out patches as described above. This was to determine whether the direct activation of BK_{Ca} channels by NO was perhaps 'uncovered' at depolarised potentials as the majority of the experiments described by Bolotina and colleagues were conducted using patches held at +40 mV (Bolotina *et al.*, 1994). This difference in holding potential could potentially explain why I never observed direct channel activation by NO. Figure 6.vi C illustrates currents activated by ramping the patch from -60 to 60, mV in symmetrical 140 mM K^+. These data were averaged from a minimum of twenty individual ramps with leak currents subtracted. By comparing the two lower ramps it is clear that the currents activated following the addition of NO were not significantly different from those activated under control conditions, even at the more positive (>40 mV) patch potentials. Therefore, differences in the holding potentials used to examine the effects of NO on BK_{Ca} channels cannot explain the startling contrast between the results presented in this study and a previous study that claimed to demonstrate direct activation of BK_{Ca} channels by authentic NO (Bolotina *et al.*, 1994).

In summary no evidence was found in this study to indicate that NO directly opened BK_{Ca} channels in rat arterial smooth muscle.

6.2.5 The effects of G-kinase were ATP-dependent

Some uncertainty currently exists as to the nature of the effect of G-kinase which ultimately produces opening of smooth muscle BK_{Ca} channels. The initial studies which reported that G-kinase catalytic subunits activated BK_{Ca} channels in arterial smooth muscle did not further investigate how this effect was transduced (Taniguchi *et al.*, 1992; Robertson *et al.*, 1993). Studies however using inside-out patches excised from GH_4C_1 cells, a rat pituitary tumour cell line, cultured hippocampal neurones and tracheal smooth muscle cells reported that the activation of BK_{Ca} channels by G-kinase catalytic subunits were inhibited by 10 nM okadaic acid, a concentration reported to selectively inhibit protein phosphatase 2A (White *et al.*, 1993; Furaukawa *et al.*, 1996). These observations led to the proposal that G-kinase activates protein phosphatase 2A via phosphorylation. Once phosphorylated and activated, protein phosphatase 2A *dephosphorylates* the BK_{Ca}, possibly by dephosphorylating the A-kinase consensus phosphorylation site located on the β subunit (Kaczorowski *et al.*, 1996), which leads to BK_{Ca} channel opening (White *et al.*, 1993). To investigate if a similar transduction pathway controlled the activity of BK_{Ca} in arterial smooth muscle cells I examined the effects of removing the source of phosphate (ATP) for any subsequent phosphorylation reactions from the cytosolic solution on channel activity. Figure 6.vii illustrates the results of such an experiment. In this experiment the inside-out patch was excised as described previously and the recording was made at a holding potential of -30 mV in symmetrical 140 mM K^+ with 1 μM free-Ca^{2+} at the cytoplasmic surface of the patch. The P_{open} of a channel, with a unitary conductance of 240 pS and a sub-conductance of 180 pS, was 0.001 when measured over approximately 30 seconds. When 500 μM cGMP and 500 units ml^{-1} of G-kinase catalytic subunits were added together with 1 μM free-Ca^{2+} the P_{open} of a channel with a unitary conductance of 253 pS and a sub-conductance state of 190 pS increased to 0.004. This small, insignificant increase may have been produced by the presence of some residual ATP present in the bath, possibly produced by lysis of smooth muscle cells. When 1 mM ATP was added to the cytosolic solution, together with G-kinase and cGMP, the activity of a channel with a unitary conductance of 255 pS and a sub-conductance of 190 pS rapidly (<5 s) increased, with the P_{open} of this channel increasing to 0.18, an increase of 180 times with respect to the control level of activity. When ATP was removed the channel P_{open} rapidly (<5 s) fell to control levels.

Figure 6.vii also provides another example of the lack of effect of authentic NO on BK_{Ca} channels, as infusing 1 ml of a 1mM solution of NO produced no increase in the activity of the large-conductance channel activated by G-kinase. When 1mM ATP was re-added a large increase occurred in the activity of a channel with a unitary conductance of 259 pS and a sub-conductance of 171pS, with the P_{open} increasing to 0.023, a increase of 23 times with respect to controls. Similar results were obtained from experiments performed on three other patches. In all experiments the P_{open} of the BK_{Ca} channel rapidly increased in the presence of ATP, cGMP and G-kinase and fell to control levels following the removal of ATP. These results indicate that a phosphorylation reaction was a prerequisite for the activation of BK_{Ca} channels by G-kinase, although to confirm whether the BK_{Ca} channel itself is phosphorylated, as was previously proposed to explain the stimulation of BK_{Ca} channels in tracheal smooth muscle by adrenergic agonists (Kume *et al.*, 1989; Kume *et al.*, 1992) or the enzyme phosphatase 2A is phosphorylated will require further experimentation using non-hydrolysable analogues of ATP, okadaic acid and temperature-inactivated G-kinase catalytic subunits. The phosphorylation and stimulation of phosphatase 2A by G-kinase would currently appear to be the more likely explanation of the results described here, as this enzyme has been recently been found to be closely associated with BK_{Ca} channels isolated from bovine tracheal smooth muscle cell membranes (Zhou *et al.*, 1996) and its presence is essential for the activation of BK_{Ca} channels by G-kinase (Zhou *et al.*, 1996). What the results in the present study and the report by Zhou and colleagues also suggest is that a currently unidentified enzyme, most likely a kinase, co-exists with BK_{Ca} channels in both rat arterial and bovine tracheal smooth muscle cells (Zhou *et al.*, 1996). The basis for this hypothesis is that in both these studies when cytosolic G-kinase was removed the level of BK_{Ca} channel activity rapidly fell to control levels, suggesting that an endogenous kinase is present which phosphorylates the BK_{Ca} channel, returning the channel to the phosphorylated low P_{open} state.

In summary, for G-kinase catalytic subunits to activate BK_{Ca} channels in membrane patches excised from rat basilar smooth muscle cells cytosolic ATP was required. It is currently unclear whether ATP provides a source of phosphate for the phosphorylation of the BK_{Ca} channels directly by G-kinase or whether G-kinase phosphorylates and stimulates protein phosphatase 2A, which then dephosphorylates and opens the BK_{Ca} channel, implying an indirect rather than a direct role for G-kinase in the activation of BK_{Ca} channels.

At the present time evidence suggests that the later mechanism is the more likely.

6.3 DISCUSSION

6.3.1 Identification of BK_{Ca} channels in cell membrane patches excised from rat basilar artery smooth muscle cells

Ca^{2+}-dependent channels were identified in membrane patches excised from rat basilar artery smooth muscle cells (see figure 6.i A). These channels were found in all patches studied, indicating that these channels are present in large numbers in rat basilar artery smooth muscle cells. Additional experiments, summarised in figures 6.ii and 6.iii, further identified these channels as BK_{Ca} channels on the basis of their large (~250 pS) unitary conductance in symmetrical 140 mM K^+ solution and the observation that depolarisation increased the P_{open} of the channel.

6.3.2 Cytosolic G-kinase subunits together with ATP and cGMP increased the P_{open} of BK_{Ca} channels in excised inside-out patches

In all patches studied (n=8), the addition of 500 units per ml^{-1} of G-kinase, together with ATP and cGMP, to the cytosolic surface of inside-out patches led to an increase in the P_{open} of channels whose unitary conductances were ~250 pS in symmetrical 140 mM K^+. Representative examples of this effect are illustrated in figures 6. iv A, 6. v A and B and 6. vii. This effect was mediated by the G-kinase sub-units rather than ATP or cGMP as no significant BK_{Ca} channel activation occurred in the absence of G-kinase. However the effects of G-kinase were dependent on the presence of both these nucleotides and cytosolic Ca^{2+}. The large-conductance channels activated by G-kinase were identified as BK_{Ca} currents on the basis of their unitary channel amplitude (~250 pS in symmetrical 140 mM K^+) and voltage-dependence. Therefore in conclusion, channels with the properties expected of BK_{Ca} channels were opened by cytosolic G-kinase catalytic sub-units together with cGMP and ATP. What therefore was the role of these two nucleotides in the activation of BK_{Ca} channels? The role of cGMP should be to activate the G-kinase catalytic sub-unit, while ATP is required to provide phosphate for a phosphorylation reaction presumably catalysed by G-kinase. This raises the question what is the target for this phosphorylation reaction and how does this reaction ultimately lead to the opening of BK_{Ca} channels?

As previously mentioned the effects of NO / G-kinase may involve an indirect role for G-kinase involving the phosphorylation and activation of protein phosphatase 2A (White *et al.*, 1993; Zhou *et al.*, 1996; Furaukawa *et al.*, 1996). Experiments are currently under way to determine whether a similar mechanism is involved in the modulation of BK_{Ca} channels in rat cerebral artery smooth muscle membranes.

6.3.3 No evidence was found to support the proposal that NO directly activated BK_{Ca} channels

A recent, and rather controversial study reported that authentic NO (0.2-5 μM) directly activated BK_{Ca} channels in cultured rabbit aortic smooth muscle cells (Bolotina *et al.*, 1994). This was potentially an exciting observation as it described a novel mechanism of BK_{Ca} channel modulation by this widely studied signalling molecule. I attempted to reproduce this effect using patches excised from freshly isolated rat basilar artery smooth muscle cells and similar conditions to those used by Bolotina and co-workers. However even when I used bath concentrations of NO approximately thirty times higher than those previously reported to directly activate BK_{Ca} channels I found no evidence to support the proposal that direct activation of BK_{Ca} channels occurred in these smooth muscle cells. However, cytosolic G-kinase, together with ATP and cGMP activated BK_{Ca} channels in all patches studied, results that agree with data in previous reports (Tanaguchi *et al.*, 1992; Robertson *et al.*, 1993; Zhou *et al.*, 1996). A number of differences exist between the present study and the study performed by Bolotina and co-workers which may explain the striking differences between these two studies.

1. The present study used smooth muscle cells freshly isolated rat basilar artery, whereas the experiments performed by Bolotina and co-workers used cultured smooth muscle cells (2-12 days primary culture) originally isolated from rabbit aorta. Could the process of cell culture somehow alter BK_{Ca} channel properties resulting in BK_{Ca} channels that were directly activated by NO? Although this explanation appears unlikely it cannot be ruled out.

2. Bolotina and her co-workers used methylene blue to inhibit GC-S and interpreted the subsequent activation of BK_{Ca} channels as evidence that direct channel activation by NO occurred. Methylene blue is an oxidising agent frequently used to inhibit GC-S, which it irreversibly oxidises, and it has the additional effect of inactivating NO by generating superoxide (Wolin *et al.*, 1990; Marczin *et al.*, 1992) which reacts with NO producing peroxynitrite (see section 2.4.1). Superoxide and peroxynitrite have previously been reported to activate BK_{Ca} and K_{ATP} channels (Wei *et al.*, 1996), therefore the activation of BK_{Ca} channels reported by Bolotina *et al.*, (1994) could in theory have resulted from the generation of superoxide or peroxynitrite. Obviously this is pure speculation and simply represents an attempt to explain the different results obtained by these studies.

In conclusion, following the addition of cytosolic G-kinase the P_{open} of BK_{Ca} channels increased. This effect required ATP, demonstrating that a phosphorylation reaction was one of the requirements for the activation of BK_{Ca} channels by G-kinase, and Ca^{2+}, indicating that ultimately the effects of G-kinase may involve increasing the Ca^{2+}-dependence of the BK_{Ca} channel.

The exact nature of this proposed phosphorylation reaction is currently unknown, although a possible target is thought to be protein phosphatase 2A, which may subsequently dephosphorylate a protein kinase A consensus phosphorylation site located on the β subunit of the BK_{Ca} channel, producing BK_{Ca} channel opening. In all the experiments conducted in this study the addition of authentic NO never increased the P_{open} of BK_{Ca} channels, indicating that this proposed mechanism of activating BK_{Ca} channels is absent in this particular artery.

CHAPTER SEVEN

Discussion

7.1 BK_{Ca} channel opening is a viable mechanism of producing smooth muscle hyperpolarisation

The initial aim of this project was to determine whether a putative BK_{Ca} channel activator, NS1619, could hyperpolarise freshly isolated arterial smooth muscle and whether this effect was due to the opening of BK_{Ca} channels. This was to establish whether BK_{Ca} channel opening actually constituted a potential novel mechanism by which vasodilation via membrane hyperpolarisation, in a manner analogous to the first generation KCOs currently in clinical use or undergoing clinical trials, could be achieved.

I found NS1619 to be a effective opener of BK_{Ca} channels which hyperpolarised isolated smooth muscle cells, suggesting that BK_{Ca} channel opening does constitute a viable mechanism by which vasorelaxation through hyperpolarisation may be produced. However, subsequent pharmacological screening studies illustrated that NS1619 was a highly non-specific compound which blocked both K_V channels and DHP-sensitive Ca^{2+} channels. Indeed this latter effect, rather than BK_{Ca} channel opening, probably accounts for the vasorelaxant effects of NS1619 (Edwards *et al.*, 1994; Holland *et al.*, 1996).

The synthesis and discovery of NS1619 and other recently described BK_{Ca} channel openers (for review see Edwards & Weston, 1996) have complemented the existing range of BK_{Ca} channel blockers. Together these compounds are potentially important to the research scientist attempting to define distinct physiological roles for BK_{Ca} channels in various systems. Specificity of action is however a necessary requirement, so while NS1619 is unquestionably an interesting and useful experimental compound, an example of its use as a positive experimental control was illustrated in chapter 5, NS1619's use in functional studies to identify and assign physiological roles to BK_{Ca} channels is somewhat limited due to the obvious difficulties in interpreting any resulting data.

7.2 Possible sites and mechanisms of action of NS1619

Having demonstrated that NS1619 activated BK_{Ca} channels in intact arterial smooth muscle cells I investigated the mechanism of action of NS1619. Previous studies, using inside- and outside-out patches of membrane excised from cultured vascular smooth muscle cells and neurones had reported that NS1619 directly activated BK_{Ca} channels (Olesen *et al.*, 1994; Sellers & Ashford, 1994). I also concluded that the activation of BK_{Ca} channels by NS1619 was due to a direct effect on the channel or a closely associated regulatory site and that the effects of NS1619 were Ca^{2+}-dependent. The rationale for investigating this latter effect was related to the mechanism of action of NS1619, as conflicting reports concerning the influence of cytosolic Ca^{2+} on the action of NS1619 have been published. One report, for example, claimed that NS1619 activated BK_{Ca} channels independently of cytosolic Ca^{2+} (Edwards *et al.*, 1994), although this study was performed using the conventional whole cell configuration exclusively, which may not allow sufficient control of the sub-membrane Ca^{2+} concentration. Another study asserted that cytosolic Ca^{2+} was required for the effects of NS1619 (Olesen *et al.*, 1994a), although this study did not actually illustrate this effect. My studies unequivocally demonstrated that the effects of NS1619 were Ca^{2+}-dependent, indicating that the mechanism of action of NS1619 does not simply involve NS1619 substituting for Ca^{2+} at Ca^{2+} binding sites located at the cytosolic surface of the BK_{Ca} channel.

I feel therefore that the mechanism of action of NS1619 probably involves altering the Ca^{2+}-sensitivity rather than voltage-dependence of the vascular smooth muscle BK_{Ca} channels. The main reason for this proposal was the finding (see chapter 3) that NS1619 never activated BK_{Ca} channels in excised patches when the cytosolic Ca^{2+} concentration was nominally zero. Support for this idea has been indirectly provided by a number of recent studies which reported that at low cytosolic Ca^{2+} concentrations (<100 nM), BK_{Ca} channels composed of either *mslo* or *hslo* α subunits co-expressed with β subunits (Cox *et al.*, 1996; Toro *et al.*, 1996; Strobaek *et al.*, Meera *et al.*, 1996), function purely as voltage-activated channels. If the mechanism of action of NS1619 involved shifting the voltage- rather than Ca^{2+}-dependence of the BK_{Ca} channel, then it is reasonable to assume that such an effect would have occurred when the cytosolic $[Ca^{2+}]$ was nominally zero and BK_{Ca} channels were functioning solely as voltage-activated channels.

This patently did not occur in the present study, a fact which has led to me to conclude that an effect on the Ca^{2+}-dependence of the channel probably best accounts for activation of BK_{Ca} channels by NS1619 described here. Opening BK_{Ca} channels by somehow increasing the Ca^{2+}-dependence of the channel may be a common mechanism of action of some BK_{Ca} channel openers. The non-steroidal anti-inflammatory drug, niflumic acid for example, and other so-called fenamates have also been reported to open BK_{Ca} channels by increasing the Ca^{2+}-sensitivity of the BK_{Ca} channel (Ottalia & Toro, 1994), via an interaction exclusively with the *slo* α subunit (Wallner *et al.*, 1995). Also the increase in BK_{Ca} channel activity produced by G-kinase catalytic subunits was abolished when the cytosolic Ca^{2+} concentration was reduced to nominally zero (see chapter 6), perhaps indicating that the action of G-kinase also involves an effect on the Ca^{2+}-dependence of the BK_{Ca} channel.

The actual mechanism of action of NS1619 as a BK_{Ca} channel opener is currently unresolved. However studies using cloned BK_{Ca} channels composed of either α subunits, or α and β subunits co-expressed, have reported that the site of action of NS1619 was located on the α channel subunit, as co-expression of the β subunit had no effect on the pharmacological profile of NS1619 (Dworetzky *et al.*, 1996; Gribkoff *et al.*, 1996). Therefore, assuming that NS1619 acts on the α subunit of the channel and its mechanism of action involves increasing the Ca^{2+}-sensitivity of the BK_{Ca} channel, then the most likely site of action is the S7-S10 'tail' of the α subunit, which has been reported to confer Ca^{2+}-sensitivity on *slo* channels (Wei *et al.*, 1994). The S7-S10 tail region results from a long intracellular loop originating from the C-terminus of the S6 helix (see figure 1.viii). The location of this loop may additionally tie in with the proposal that the site of action of NS1619 may be intracellular (Olesen *et al.*, 1994; chapter 3 of the present study). If the site of action of NS1619 is intracellular and involves increasing the Ca^{2+}-sensitivity of the BK_{Ca} channel, then an effect on the intracellular S7-S10 tail region would explain both the Ca^{2+}-dependence of action of NS1619 and the characteristic slow onset of action of this drug during whole cell experiments probably because of the time taken for sufficient intracellular concentration of NS1619 to be reached (Olesen *et al.*, 1994; Edwards *et al.*, 1994; Holland *et al.*, 1996).

The precise mechanism of action of NS1619 may only be described using a molecular approach and cloned BK_{Ca} channels.

By deleting specific sections of the α subunit, areas vital to the effect of NS1619 may be identified which may reveal information regarding the mechanism of action of NS1619 and also how BK_{Ca} channel opening as a whole can be produced. I would be interested, for example, to identify the importance of the S7-S10 'tail' section of the channel α subunit with regard to the mechanism of action of NS1619.

In summary, experimental evidence presented in this thesis suggests that the mechanism of action of some agents which open BK_{Ca} channels, including NS1619, may involve increasing the Ca^{2+} sensitivity of BK_{Ca} channels by a currently unknown mechanism. This leads to an increase in channel P_{open}, at a fixed cytosolic Ca^{2+} concentration.

7.3 NO activated BK_{Ca} channels in arterial smooth muscle

The second section of this study focused on the effects of NO and cGMP-dependent protein kinase, an enzyme which is activated in vascular smooth muscle cells following the addition of NO (Schmidt *et al.,* 1993), on the activity of K^+ channels in arterial smooth muscle cells. Functional and electrophysiological studies have previously reported that the vasorelaxant mechanism of NO may involve hyperpolarisation of vascular smooth muscle cells via the opening of K^+ channels. One vascular bed where this may be true is the rat cerebral circulation, where a nitrovasodilator-induced, glibenclamide-insensitive hyperpolarisation has previously been reported (McPherson & Stork, 1992).

The aims of this section of the study were to initially examine the effects of NO on whole cell currents using holding potentials comparable to the RMP of vascular smooth muscle. NO, administered in the form of a solution or as a donor compound, led to the activation of IbTX and CTX-sensitive currents in ~40 % of cells studied. This effect occurred in the presence of superoxide dismutase, ensuring that these effects were not simply due to the generation of reactive superoxide or peroxynitrite ions from NO, which have previously been reported to activate BK_{Ca} channels in feline cerebral arteries (Wei *et al.,* 1996). Initial experiments also illustrated that a membrane-permeant activator of G-kinase, 8-bromo cGMP, activated whole cell currents that resembled those produced by SIN-1A and authentic NO. Perhaps indicating that where NO has been reported to produce BK_{Ca} channel opening this effect may be via the protein kinase G signalling pathway rather than by a direct effect of NO on the channel.

Further experiments to confirm whether NO produced BK_{Ca} channel opening via activation of guanylyl cyclase and the subsequently activation of G-kinase could potentially be approached from a number of angles. The most obvious approach would be to examine the effects of specific inhibitors of guanylyl cyclase such as LY-83583 or ODQ, or G-kinase inhibitors such as KT5823, on NO-induced whole cell currents and determine if these compounds could reverse the effects of NO. A slightly different approach could involve including G-kinase catalytic subunits in the pipette solution and using the conventional whole cell configuration to dialyse cells with the G-kinase catalytic subunits. This approach may determine if G-kinase catalytic subunits open BK_{Ca} channels in the whole cell configuration as well as in excised patches. A similar method to this was previously used to illustrate the stimulatory effects of protein kinase A on K_{ATP} channels in rabbit mesenteric artery smooth muscle cells (Quayle *et al.*, 1994).

A study of the effects of peroxynitrite and superoxide ions may also be potentially interesting, as I feel the production of these ions may potentially explain the direct activation of BK_{Ca} channels by NO.

7.4 G-kinase catalytic subunits opened BK_{Ca} channels in excised inside-out patches

To further support the hypothesis that NO opened BK_{Ca} channels via the cGMP signal transduction pathway, I found that protein kinase G catalytic subunits, together with ATP and cGMP, opened BK_{Ca} channels in all excised patches studied. Direct application of authentic NO solutions, on the other hand, never produced BK_{Ca} channel activation, even when the concentration of NO used far exceeded the concentration required to fully relax pre-contracted rat basilar artery rings *in vitro*.

Cytosolic ATP was required for BK_{Ca} channel opening in response to protein kinase G catalytic subunits, indicating that protein kinase G was probably participating in a crucial phosphorylation reaction which then produced BK_{Ca} channel opening. As discussed in detail in chapter 6 this phosphorylation reaction is currently thought to activate protein phosphatase 2A, which is presumably functionally associated with the channel. Activated protein phosphatase 2A then presumably dephosphorylates the BK_{Ca} channel resulting in channel opening.

Future work to confirm the requirement for cytosolic ATP and to illustrate that a phosphorylation reaction is a pre-requisite for BK_{Ca} channel opening by protein kinase G, will involve using non-hydrolysable analogues of ATP to establish if removing the source of phosphate will inhibit the stimulatory action of protein kinase G on BK_{Ca} channels.

A BK_{Ca} channel regulatory site is currently thought to exist, which, when *dephosphorylated,* possibly by protein phosphatase 2A, leads to BK_{Ca} channel opening (Zhou *et al.,* 1996). This site may correspond to a consensus protein kinase A phosphorylation site located on the β subunit of the BK_{Ca} channel (Kaczorowski *et al.,* 1996). Phosphorylation of this site by protein kinase A, an enzyme which possesses a 'basal' level of activity in smooth muscle due to the constant turnover of cAMP in smooth muscle cells, may occur, so that the P_{open} of BK_{Ca} channels in resting smooth muscle cells would be low. The potential role of protein kinase A in the regulation of BK_{Ca} channels contained in vascular smooth muscle could be investigated using cAMP-dependent protein kinase A catalytic subunits and an experimental approach similar to those described in chapter 6. These experiments could potentially clarify what has recently become a fairly confused area following the publication of a number of studies which directly contradict a previously accepted and frequently cited hypothesis; namely that protein kinase A catalytic subunits phosphorylate and activate BK_{Ca} channels in airways smooth muscle (Kume *et al.,* 1989; Kume *et al.,* 1992). A recent study for example, reported protein kinase A catalytic subunits *reduced* the P_{open} of cloned BK_{Ca} composed of α and β subunits, but increased the P_{open} if the channels were composed of α subunits alone (Dworetzky *et al.,* 1996). It could be argued that the BK_{Ca} channels in tracheal smooth muscle are composed of only α subunits therefore explaining the stimulatory effect of protein kinase A catalytic subunits on BK_{Ca} channels (Kume *et al.,* 1989; Kume *et al.,* 1992). This is an unlikely explanation however, as the BK_{Ca} channel β subunit was originally identified using a tracheal smooth muscle preparation and it is likely that BK_{Ca} channels in tracheal smooth muscle cells are heteromultimers composed of both α and β subunits. Also a study using patches excised from tracheal smooth muscle cells reported that okadaic acid (10 nM) *inhibited* the increase in BK_{Ca} channel P_{open} produced following the addition of protein kinase G catalytic subunits (Zhou *et al.,* 1996). This finding directly contrasts with the study by Kume *et al.,* (1989) which reported that okadaic acid stimulated the opening of BK_{Ca} channels by inhibiting a protein phosphatase and maintaining the channel in a phosphorylated state. This conclusion now appears to be questionable and the whole area of protein kinase regulation of BK_{Ca} channels requires clarification.

To confirm that protein phosphatase 2A is intimately involved in the regulation of BK_{Ca} channels in this vascular bed as it appears to be in tracheal smooth muscle (Zhou *et al.*, 1996), okadaic acid or calyculin A, specific inhibitors of protein phosphatase 2A, could be used together with G-kinase catalytic subunits to determine if inhibition of protein phosphatase 2A does inhibit the BK_{Ca} channel opening in response to protein kinase G catalytic subunits.

The identification of a possible regulatory site on the β subunit may help explain the stimulatory effects of G-kinase on BK_{Ca} channels reported in the present study (see sections 1.8.1 and 1.8.2). The β subunit has been recently been proposed to act as a regulatory 'switch' which serves to increase the Ca^{2+}-sensitivity of the BK_{Ca} channel which decreases the voltage-dependence of the α subunit of the BK_{Ca} channel by ~100 mV (Meera *et al.*, 1996). This protein kinase A phosphorylation site may regulate this 'switch' effect of the β subunit on the α subunit. When dephosphorylated it exerts a powerful effect on the α subunit increasing the Ca^{2+}-sensitivity and reducing the voltage-dependence of the BK_{Ca} channel substantially an effect which is reversed when it phosphorylated. Again satisfactory answers to these intriguing questions may only be obtained using a molecular approach.

The question remains whether G-kinase is present in sufficient quantities in vascular smooth muscle cells to produce BK_{Ca} channel activation following the activation of GC-S by NO?. Unfortunately I do not have the answer to this question, although a considerable amount of evidence from both functional and electrophysiological studies suggests that G-kinase is indeed produced in sufficient quantities to activate BK_{Ca} channels, as illustrated by the antagonism of NO-induced hyperpolarisation and its vasorelaxant effects by BK_{Ca} channel blockers (Williams *et al.*, 1989; Taniguichi *et al.*, 1992; Robertson *et al.*, 1993; Cowen *et al.*, 1993; Khan *et al.*, 1993; Bolotina *et al.*, 1994; Chen & Rembold, 1996; Banks *et al.*, 1996; Plane *et al.*, 1996). A recent report which attempted to determine the relative importance of the NO-induced hyperpolarisation to the overall relaxation of vascular smooth muscle by NO revealed that concentrations of NO 40-fold higher than those required to produce full relaxation of pre-contracted arteries were required to hyperpolarise smooth muscle (Parkington *et al.*, 1995). A concentration of NO of this magnitude may only be produced during certain pathological conditions such as septic-induced circulatory shock where iNOS in smooth muscle cells produces a large continuous stream of NO (Gross & Wolin, 1995).

This report by Helena Parkington and her co-workers concluded that the ability of NO to hyperpolarise vascular smooth muscle is probably therefore of secondary importance to the other vasodilatory pathways activated by G-kinase (see section 5.1.2 chapter 5)..

In conclusion, the opening of BK_{Ca} channels by NS1619 hyperpolarises vascular smooth muscle cells, and as such presumably constitutes a potentially useful mechanism of producing hyperpolarising vasodilation, providing the problem of selectivity can be overcome.

Activated protein kinase G catalytic subunits also activate BK_{Ca} channels which may explain how part of the vasorelaxation by NO and nitrovasodilators is produced. Both the mechanisms of action of these very different agents may involve somehow increasing the Ca^{2+} sensitivity of the BK_{Ca} channel allowing it to open at less depolarised potentials.

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