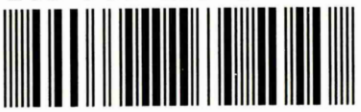
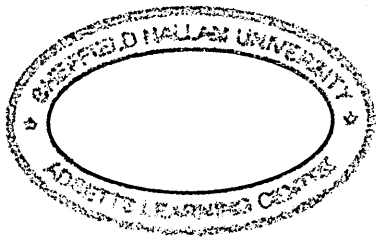


Investigation of the pharmacophore of BK_{Ca} potassium channel openers

Robert William Kirby

A thesis submitted in partial fulfilment of the requirements of
Sheffield Hallam University
for the degree of Doctor of Philosophy



April 2008

I Abstract

Large conductance voltage-activated calcium-sensitive potassium channels (BK_{Ca}) are fundamental in the control of cellular excitability which is critical in the regulation of many physiological processes. Agents that activate the channel (openers) have been proposed to be potential therapeutics for a number of un-met clinical conditions. Several heterogeneous classes of compounds have been described as BK_{Ca} channel openers and preliminary pharmacophore data has arisen for synthetic molecules based on the structure of benzimidazolones.

The project aimed to explore a series of novel compounds based on a benzanilide template for BK_{Ca} channel opener properties and to further probe the pharmacophore of BK_{Ca} channel openers. This was achieved by the validation and optimisation of a medium through-put, non-radioactive, rubidium (Rb^+) efflux assay using recombinant HEK293 cells expressing BK_{Ca} channel subunits. From which novel benzanilides have been identified as BK_{Ca} channel openers that display varying degrees of potency, efficacy and co-operativity. The Rb^+ efflux stimulated by each compound was blocked by use of the BK_{Ca} channel blockers paxilline and iberiotoxin demonstrating specificity to BK_{Ca} channels in the cell lines studied. Furthermore initial data demonstrates that some of these compounds showed selectivity for the BK_{Ca} channel α-subunit.

The benzanilide compounds were also examined using whole cell electrophysiology; all compounds tested, relative to control, produced shifts in V_{50} values in the hyperpolarising direction, indicative of BK_{Ca} channel activation. The benzanilides did not affect the kinetics of activation or deactivation. Furthermore, the order of effectiveness determined using whole cell electrophysiology showed good correlation with that obtained using the Rb^+ efflux assay. Using both the electrophysiology and Rb^+ efflux techniques BKOEt1 was identified as a novel and potent BK_{Ca} channel opener and selected as a lead compound.

In single channel electrophysiology recordings of cloned cell lines expressing BK_{Ca} channel subunits application of BKOEt1 promoted significant, paxilline sensitive, BK_{Ca} channel activation. BKOEt1 increased open pore probability rapidly for small changes in voltage and increased the voltage-sensitivity of the channel. In addition, BKOEt1 did not affect single channel conductance. BKOEt1 activated BK_{Ca} channels in the near absence of intracellular calcium and its effects were not additive. Furthermore, the compound did not affect the level of intracellular calcium. It was concluded that BKOEt1 acts directly on the channel at a site located on the α-subunit. A Hill slope of unity suggested one binding site per tetrameric channel complex and either an intracellular or transmembrane site of interaction was proposed. BKOEt1 also stimulated paxilline sensitive Rb^+ efflux from rat bladder myocytes and initial Rb^+ efflux studies demonstrated that BKOEt1 could activate K_V and SK channels.

Molecular modelling of the series of benzanildies provided clues as to the chemical or structural features required, in particular for BKOEt1, to retain potent channel opener properties. In addition, physicochemical properties were determined and revealed commonalities for compounds to retain potent BK_{Ca} channel opener properties. A confirmatory pharmacophore was proposed with compounds requiring two substituted phenyl rings, the presence of an oxygen containing group, an amide group provided by the linker region and hydrophobic moieties.

II Acknowledgments

I would firstly like to thank Dr Neil McKay and Dr Kim Lawson for all their supervision, guidance and support; this has been much appreciated through-out my studies. I also express my gratitude to Dr Donna Sellers for her advice and help and also Dr Anne-Marie Harrison for her helpful comments with the pharmacophore modelling. All my love to my mum and dad for all their continual love and support and encouragement though-out my life and to Lindsey for all her love and for always being there for me through the ups and downs, smiles and frowns. Finally, thanks to fellow BMRC PhD students and post docs past and present for all the fun times.

III Publications

McKay N.G., Kirby R.W., Lawson K., (2008). Rubidium efflux as a tool for the pharmacological characterisation of compounds with BK channel opening properties. Methods in Molecular Biology. **491**, 267-277.

Published conference proceedings and oral communications

July 2007: LifeSciences 2007, Glasgow, U.K.

Kirby RW *et al* (2007) Assessment of a series of compounds as BK_{Ca} channel openers using an Rb^+ efflux assay. Proc Life Sciences PC154 published online at http://www.physoc.org/publications/proceedings/archive/article.asp?ID=Proc%20Life%20SciencesPC154

Kirby RW *et al* (2007) Identification of the N-arylbenzamide, BKOEt1 as a novel BK_{Ca} channel opener. Proc Life Sciences, PC153 published online at http://www.physoc.org/publications/proceedings/archive/article.asp?ID=Proc%20Life%20SciencesPC153

December 2006: British Pharmacological Society, Winter meeting, Oxford, U.K.

Kirby RW *et al* (2006). Identification of a series of novel N-aryl-benzamide compounds as BK_{Ca} channel openers. Proc Br Pharml Soc at http://www.pA2online.org/abstracts/Vol4Issue2abst107P.pdf

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March 2006: Science, Engineering and Technology (SET) for Britain, London, U.K.

Selected to present a poster at a special parliamentary reception for Britains top young researchers in UK Bioscience and to compete for the 2006 GJ Mendal medal. Presented a poster entitled 'BK Channel Activators: A Therapeutic Approach for unmet clinical conditions'.

September 2007: 17th Annual Cannaux Ioniques, Presqu'ile de Giens, France

Selected to give an oral presentation entitled 'Identification of novel compounds as BK_{Ca} channel openers'

Contents

Chapter 1: Introduction

Chapter 5: Characterisation of BKOEt1 as a novel BK_{Ca} channel opener

Chapter 6: Pharmacophore modelling of benzanilides and general discussion

IV List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

V List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 5

Chapter 6

VI List of Abbreviations

Ab	Antibody
4-AP	4-aminopyridine
ACTZ	Acetazolamide
AAS	Atomic Absorption Spectrometry
ANOVA	Analysis of Variance
BK_{Ca}	Large conductance voltage activated calcium sensitive potassium channel
BK_{Ca} α	HEK293 cells expressing the human BK_{Ca} channel α-subunit only
BK_{Ca} α + β1	HEK cells expressing both the human BK_{Ca} channel α and β1 subunit
BK_{Ca}CO	BK_{Ca} channel opener
BMS	Bristol Myers Squibb
CA	Carbonic Anhydrase
CF_3	Trifluoromethyl group
ChTX	Charybdotoxin
CRC	Concentration response curve
DHAA	dihydroabetic acid
DHS-1	Dehydrosoyasaponin
di-Cl-DHAA	di-chloro dihydroabetic acid
DMEM	Dulbecco's Modified Eagles Media
DMSO	Dimethylsulphoxide
$EC_{40\%}$	Potency; concentration required to increase Rb^+ efflux by 40%
EC_{50}	Effective concentration 50%
ED	Erectile dysfunction
E_{max}	Efficacy: Maximum effect
EWG	Electron withdrawing group
FCS	Foetal Calf Serum
G	Conductance
Gmax	Maximum conductance
G/Gmax	Normalised conductance
Glib	Glibenclamide
H-bond	Hydrogen bond

HBSS	Hank's balanced salt solution
HEK293	Human embryonic kidney cells
hERG	human *ether-a-go-go* related gene
HTS	High throughput screening
hSlo	Human BK_{Ca} channel α-subunit gene
hypoPP	Hypokalemic periodic paralysis
I	Current
ICC	Immunocytochemistry
I_k	Potassium channel current
IK	Intermediate conductance potassium channel
IbTX	Iberiotoxin
IC_{50}	Inhibitory concentration 50%
K_{ATP}	ATP sensitive potassium channel
KCO	Potassium channel opener
K_{Ca}	Calcium-activated potassium channels
K_v	Voltage dependent potassium channel
logP	Octanal/Water partition co-efficient
Marg	Margatoxin
MCAO	Middle cerebral artery occlusion
mSlo	Mouse BK_{Ca} channel m*Slo* α-subunit
Ω	Ohms
OH	Hydroxyl group
Ouab	Ouabain
p-	Para position
Pax	Paxilline
PBS	Phosphate buffered saline
PDE	Phosphodiesterase Inhibitor
PiMA	Pimaric acid
pKa	logarithm of the acid dissociation constant
P_o	Open pore probability
pS	picosiemens
Rb^+	Rubidium
SEM	Standard error of the mean
sGC	Soluble guanylyl cyclase

SK	Small conductance potassium channel
STOC	Spontaneous transient outward currents
TEA	Tetraethylammonium
TPSA	Total polar surface area
VDCC	Voltage-dependent calcium channels
V_{50}	Half maximal voltage of activation
WCR	Whole cell recording

Chapter 1

Introduction

1.1 Potassium channels

Potassium (K^+) selective ion channels are a diverse group of membrane spanning proteins of which there are at least 100 different types (Seebohm 2005). K^+ channel activation leads to the efflux of K^+ ions out of the cell resulting in hyperpolarisation of the cell membrane or returning the membrane potential towards the equilibrium potential of K^+ (E_K), this provides a powerful means of controlling the level of cellular excitability following a depolarisation stimulus (Figure 1.1). Opening of K^+ channels by endogenous mediators (ions, hormones, neurotransmitters etc) provides a negative feedback mechanism leading to the inhibition of several physiological functions including muscle contraction and action potential propagation (Lawson 2000a,b; Lawson and Dunne 2001; Lawson and McKay 2006).

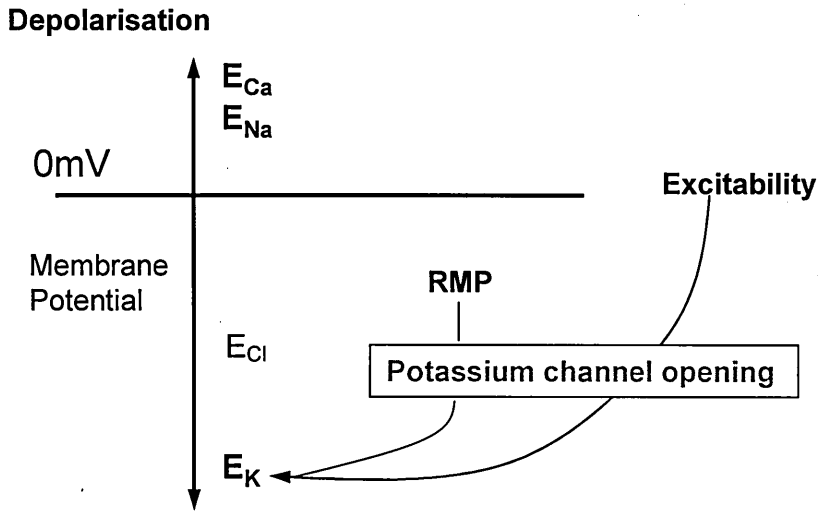


Figure 1.1: Effect of potassium channel opening on membrane potential
Potassium channel opening leads to efflux of K^+ ions resulting in hyperpolarisation of the membrane of the cell dampening or decreasing cell excitability as a consequence of depolarisation. RMP=resting membrane potential, E=equilibrium potential for the specified ion. Modified from Lawson (2000a).

Due to their critical role in physiology there have been many rapid advances in the investigation of K^+ channels and the development of potassium channel openers (KCO) during the past decade (Coghlan and Carroll 2001).

A sub-group of the K^+ channel family are the calcium (Ca^{2+}) activated potassium channels (K_{Ca}). They were first postulated by Gardos *et al* (1958) following observations that changes in potassium permeability across the plasma membrane of erythrocytes occurred in response to minor changes in intracellular calcium. Since then three groups of K_{Ca} channels have been identified that differ in their voltage-dependence and calcium sensitivity and are classified based on their single channel conductance (Garcia and Kaczorowski 1992); large-conductance voltage-activated calcium-sensitive potassium channels (BK_{Ca}), intermediate conductance K^+ channels (IK) and small conductance K^+ channels (SK).

BK_{Ca} channels have received the most attention for their physiological roles and as a potential pharmacological target for un-met clinical conditions.

1.2 Large conductance voltage-activated calcium-sensitive potassium channels (BK_{Ca})

Single channel recordings of BK_{Ca} channel currents were first reported in chromaffin cell membranes (Marty *et al* 1981), cultured nerve cells (Pallota *et al* 1981) and membrane preparations of skeletal muscle t-tubules (Latorre *et al* 1982). BK_{Ca} channels are unique among K^+ channels in demonstrating a large single channel conductance of around 200-300pS in symmetrical 0.1M potassium chloride (KCl), (Marty *et al* 1981; Pallota *et al* 1981; Latorre *et al* 1982). BK_{Ca} channels display a high degree of potassium selectivity with a permeability ratio >50 relative to sodium (Na^+) and to rubidium (Rb^+) and >200 for caesium (Cs^+), (Blatz and Magleby 1984; Latorre *et al* 1989; Gutman *et al* 2003).

BK_{Ca} channels have been characterised pharmacologically by their specific sensitivity to block by the peptide iberiotoxin (IbTX) and the tremorgenic mycotoxin paxilline (pax). Other non-specific blockers of BK_{Ca} channels include tetraethylammonium (TEA) and 4-aminopyridine (4-AP), (Gutman *et al* 2003). The unique feature of BK_{Ca} channels is its 'dual' ability to be activated by both changes in intracellular Ca^{2+} and plasmalemmal membrane voltage.

BK_{Ca} channels are present in many different species such as nematodes and insects (Ghatta *et al* 2006) and the gene was first identified from the slopoke locus (d*Slo*) of *Drosophila melanogaster* (Atkinson *et al* 1991) from which subsequent homologues were cloned from human brain (h*Slo*), mouse (m*Slo*) and skeletal muscle (Butler *et al* 1993; Dworetsky *et al* 1994).

BK_{Ca} channels have since been shown to be ubiquitously expressed in nearly all excitable and non-excitable cell types (Korovkina *et al* 2002). Expression has been reported in various brain regions and within axons, nerve terminals, glioma cells (Tseng-Crank *et al* 1996; Knaus *et al* 1996; Liu *et al* 2002) and within the inner mitochondrial membrane of neurons and cardiomyocytes (Douglas *et al* 2006). BK_{Ca} channels have also been shown to be expressed in smooth muscle of the vasculature (Tanaka *et al* 1997), uterus (Khan *et al* 2001), stomach and bladder. Skeletal muscle (Tseng-Crank *et al* 1994; Tanaka *et al* 1997; Jiang *et al* 1999), epithelial cells (Grunnet *et al* 2005), endothelial cells (Nilius and Droogmans 2001), endocrine cells (Xie and Mccobb 1998; Prakriya and Lingle 1999), neutrophils and eosinophils (Ahluwalia *et al* 2004) also express BK_{Ca} channels.

1.3 BK_{Ca} channel structure

Cloning revealed BK_{Ca} channels to be members of the S4 superfamily of voltage activated ion channels similar to voltage dependent K^+ (K_v), Na^+ (Na_v) and Ca^{2+} (VDCC), (Diaz *et al* 1998). Structurally BK_{Ca} channels consist of a pore forming α-subunit and a regulatory β- subunit. Functional channels can be formed by a tetrameric complex of four α-subunits alone or found in a 1:1 stoichometry associated with four β-subunits (Garcia-Calvo *et al* 1994; Toro *et al* 1998), (Figure 1.2).

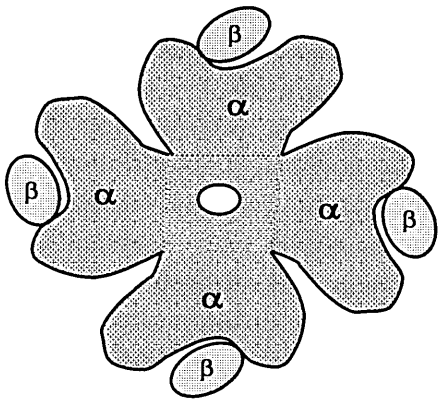


Figure 1.2: Topological structure of BK_{Ca} channels

Proposed topological structure of BK_{Ca} channels highlighting the 1:1 stoichometry of the α and β-subunit. The channel is arranged with the pore formed at the centre by four α-subunits. Modified from and Lu *et al* (2006) and Cox (2007).

1.3.1 BK_{Ca} channel β-subunit

Currently one group of β-subunits (β1-β4) have been identified from mammalian tissues with each subunit encoded by a separate gene (KCNMB1-4), (Uebele *et al* 2000). β-subunits expressed on their own cannot form functional channels (Knaus *et al* 1994a; McManus *et al* 1995) but when co-expressed with BK_{Ca} channel α-subunits have a diversity of regulatory effects on channel function as described in Table 1.1 (Dworetsky *et al* 1996; Meera *et al* 1996; Nimingean and Magleby 1999; Orio *et al* 2004; Orio and Latorre 2005).

β-subunits are smaller than α-subunits and are composed of two transmembrane segments of between 191-270 amino acids and are arranged with intracellular N- and C-termini (Figure 1.3). The two transmembrane segments are linked by a long (116-128 amino acid) extracellular loop containing four putative glycosylation sites and four cysteine residues enabling disulphide bridge formation (Knaus *et al* 1994a; McManus *et al* 1995).

Subunit	Expression	Physiological Effect on α-subunit	Function
β1	Smooth muscle and Cardiac Tissue	↑ Ca^{2+} sensitivity ↓ in voltage dependence of activation Slowing of activation & deactivation kinetics ↓ in potency to block by ChTX	Regulation of vascular smooth muscle tone Stabilisation of voltage sensor ↑ coupling strength between Ca^{2+} spark and STOC
β2	Chromaffin cells, Ovary, uterus and brain	↑ Ca^{2+} sensitivity similar to β1 subunit effect Rapid and complete voltage dependent inactivation Slowing of deactivation kinetic	Repetitive firing of action potentials Regulation of neuro-secretion
β3	Brain, Testis, Spleen Pancreas, 4 splice variants (β3a-d)	Variant specific voltage dependent inactivation usually rapid but not complete ↓ in potency to block by ChTX	Neurotransmitter release Neuronal excitability Mutations associated with idiopathic generalised epilepsy
β4	Abundant distribution in the brain	↓ Ca^{2+} sensitivity Slowing of activation kinetics and rapid deactivation kinetics ↓ in potency to block by ChTX	Neuronal excitability

Table 1.1: The pharmacology and physiology of β-subunits

ChTX=Charybdotoxin, STOC=Spontaneous transient outward currents

The presence of β2 subunit and some splice variants of the β3 subunit impart rapid inactivation properties to BK_{Ca} channels; this is conferred by the presence of an inactivation peptide at the NH_2 terminal of subunits. The inactivation peptide is a hydrophobic domain followed by a region of positive charged residues and is similar to that of Shaker channels. The removal of the NH_2 domain eliminated inactivation properties of these subunits (Zeng *et al* 2001; Orio *et al* 2002). Inactivation has been proposed to occur by the entry of the first 2-4 residues of the cytosolic NH_2 terminus into the pore occluding permeation of K^+ ions (Xia *et al* 2003).

1.3.2 BK_{Ca} channel α-subunit

The α-subunit is derived from a single gene (KCNMA-1) located on chromosome 10 at locus 10q22 that encodes an 1154 amino acid residue subunit. This forms a pore forming α-subunit composed of an exoplasmic N' terminus sequence, a non-conserved linker sequence and eleven hydrophobic domains. These domains contain a voltage sensor, a p-loop pore region and form part of a large intracellular C' terminus tail region (Meera *et al* 1997; Magleby 2003). The core region of the channel is composed of six transmembrane spanning domains (S1-S6) that exhibit high homology to the α-subunit of K_v channels (Toro *et al* 1998). However, BK_{Ca} channels have an additional S_0 transmembrane domain that places the N'terminus extracellular (Wallner *et al* 1996; Meera *et al* 1997). The S_0 domain forms a site for functional coupling with the β-subunit and its consequent facility to modulate BK_{Ca} channel activity (Meera *et al* 1997; Toro *et al* 1998), (Figure 1.3).

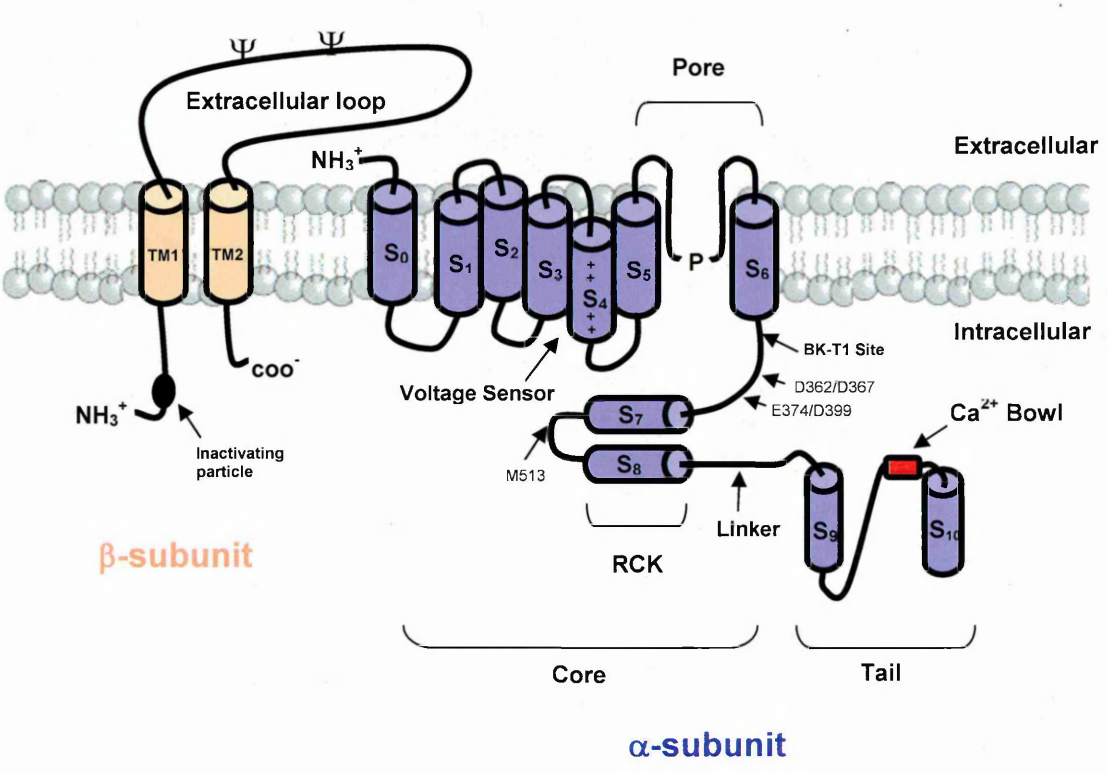


Figure 1.3: **The structure of the BK_{Ca} channel α and β subunit**

Schematic representation of the structure of α- and β-subunits highlighting major structural components, ψ= site of glycosylation. Re-drawn with reference to Magleby 2003 and Ghatta *et al* 2006.

The potassium selective pore is formed at the centre of four α-subunits from a motif that comprises the S_5 and S_6 domains and the p-loop domain. The p-loop from each subunit contains the characteristic K^+ channel selectivity filter motif of the amino acid residues 'TVGTG' which provide carbonyl oxygen atoms arranged to form four binding sites for potassium (Latorre 1989; Orio *et al* 2002; Yellen 2002). The crystal structure of BK_{Ca} channels remains as yet un-resolved, however the crystal structures of five different bacterial potassium channels (MthK, KirBac1.1, K_v1.2, K_vAP) have been reported (Cox 2007). These channels have different transmembrane and cytosolic structures, yet they all share (structurally) identical selectivity filters that span about a third of the way through the bi-layer (MacKinnon 2003; Cox 2007). The large conductance of BK_{Ca} channels equates to ~90 million ions passing through the pore per second, these traverse in single file separated by a water molecule. The mechanism of large conductance is not fully known; although, two negatively charged rings of glutamate residues on the inner pore helix of BK_{Ca} channels increase the local concentration of K^+ from 150 to 500mM (Brelidze *et al* 2003; Cox 2007). Therefore due to the high conductance only a few BK_{Ca} channels need to open to produce a major physiological response.

1.3.3 BK_{Ca} channel α-subunit: tail region

The α-subunit has a large intracellular C'terminus region whose structure is unique to BK_{Ca} channels. This has approximately twice as many amino acid residues (~800) as present in the core region and consists of hydrophobic domains (S7-S10) and sites for regulation by calcium (Wei *et al* 1994; Ghatta *et al* 2006), (Figure 1.4).

The C'termius contains a tetramerisation domain termed BK-T1 which is located near the S6 domain. The exact mechanism of tetramerisaton of BK_{Ca} channels remains unknown (Quirk and Reinhart 2001). The C'terminus also contains sites for regulation by intracellular mediators in addition to binding sites for a number of protein partners. For example, binding of the protein cereblon results in suppression of ionic currents through a decrease in surface expression of the channel (Jo *et al* 2005). Furthermore, a *d*Slo binding protein, dSlip1 expressed throughout the Drosophila nervous system can result in a decrease in BK_{Ca}

channel currents (Lu *et al* 2006) although no mammalian equivalent has been identified. The carboxy terminus has multiple phosphorylation sites and a leucine zipper domain for interaction with other protein structures including protein kinases (Zhou *et al* 2001; Tian *et al* 2003).

1.3.4 Phenotypic diversity of BK_{Ca} channels

BK_{Ca} channels display tissue specific expression of different α-subunit splice variants, moreover these can combine with different β-subunits to form homo or hetero oligomeric combinations of BK_{Ca} channels even within the same cell (Korovkina and England 2002). An example of this has been extensively studied in the avian tonotopic axis, where it was revealed that individual hair cells express multiple subsets of different splice variants of BK_{Ca} channels that are different in composition to adjacent cells lining the cochlear (Fettiplace *et al* 1999; Ramanathan *et al* 1999). Phenotypic variation also occurs as a result of multiple nuclear transcription promoters (Bohm *et al* 2000) and transcriptional regulatory elements such as Ca^{2+}/calmodulin protein kinase and Ca^{2+} regulatory response elements (Xie and Black 2001).

Numerous cellular mechanisms contribute to changes in physical and physiological properties of the channel (Lu *et al* 2006), these include phenotypic variation that can occur at the post transcriptional level such as glycosylation (Hagen and Sanders 2006) and through mechanisms of alternative splicing of RNA (Shipston 2001). Over 10 different splice site variants have been identified, of which most occur within the intracellular C-terminus of the α-subunit (Shipston 2001; Qian *et al* 2002) although only few have shown to have major physiological effects. The most widely characterised example is 'stress axis' regulated exon (STREX) which is a cysteine rich insert of 59 amino acids whose expression has been detected in a variety of excitable cells (Shipston *et al* 1999). Inclusion of STREX leads to an increase in calcium and voltage sensitivity of the channel and confers inhibition on the channel by protein kinase A (PKA) phosphorylation. Investigation into the dynamic regulation of the inclusion of this splice variant in the channel and its regulation by hormones, protein kinases and response elements that have been described by Shipston *et al* (2001), Tian *et al* (1998) and Erxleben *et al* (1999).

1.4 The functional role of BK_{Ca} channels

The unique feature of BK_{Ca} channels is its 'dual' ability to be activated by both changes in intracellular Ca^{2+} and plasmalemmal membrane voltage. Meech (1978) was the first to propose that K_{Ca} channels could provide a role in 'linking cellular metabolism to electrical activity'. BK_{Ca} channels provide this via a negative-feedback mechanism to control the level of cellular excitability by returning the membrane to a hyperpolarising state reducing the activity of voltage dependent Ca^{2+} channel activation (VDCC), (Figure 1.4), (Latorre *et al* 1989; Lawson 2001a; Lawson 2001b; Latorre and Brauchi 2006). BK_{Ca} channels lend themselves to facilitating this process due to their large conductance.

Further it has been identified that BK_{Ca} channels co-localise with VDCC (L-type, N-type, P/Q type) in rat brain forming a Ca^{2+} nanodomain, where Ca^{2+} influx through VDCC activates BK_{Ca} channels in a physiological voltage range with sub-millisecond kinetics (Prakriya and Lingle 1999; Berkefeld *et al* 2006; Loane *et al* 2007).

An increase in BK_{Ca} channel activity can be induced by the localised release of Ca^{2+} from the sarcoplasmic reticulum (Ca^{2+} sparks) that produce spontaneous transient outward currents (STOC) generated by BK_{Ca} channel activation. This then leads to hyperpolarisation of the membrane and prevention of influx of Ca^{2+} (Benham and Bolton 1986; Brenner *et al* 2000; ZhuGe *et al* 2000), (Figure 1.4).

Therefore, BK_{Ca} channel function is important in the regulation of depolarisation and firing patterns in neurons (Gribkoff *et al* 2001a; Sah and Faber 2002; Berkefeld *et al* 2006), neurotransmitter release (Robitaille *et al* 1993; Gribkoff *et al* 2001a; Wang *et al* 2001), chemosensing control in the vasculature (Brayden and Nelson 1992; Neslon and Bonev 2004), smooth muscle contraction (Giangiacomo *et al* 1995; Korovkina *et al* 2002) and electrical tuning of non-spiking properties of inner ear hair cells (Ramanathan *et al* 1999). Also BK_{Ca} channels are important in regulating steroidogenesis (Kunz *et al* 2002), hormone secretion and phagocytic killing in neutrophils (Ahluwalia *et al* 2004).

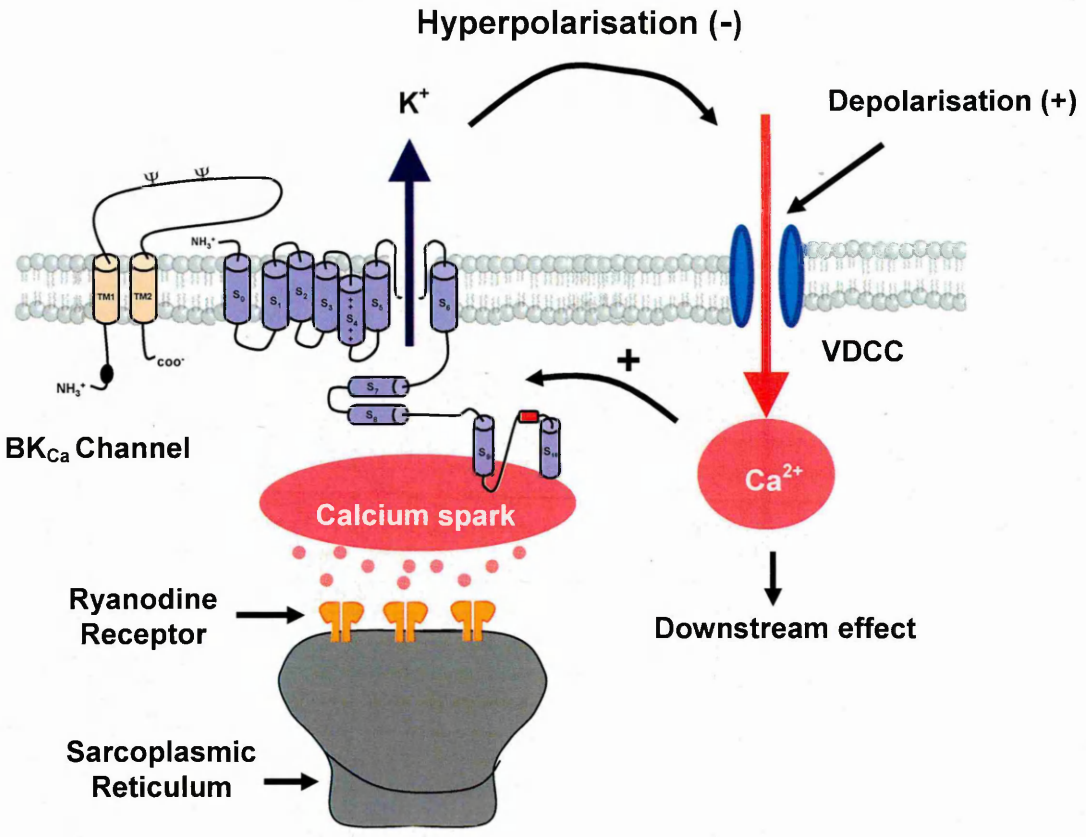


Figure 1.4: BK_{Ca} channel as a regulator of cellular excitability

Schematic representing pathways of BK_{Ca} channel activation in smooth muscle. Depolarisation activates VDCC that leads to Ca^{2+} entry in the cell and resultant physiological effect. Intracellular Ca^{2+} build up via VDCC activation or from Ca^{2+} sparks activates BK_{Ca} channels leading to efflux of K^+ resulting in hyperpolarisation and inhibition of cellular excitability. Re-drawn and modified from Nelson and Bonev (2004).

The physiological importance of BK_{Ca} channels has been highlighted through the use of genetically modified (knock-out) animals by removing particular BK_{Ca} channel subunit genes (Magleby 2003). The effect of deleting the β-subunit gene has been widely studied in smooth muscle of the vasculature. β1 subunit knock-out mice demonstrate a decreased level of STOC's with functional coupling of calcium sparks to STOC's shifted to more depolarised potentials, that consequently resulted in hypertension in the animal models (Brenner *et al* 2000; Pluger *et al* 2007), (Table 1.1). Thus the β1 subunit is critical to the negative-feedback regulation of vascular tone. This coupled to recent evidence

that β1 subunit expression becomes decreased with age (Marijic *et al* 2001; Nishimaru *et al* 2004) has identified BK_{Ca} channel modulation as a therapeutic approach for hypertension.

Human genetic studies have also implicated the β1 subunit in hypertension; Fernandez-Fernandez *et al* (2004) in a larger human study showed that a single-nucleotide substitution (G352A) in the β_1 subunit gene (*KCNMB1*) resulted in an E65K gain of function mutation. The frequency of the E65K mutation decreased with increasing diastolic blood pressure leading to a low prevalence of moderate to severe diastolic blood pressure. In a recent larger term study the gain-of-function mutation reduced the incidence of stroke and myocardial infarction in women (Senti *et al* 2005).

1.5 Modulation of BK_{Ca} channel function

Sequence analysis and topological determination of the BK_{Ca} channel revealed that certain structural motifs could account for regulation of the channel by voltage and calcium. As a result there has been an increased interest into the regulatory effects of voltage and calcium on BK_{Ca} channel activation (Magleby 2003; Cox 2007). Recent research has also highlighted a number of different classes of endogenous physiological stimuli that can modulate BK_{Ca} channel function.

1.5.1 Voltage sensitivity

The S_4 subunit has shown to confer the channel with its intrinsic voltage sensitivity (Stefani *et al* 1997), with partial contribution by acidic residues present in the S_2 domain (Seoh *et al* 1996) and S_3 domain (Papazain *et al* 1995). Voltage sensitivity is imparted by a repeated triple sequence of positively charged amino acids (either argenine or lysine) separated by two hydrophobic residues (Stefani *et al* 1997; Diaz *et al* 1998). The exact molecular mechanism of voltage dependent activation of BK_{Ca} channels is thought to be similar to that of other K^+ channels. Depolarisation results in the movement of the charged residues that provide gating currents that result in a conformational change causing a bend in the inner helix lining the pore at a conserved glycine gating hinge point (Stefani *et al* 1997; Ghatta *et al* 2006). The possession of a voltage sensor domain confers BK_{Ca} channels with an ability to open in the absence of

calcium but only at high depolarising voltages (Toro *et al* 1998; Cui and Aldrich 2000). It has been proposed that calcium acts to decrease the intrinsic configuration energy difference that separates the closed from the open state (Diaz *et al* 1998).

1.5.2 Calcium sensitivity

Wei *et al* (1994) showed that cutting the channel of *mSlo* and *dSlo* at a non-conserved linker region between S_8 and S_9 domains yielded two molecular regions, a core (S_0-S_8) and tail (S_9-S_{10}), that when expressed together produced identical calcium sensitivity to wild type channels. This led to the identification of a site of calcium regulation in the intracellular C-terminus of the channel. This was confirmed by the work of Schreiber and Salkoff (1997), in that the intrinsic sensitivity to activation by intracellular calcium ions was conferred upon the channel by a characteristic calcium binding EF hand motif in the cytoplasmic tail. This "calcium bowl" region contained high affinity calcium regulatory sites. The calcium bowl region occurs prior to the hydrophobic S_{10} subunit and consists of ten negative charges, five of which are derived from five consecutive aspartate residues (Schreiber and Salkoff 1997), (Figure 1.5). This sequence is conserved (except residue 889) between nematode, mouse, fly and humans. Removing or replacing negative charges within the calcium bowl greatly decreased calcium sensitivity (Magleby 2003). Additionally binding experiments confirmed that the calcium bowl was a high affinity calcium binding site and that each functional calcium bowl (of the tetrameric channel) added a stepwise increase to the Hill co-efficient, (a measure of co-operativity in ligand binding) for calcium binding of 0.3-0.8 (Schreiber and Salkoff 1997; Schreiber *et al* 1999; Niu and Magleby 2002).

883 TELVN**D**TNVQFL**DQDDDDD**P**D**TELYTQ

Figure 1.5: The primary amino acid structure of the Ca^{2+} bowl
Sequence shows the highly conserved calcium bowl amino acid sequence. Bold letters Indicate negative charged residues. E=gluatmic acid, D=aspartic acid. Re-drawn and adapted from Magleby 2003.

BK_{Ca} channels can respond to calcium over four fold orders of magnitude from 500nM to 50mM (Schreiber *et al* 1999; Xia *et al* 2002; Magleby 2003); this would support more than one site of Ca^{2+} regulation of the channel. Schreiber *et al* (1999) replaced the tail of BK_{Ca} channel α-subunit (Slo1) with the tail of the *Slo3* channel (that lacks a calcium bowl), the chimeric channel was insensitive to low calcium concentrations, however was activated at higher levels of calcium (>300μM) suggesting that sites separate from the calcium bowl contribute to the broad sensitivity of BK_{Ca} channels to Ca^{2+} (Schreiber *et al* 1999). The mechanisms of calcium regulation of BK_{Ca} channel remain unknown; in an attempt to further understand calcium regulation of the BK_{Ca} channel, site directed mutagenesis has been extensively used and has revealed different types of calcium sensors that act independently from one another to increase the activity of BK_{Ca} channels. In addition to the calcium bowl other high affinity sensors include the D36S/D367 and D81E374/E399 in addition to low affinity calcium/magnesium site (E374/E399), (Figure 1.3), (Magleby 2003).

A second region to the calcium bowl the 'regulator of conductance' for potassium (RCK) domain was identified by Jiang *et al* (2001) following alignment of C' terminal sequences of BK_{Ca} channels to that of different bacterial K^+ channel sequences. The RCK domain adopts a Rossmann fold structure that is conserved among BK_{Ca} channels of eukaryotic and prokaryotic potassium channels (Hu *et al* 2003). Xia *et al* (2002) showed that the RCK domain contained residues that enable two sites of regulation of the BK_{Ca} channel, the first site in association with the calcium bowl accounts for the physiological regulation of BK_{Ca} channels by calcium and the second site contributed to the effects of other divalent cations particularly milli-molar magnesium on channel activation (Niu and Magleby 2002), this site has been shown to include the E374 and E399 low affinity regulation site (Magleby 2003), (Figure 1.3).

Therefore, the calcium bowl has a high-affinity (<10μM) for calcium and the RCK site has a low affinity for calcium and magnesium (>100μM), it is thought that regulation of these channels over many sites enables BK_{Ca} channels to respond to a broad range of calcium concentrations. Hill co-efficient of 2-5 for BK_{Ca} channels indicate that calcium acts in a co-operative manner and

suggests that 2-5 calcium ions must be bound to allosteric activators to maximally activate the channel (Niu and Magleby 2002). In complete contrast to the extensive work on sites of Ca^{2+} regulation of channel function, Piskorowski and Aldrich (2002) showed that when the whole cytoplasmic tail is deleted Ca^{2+} and voltage dependence is unaltered implying Ca^{2+} sensitivity is located within the 'core' S0-S6 region. Therefore, a lack of understanding still remains in the mechanism of Ca^{2+} activation of the channel, this is in part down to a lack of crystal structure of BK_{Ca} channels in particular of the Ca^{2+} bowl region (Moczydolowski 2004).

Cui and Aldrich (2000) propose that calcium and voltage have additive effects upon channel stimulation and opening. It has been proposed that the calcium binding sites and voltage sensors do not directly interact and that an allosteric mechanism of channel opening occurs that could facilitate the open and closed conformations of the pore (Cui and Aldrich 2000; Moss and Magleby 2001).

1.5.3 Biochemical and physiological mediators

BK_{Ca} channels can be modulated by a plethora of other physiological stimuli including sulphatides (Chi and Qi 2006), glucocorticosteroids (Tian *et al* 1998), 17β-oestradiol (Valverde *et al* 1999), insulin (O'Malley and Harvey 2004), nitrous oxide (NO), (Gruhn *et al* 2002), bile acids (Dopico *et al* 2002), fatty acids (Clarke *et al* 2003) the inhibitory amino acid γ-aminobutyric acid (GABA) (Baragatti *et al* 1999), ethanol (Brodie *et al* 2007) hypoxia (Lewis *et al* 2002), cannabinoids (Sade *et al* 2006). More recently numerous protein partners to BK_{Ca} channels have been identified (Lu *et al* 2006) and include calveolin-1 (Wang *et al* 2005) and haemoxygenase (Williams *et al* 2004). BK_{Ca} channels have also shown to form macromolecular complexes with β2 adrenergic receptors in smooth muscle (Liu *et al* 2004; Lu *et al* 2006).

BK_{Ca} channels have shown diversity in their sensitivity to protein phosphorylation (Schubert and Nelson 2001) and with association of G-proteins and intracellular signalling pathway intermediates such as cyclic AMP and cyclic GMP (Zhou *et al* 2001) and protein kinases (Shubert and Nelson 2001).

The function of BK_{Ca} channels can also be governed by metabolic regulation in that vasoconstrictors such as angiotensin II and thromboxane A2 have shown to inhibit BK_{Ca} channels via src tyrosine kinase phosphorylation of the channel (Alioua *et al* 2002; Lu *et al* 2006). Whereas smooth muscle vasodilators such as prostaglandin, NO and arachdonic acid induce Ca^{2+} sparks that activate BK_{Ca} channels (Schubert and Nelson 2001; Lu *et al* 2006), (Figure 1.4).

1.6 BK_{Ca} channels: A therapeutic target for clinical conditions

Rapid progress into the pharmacology of potassium channels has been facilitated by the introduction of modern ion channel research technologies. This coupled with advances in bioinformatics and molecular biology has enabled better characterisation of ion channel structure, distribution and the regulatory mechanisms involved in the modulation of gating and physical properties. This in turn has enabled the development of improved pharmacological tools to study ion channels (Calderone 2002).

Over the last couple of decades there has been rapid progress in the development of therapeutic ion channel modulators. Ion channel drugs represent 15 of the top 100 selling drugs world-wide totalling $5 billion in global sales (England 1999; Cox *et al* 2002). Examples include sodium channel blockers used as local anaesthetics or class I antiarrhythmic agents including lidocaine and disopiramide. Calcium channel blockers such as verapamil and nifedipine have been used as major drugs in the treatment of hypertension and angina. In addition, GABA-activated channels are the targets for barbiturates and benzodiazepines and are utilised in the treatment of conditions associated with excitability of the nervous system (Kazic *et al* 1999).

The structural diversity of BK_{Ca} channels coupled with their response to a plethora of physiological stimuli and their central role in physiological processes has highlighted them as potential 'tissue specific' targets. Numerous reports have suggested that synthetic openers of the BK_{Ca} channel could provide therapeutic agents in a number of pathophysiological conditions including several un-met clinical conditions. These are stroke (Gribkoff *et al* 2001b; Jensen *et al* 2002), coronary artery spasm (Marijic *et al* 2001), epilepsy (Graves 2006), progressive hearing loss (Ruttiger *et al* 2004), bladder over-reactivity and

urinary incontinence (Hewawasam *et al* 2002d; Turner *et al* 2003; Meredith *et al* 2004), asthma (Ise *et al* 2003), chronic cough (Fox *et al* 1996; Belvisi and Geppetti 2004), hypertension (Korovkina *et al* 2002; Nelson and Bonev 2004), erectile dysfunction (Hewawasam *et al* 2004; Boy *et al* 2004), colonic and gastric hypermotility (Sivarao *et al* 2005), dup(3q) syndrome (Riazi *et al* 1999), hypokalemic periodic paralysis (hypoPP) (Tricarico *et al* 2004) and psychoses (Kaczorowski *et al* 1996; Coghlan and Carroll 2001; Gribkoff *et al* 2001b).

Paroxymal dyskinesia was recently identified as the first BK_{Ca} channelopathy and is caused by single missense mutation in the regulator of conductance (RCK) domain of the channel protein (Du *et al* 2005). BK_{Ca} channel function has been implicated in the pathology of schizophrenia (Zhang *et al* 2006), Alzheimer's disease (Chi *et al* 2000) and age related disease in the cardiovascular system (Marijic *et al* 2001; Nishimaru *et al* 2004).

Christ and colleagues have investigated the use of delivering cDNA encoding BK_{Ca} channels as a gene therapy technique (Christ *et al* 2001; Christ 2002). Examining the streptozotocin induced diabetes rat model it was observed that a decline in BK_{Ca} channel expression occurred in corporal tissue with age and that it was associated with the development of erectile dysfunction (ED). Further, that gene transfer of cDNA encoding h*Slo* fully restored age or diabetes induced ED with relatively low levels of h*Slo* transfection rates. The BK_{Ca} channel was thought to produce enhanced hyperpolarising ability of corporal smooth muscle in response to cellular activation by neurotransmitters (Christ 1998; Christ 2002). Christ *et al* (2001) also showed that injection of h*Slo* cDNA ameliorated obstruction-associated bladder hyperactivity. Therefore, gene transfer of BK_{Ca} channel subunits could offer a therapeutic strategy for treatment of bladder ovaeractivity and urinary incontinence (Christ *et al* 2001; Christ 2002).

Compound	Clinical benefit	Clinical trial stage
[a] Bristol Mayer Squib **BMS-204352/MaxiPost**	post stoke neuroprotection	Discontinued (2001) after phase III
[b] Nippon Shinyaku and Apogepha Arznemittel **NS-8**	Overactive Bladder	Discontinued (Jan 2007) after completion of phase I
[c] Tanabe and GlaxoSmithKline **TA-1702**	Overactive Bladder	Phase I
[d] Rottapharma **CR-2039/Andolast**	Bronchial Asthma Allergic Rhinitis COPD	Phase II allergic rhinitis and COPD and Phase III for Bronchial Asthma

Table 1.2: BK_{Ca} channel opener compounds submitted for clinical trials
(a) Jensen (2002), (b) Japan Corporate News (2007), (c) Mitsubishi Tanabe Pharma (2007), (d) Rottapharm (2007).

The development of medium to high throughput pharmacological screening assays including rubidium efflux, membrane potential and binding assays, (Bennett and Guthrie 2003) has facilitated industry led developments in the identification of synthetic potassium channel openers (KCO) with a recent focus, over the past decade, into the development of BK_{Ca} channel openers ($BK_{Ca}CO$). As a consequence a few synthetic BK_{Ca} channel openers have been advanced to clinical trials for the treatment of stroke, bladder over-activity and asthma (Table 1.2). Neurosearch A/S and TopoTarget have developed the therapeutic use of $BK_{Ca}CO$ to enhance the transport of anti-cancer drugs into brain tumours and are testing certain a compound termed NSD-551 (structure undisclosed) to pre-clinical testing (Neurosearch A/S 2005).

The most well known compound advanced to clinical trail as a $BK_{Ca}CO$ was Maxipost (BMS-204352) developed by the work of Gribkoff and colleagues at Bristol Myers Squibb (BMS) for use in post-stroke neuroprotection. In the middle cerebral artery occlusion (MCAO) rat stroke model Maxipost was able to reduce

cerebral oedema and improve neuromotor function by preventing toxic Ca^{2+} build up within the cell that is associated with the pathogenesis of this condition (Gribkoff *et al* 2001a; Gribkoff *et al* 2001b). However, following phase III clinical trials Maxipost was withdrawn because of a lack of clinical efficacy (Jensen 2002), although a second phase III study is being considered (Davis 2006). There has been a lack of published *in-vivo* work studying the potential of $BK_{Ca}CO$ to provide therapeutic benefit in different animal models, this in part is preventing the potential therapeutic benefits of $BK_{Ca}CO$ from being realised (Lawson and McKay 2006).

1.7 BK_{Ca} channel openers ($BK_{Ca}CO$'s)

A number of $BK_{Ca}CO$'s have been discovered through an empirical approach; where compounds are selected from a large pool of molecules such as found in fermentation broths and plant extracts or identified through serendipitous means (Lawson 2001a,b). The advent of structure based ligand/drug design has led to a decline in the empirical selection of pharmacological agents. However, Van Regenmortel (2000) argues that design and empirical selection should not be seen as separate but should be complimentary. For example, molecules identified as $BK_{Ca}CO$'s, using an empirical approach, could be used as templates to guide the design of other compounds. Further, using similar structured compounds the requirements of a particular compound to be BK_{Ca} channel opener can be determined. Due to the lack of 3D structural information of BK_{Ca} channel function ligand-protein modelling has been limited (Li and Harte 2002). One such approach for the elucidation of $BK_{Ca}CO$'s is to define and develop pharmacophores. Langer and Wolber (2004) define a pharmacophore as 'an ensemble of steric and electrostatic features of different compounds which are necessary to ensure optimal supramolecular interactions with a specific biological target structure to trigger or block a biological response'

Therefore, the ability to develop subtype specific BK_{Ca} channel openers through pharmacophore identification will be useful in understanding the basic pharmacology of these specific subsets and could provide selective tissue specific therapeutics in numerous diseases. This approach has been used to guide the design and development of a number of novel heterogeneous classes

of compounds. The development of pharmacophores through rationale design could infer knowledge about the structure of BK_{Ca} channels and provide further understanding of the function of BK_{Ca} channels at the molecular level.

The development of high throughput screening technology as alternative to functional tests in combination with developments in combinatorial chemistry libraries has enabled rapid development in drug discovery of ion channel modulators. The progress in the identification and the elucidation of $BK_{Ca}CO$ has lagged behind that of the development of K_{ATP} channel modulators (Lawson and Dunne 2001) where detailed descriptions of pharmacophores and differential specificity profiles of different K_{ATP} channel openers including cromakalim, pinacidil and nicorandil has been described (Lawson 2000; Coghlan and Carroll 2001).

Compounds identified as $BK_{Ca}CO$ can be grouped broadly based on the source of their discovery. For example compounds have been discovered through empirical selection from natural sources including plant extracts and fermentation broths or identified as ancillary effects of other drugs and pharmacological agents, but over the past decade there have been many rapid advances in the development of novel synthetic compounds as $BK_{Ca}CO$'s.

This thesis will review BK_{Ca} channel openers discovered and disclosed to date. Furthermore, identify the synthetic pharmacophores being studied by different research groups and highlight the particular features of these compounds that are intrinsic to them displaying $BK_{Ca}CO$ properties and describe issues concerned with the development and elucidation of $BK_{Ca}CO$'s.

Chemical structures displayed in the following figures have been drawn using Chemdraw version 10 software (Cambridgesoft, Cambridge, U.K).

1.8 $BK_{Ca}CO$ isolated from natural sources

A pharmacognosy approach has identified $BK_{Ca}CO$ properties for a number of compounds isolated from natural sources that had historically been shown to treat symptoms that could be therapeutically relieved by BK_{Ca} channel modulation (Wu *et al* 2006).

1.8.1 Terpenes

Researchers from Merck Laboratories, using a ligand binding assay based on the use of the radiolabelled BK_{Ca} channel blocker, charybdotoxin (^{125}I-ChTX), were the first to isolate and identify compounds with $BK_{Ca}CO$ properties from natural sources. The first example, dehydrosoyasaponin (DHS-1), (Figure 1.6) was isolated from the medicinal herb *Desmodium adscendes* that was traditionally used in Ghana as a powerful remedy against asthma (McManus *et al* 1993). Application of low concentrations of DHS-1 (10nM range) led to a 50-fold increase in open pore probability by stabilising the open state conformation of the channel (Giangiacomo *et al* 1998). Interestingly, DHS-1 only activated channels when applied to the intracellular aspect of the channel and could not activate the channel in the absence of intracellular calcium (McManus *et al* 1993), (Figure 1.9). DHS-1 activation is dependent upon the co-expression of the β-subunit as BK_{Ca} channels composed of only four α-subunits were insensitive to DHS-1 up to concentrations of 500nM (McManus *et al* 1995).

McManus *et al* (1993) proposed that the binding site is probably as long as the length of the molecule and could be allosterically coupled between the α-subunit and to a region within the β-subunit and suggested the rigid triterpene portion could bind to a hydrophobic site on the channel. Chemical modifications to the structure have showed that the first two sugar residues are required for maximal activation and proposed that the molecule aligns orthogonal to the plane of the membrane with the sugar molecules facing the hydrophillic cytoplasmic side (McManus *et al* 1993; Giangiacomo *et al* 1998). Furthermore, DHS-1 did not display any ancillary pharmacology to SK channels, K_{ATP} channels and L-type Ca^{2+} channels (McManus *et al* 1993).

Another compound identified by researches at Merck was MaxiKdiol (1,2-dihydroxyisoprimane diterpenoid), (Figure 1.6) isolated from the fermentation broth of an unidentified coelomycete. Although structurally dissimilar to DHS-1, MaxiKdiol, like DHS-1, only activated BK_{Ca} channels when applied to the cytoplasmic side of the channel, however unlike DHS-1 it was less potent with a higher threshold of activation (1-10μM) and activation of BK_{Ca} channels was not dependent upon the presence of a β-subunit (Singh *et al* 1994), (Figure 1.9).

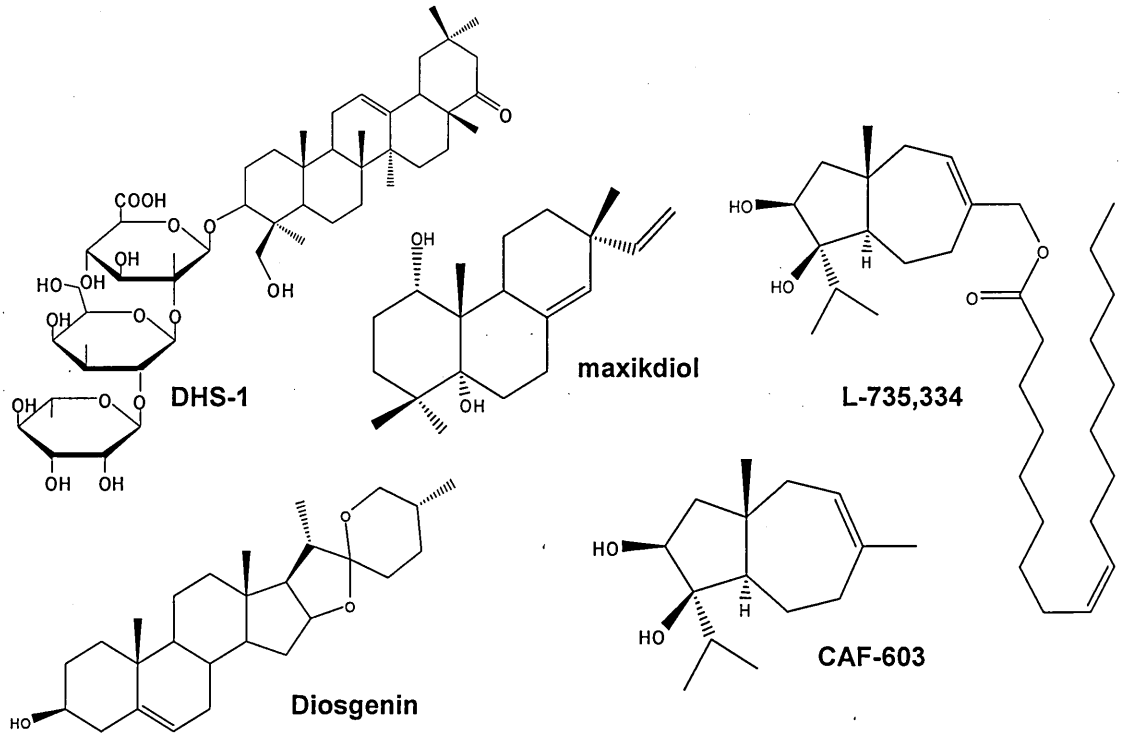


Figure 1.6: Terpinoids

Other terpene derivatives identified by Merck Research Laboratories using ^{125}I-ChTX assay include isolation of the carotene sesquiterpene CAF-603 (Figure 1.6) from the fermentation products of *Arthinium Phaesospermum* whose structure was identical to a product with anti-fungal activity isolated from *Trichoderma Virens*. Although, CAF-603 caused significant displacement of radio-labelled ChTX it did not demonstrate $BK_{Ca}CO$ effects (Ondeyka *et al* 1995). However an oleic acid ester, L-735,334 (Figure 1.6) isolated from the same culture of *T.Virens* showed slow effects at activating BK_{Ca} channels and was only effective, like maxiKdiol, when applied to the intracellular aspect of the channel (Lee *et al* 1995c), (Figure 1.9). Poor cell membrane permeability of this

group of terpenoids limited their use as *in-vitro* experimental tools and as therapeutic agents (Nardi *et al* 2003).

Another terpenoid investigated for $BK_{Ca}CO$ properties was diosgenin (3β-hydroxy-5-spirostene), (Figure 1.6). This a plant steroid isolated from the root of wild yam that can be enzymatically converted *in-vivo* into human steroid hormones progesterone and oestrogen and has been used to treat post-menopausal symptoms. Wang *et al* (2006) investigated its effects on BK_{Ca} channels in human cortical neuron cells (HCN-1A). Diosgenin failed to directly activate BK_{Ca} channels when applied to the intracellular aspect of the channel during inside-out patch recordings. This coupled to it not having an effect on single channel conductance led them to conclude that it must activate BK_{Ca} through an indirect mechanism. They showed that diosgenin increased P_o of BK_{Ca} channels in HCN-1A cells resulting from an increase of calcium within the cell, potentially as a consequence of VDCC activation (Wang *et al* 2006), (Figure 1.9).

1.8.2 Phenols

A number of BK_{Ca} CO's have been discovered from plant extracts, in particular a number of phenolic based compounds (Figure 1.7). Magnolol isolated from the Chinese medicinal herb *Magnolia officinalis* is one of the active components in the herbal medicine Saiboku-to traditionally used to treat bronchial asthma (Wu *et al* 2002). Trans-resverstrol is a naturally occurring phytoalexin present in grapes and wine thought to have potent antioxidant and cardio-protective effects (Li *et al* 2000). These phenols activated BK_{Ca} channels concentration dependently,by causing a shortening in the closed and a lengthening in the open time of the channel. In addition both compounds did not affect the affinity of Ca^{2+} binding or single channel conductance. Therefore, activation of BK_{Ca} channels by these compounds may underlie their anti-asthmatic and potential cardio-protective effects (Li *et al* 2000; Wu *et al* 2002).

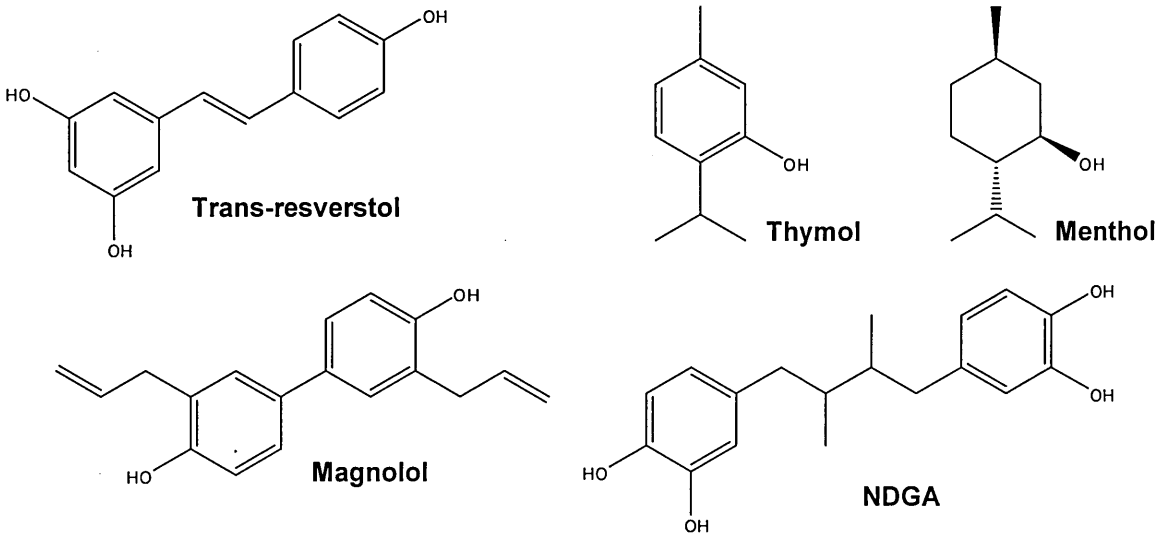


Figure 1.7: Phenolic compounds

The phenolic nordihydroguaiaretic acid (NDGA) is a natural product contained in the Creosote bush that is used as a natural antioxidant in fats and food oils and as a nutritional supplement (Figure 1.7). NDGA has been demonstrated to be an inhibitor of lipoxygenase and cyclooxyenase and at high concentrations will inhibit mitochondrial respiration. In smooth muscle cells NDGA directly activated BK_{Ca} channels independent of its effects on lipoxygenase by association with the α-subunit increasing the channels sensitivity to calcium (Yamamura *et al* 2002). At higher concentrations, NDGA could release Ca^{2+} from caffeine/ryanodine sensitive Ca^{2+} storage sites, it affects on Ca^{2+} storage site were thought to be associated with its ability to inhibit mitochondrial respiration (Yamamura *et al* 2002), (Figure 1.9).

Thymol (2-isopropyl-5-methylphenol) is a phenolic monoterpene found in plant oil of *Thymus Vulgaris* (Thyme) and is used widely in medical and industrial applications in particular as a stabilising reagent for volatile anaesthetics (Figure 1.7). Thymol activated BK_{Ca} channel currents from GH_3 rat pituitary cells increasing P_o with an EC_{50} of 75μM. However, in cell attached recording, thymol applied to the bath was inhibited upon extracellular application of the Ca^{2+} chelator BAPTA, whether thymol affects Ca^{2+} binding on the channel remains unknown. However, application of thymol to the intracellular aspect of excised patches resulted in activation of BK_{Ca} channels whereas the structurally similar menthol did not, therefore the underlying the mechanisms of thymol activation

of BK_{Ca} channels could be helpful in understanding neuronal or endocrine functions of the medicinal herb (Huang *et al* 2005).

1.9 BK_{Ca}CO identified as ancillary effects of other agents

As well as the identification of BK_{Ca} channel openers from natural sources there has more recently been a number of synthetic enzyme inhibitors, cell signalling activators and therapeutic agents reported as BK_{Ca}CO's.

1.9.1 The effect of enzyme inhibitors and cell signalling activators

A number of synthetic pharmacological agents used in the study of intracellular signalling enzymes and pathways have been determined as potential BK_{Ca}CO's (Wu 2003; Wu *et al* 2006). These include xanthine and non-xanthine based phosphodiesterase (PDE) inhibitors, protein kinase activators and soluble cyclic guanylyl cyclase (sGC) activators (Table 1.3); all have shown differential effects on BK_{Ca} channel activation (Figure 1.9). These agents can act via direct association with the channel or as an indirect consequence of their effects on intracellular cell signalling enzymes and pathways that lead to BK_{Ca} channel activation (see Figure 1.8). These ancillary agents activate BK_{Ca} channels at concentrations similar to their primary pharmacological effect. Therefore these agents as therapeutic BK_{Ca}CO's are disadvantageous as they display various ancillary pharmacology and are not specific to BK_{Ca} channels.

SCA-40, an imidazopyrazine, was developed by Zeneca as a smooth muscle relaxant for the treatment of asthma (Lawson 1997). It was initially thought to exert its vasorelaxant effect via BK_{Ca} channel activation in smooth muscle airways (Bonnet *et al* 1992; Cortijo *et al* 1997). However subsequent to this, SCA-40 has demonstrated differential IbTX sensitive effects in different tissues with organ bath experiments and when tested with electrophysiology or ^{86}Rb efflux it did not activate BK_{Ca} channels (MacMillan *et al* 1995; Cook *et al* 1995; Lawson 1997). Around the same time it was demonstrated that like structurally related theophylline, SCA-40 was a potent PDE inhibitor. Inhibition of PDE and consequent elevation of cyclic nucleotides could lead to BK_{Ca} channel activation without SCA-40 having direct effects upon channel activation (Lawson 1997; Cortijo *et al* 1997).

Compound	Pharmacological Action	Therapeutic Use	Identification as $BK_{Ca}CO$ (Reference)
YC-1	sGC activator	Initially developed for treatment of circulatory disorders	Wu *et al* 2000 [a]
BAY-41-2272	sGC activator	Initially developed for treatment of erectile dysfunction	Liu and Wu 2005
KMUP-1	PDE Inhibitor	none	Wu *et al* 2005
Theophylline	PDE Inhibitor	Bronchodilator Treatment of COPD	Ise *et al* 2003
Vinpocetine	PDE Inhibitor	Isolated from leaves of Vinca minor used as for its neuroprotective properties	Wu *et al* 2000[b]
Cilostazol	PDE Inhibitor	Treatment of symptoms of intermittant claudation	Wu *et al* 2004
LY-171883	Leukotriene D4 antagonist	Initially proposed as a bronchodilator	Li *et al* 2002
Rottlerin/Mallotoxin	PKCδ inhibitor	Patent filed as a $BK_{Ca}CO$ for treatment of hypertension	Zakharov *et al* 2005 Marx and Zakharov 2006 Wu *et al* 2007

Table 1.3: Synthetic cell signalling enzyme activators and inhibitors identified as BK_{Ca} channel openers.

COPD=Chronic obstructive pulmonary disease

Compound	Pharmacological Action	Therapeutic Use	Identification as a $BK_{Ca}CO$ (Reference)
Chloroxazone	modulation of polysynaptic reflexes in spinal cord	Centrally acting muscle Relaxant	Liu *et al* 2003
Zonisamide	Potential block of Na_V channels or GABA interaction	Antiepileptic	Huang *et al* 2007
Amiodarone derivative KB130015	Multiple actions on ion channels	Cardiac anti-arrhythmic	Gessner *et al* 2007
Unoprostone	FP receptors in trabecular network	Glaucoma: reduction of intraoccular pressure	Cuppoletti et al 2007
Evans Blue	Associates with albumin in circulation	Dilution indicator for measurement of blood volume and vascular permeability	Hollywood *et al* 1998 Yamada *et al* 2002
Fenamates	Inhibition of cyclo-oxygenase	Common non steroidal anti Inflammatory drugs	Ottolia and Toro 1994 Wu *et al* 2001
Tamoxifen	Oestrogen receptor modulator	Anti cancer drug	Dick et al 2001 Duncan 2005 Coiret et al 2007
ICI-187,780	Oestrogen receptor antagonist	Anti cancer drug	Dick 2002

Table 1.4: Therapeutic agents identified as having $BK_{Ca}CO$ properties

FP= prostaglandin F receptor

1.9.2 Ancillary effects of therapeutics and agents

There have been a few reports of currently used therapeutics for a number of different conditions that have also shown to be able to activate BK_{Ca} channels. Further that BK_{Ca} channel activation could account for the mechanism of action of these drugs in relieving symptoms, such as zonisamide and chloroxazone (See table1.4). In addition, certain drugs such as tamoxifen and fenamates have shown to also activate BK_{Ca} channels as a secondary effect to their primary site of action and that activation of BK_{Ca} channels may underlie the clinical benefit or adverse effects of these drugs. The mechanism of actions of these BK_{Ca}CO isolated or identified from natural sources or as ancillary effects have been well characterised and is summarised in Figure 1.8 and Figure 1.9.

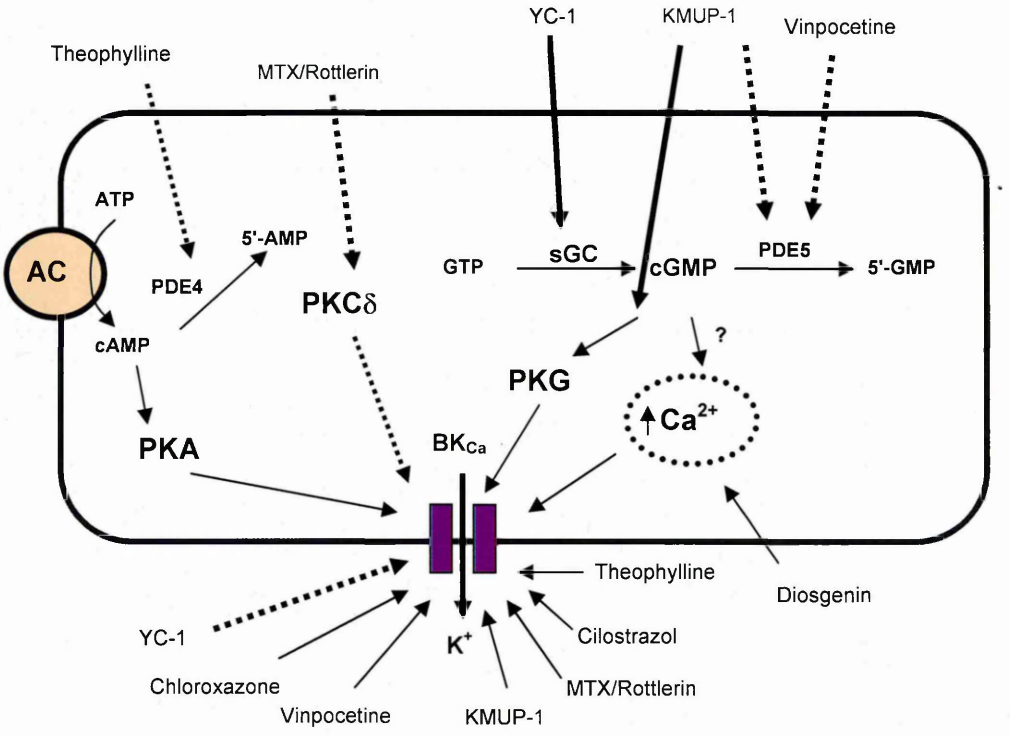


Figure 1.8: The mechanisms of BK_{Ca} channel activation by cell signalling enzyme activators and inhibitors

Solid arrows represents that particular compound/intracellular intermediate having an activating effect on the other; Dashed arrows represent an inhibitory effect. ?=unknown thought may involve activation of cyclic nucleotide gated channels or the phosphorylation effects of PKG on VDCC the effects of these molecules are independent of intracellular Ca^{2+} release from stores. Note; PKCδ phosphorylation inhibits BK_{Ca} channels.

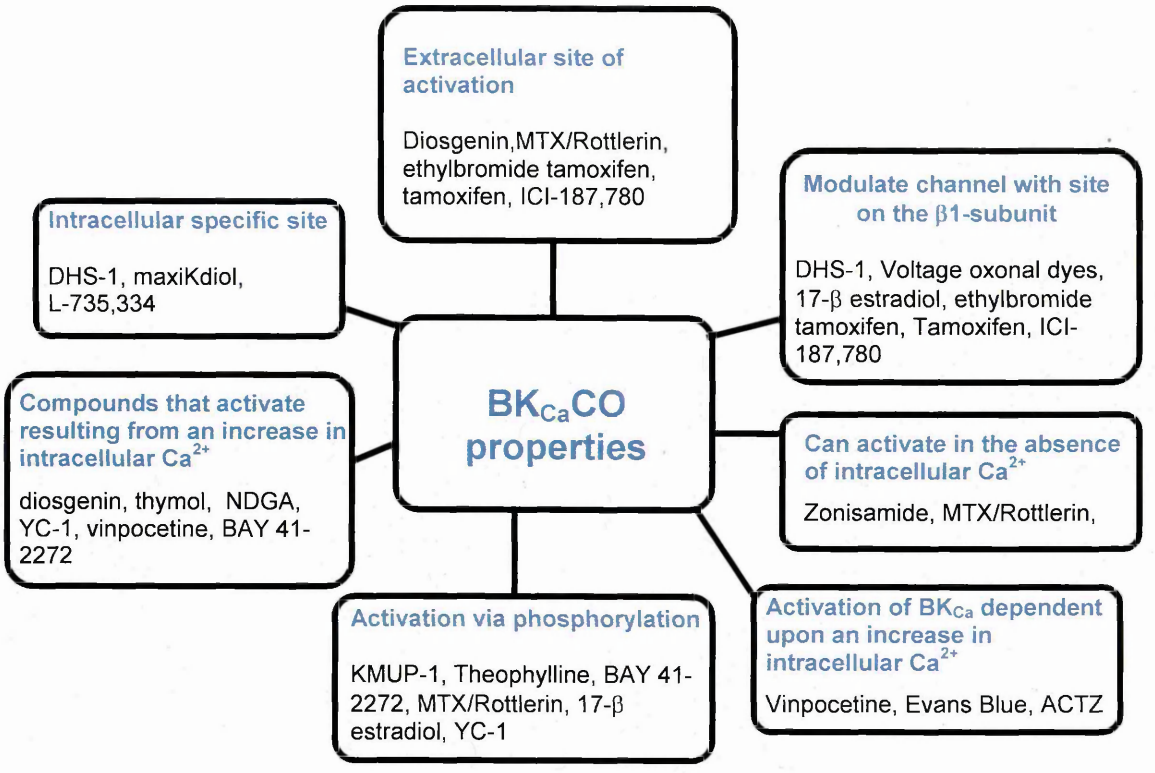
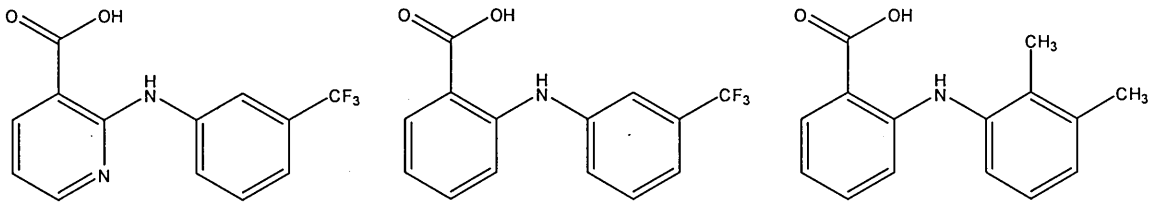


Figure 1.9: $BK_{Ca}CO$ properties of compounds identified from natural sources, therapeutic agents or enzyme inhibitors

1.9.3 Fenamates

The first $BK_{Ca}CO$'s identified as an ancillary effect of a therapeutic were the fenamates (Figure 1.10). These are derivatives of N-phenylanthrancilic acid that are used therapeutically as non-steroidal anti-inflammatory drugs (NSAID) whose primary mechanism of action is via the inhibition of cyclo-oxygenase. In a study by Ottolia and Toro (1994) the effects of niflumic, flufenamic and mefenamic acid were assessed. Niflumic acid (EC_{50} 20μM) activated the channel by increasing its affinity for Ca^{2+} and by causing a decrease in the time the channel remains in the closed state. An external binding site was proposed that was not associated with TEA or ChTX and a decrease in potency was observed when it was applied to the intracellular aspect of the channel.



Niflumic Acid **Flufenamic Acid** **Mefenamic Acid**

Figure 1.10: Fenamates

The shift in voltage sensitivity was proposed to be due to the anionic form of niflumic acid having non-specific surface charge effects on the external side of the membrane resulting in local depolarisation and an increase in channel activity; however at higher concentrations niflumic acid (1mM) did not activate VDCC. Further a rank order of potency was achieved; Niflumic acid=Flufenamic acid>>Mefenamic acid (Ottolia and Toro 1994), this order of potency and a similar EC_{50} for niflumic acid was calculated for the effect of these fenamates on BK_{Ca} currents in MG-63 osteoblast like cells (Wu *et al* 2001). If these compounds were causing non-specific membrane effects resulting in BK_{Ca} channel activation then the increased hydrophobicity of mefenamic acid (di-methyl groups) relative to niflumic and flufenamic acid would result in this being the most active compound. However the amphipathic nature of niflumic and flufenamic acid and the commonality of the presence of a trifluoromethyl group (CF_3) group supported the hypothesis that these two compounds bind the channel at a receptor confined to a hydrophobic pocket with the rest of the molecule facing the aqueous phase (Ottolia and Toro 1994).

1.10 Development of synthetic BK_{Ca} channel openers: Benzimidazolones

The breakthrough in the discovery of synthetic BK_{Ca} channel openers was the development of NS004 (5-trifluoromethyl-1- (5-chloro-2-hydroxyphenyl)-1,3-dihydro-2H-benzimidazole-2-one) and NS1619 (1-(2'-hydroxy-5'-trifluoromethylphenyl)-5-trifluoromethyl-2(3H) benzimidazolone) (Olesen *et al* 1994a; Olesen *et al* 1994b), (Figure 1.11). Since then their use has been

reported in over 100 published articles (Nardi *et al* 2006). These compounds have also formed the basis for the generation of several novel pharmacophores (Coghlan and Carroll 2001; Lawson and Dunne 2001).

1.10.1 NS1619 and NS004

NS1619 has been extensively studied in many different tissues including the myometrium, trachea, smooth muscle, vascular tissues and neurons (Edwards *et al* 1994; Olesen *et al* 1994a; Olesen *et al* 1994b; Lee *et al* 1995; Holland *et al* 1996; Hu and Kim 1996; Sheldon *et al* 1997; Huang *et al* 1997; Khan *et al* 1998; Zhang *et al* 2003; Malysz *et al* 2004).

NS1619 has been shown to hyperpolarise cells moving the membrane potential close to the equilibrium potential for K^+ (E_K), a common feature for BK_{Ca} channel openers. NS1619 activated BK_{Ca} channels equi-effectively when applied to either side of the channel (Olesen *et al* 1994b; Holland *et al* 1996) with an EC_{50} of around 10-30μM, the responses to NS1619 were eliminated by co-application of IbTX (Olesen *et al* 1994b). NS1619 is thought to associate with a binding site on the α-subunit, and due to its differential effects with time to activate BK_{Ca} channels in whole cell vs excised inside-out patches suggested an intracellular binding site (Edwards *et al* 1994; Olesen *et al* 1994b; Holland *et al* 1996), a similar mechanism has been proposed for NS004 (McKay *et al* 1994).

NS1619 affected the open channel properties (Olesen *et al* 1994a,b; Sheldon *et al* 1997) in cell-attached patches at the single channel level by increasing the number of open channels within the patch but not affecting the open time (Lee *et al* 1995b) or unitary conductance. Whether NS1619 can activate BK_{Ca} channels in the absence of calcium has not been fully determined as Edwards *et al* (1994) showed that in a calcium free system NS1619 resulted in activation of BK_{Ca} channels from whole cell recording of rat portal vein suggesting that NS1619 could substitute for intracellular calcium. Whereas it has been shown that removal of calcium from inside out patch configurations reversed the effects of channel activation in the presence of NS1619 (Olesen *et al* 1994b; Holland *et al* 1996). NS1619 effects were independent of channel phosphorylation as application of protein kinases, guanosine-5'-triphosphate (GTP) and the un-

coupling of G-proteins using pertussis toxin did not affect the NS1619 response (Olesen *et al* 1994b; Edwards *et al* 1994). However, Yammamura *et al* (2001) showed that NS1619 effects could be mediated by stimulating an increase in intracellular calcium release from the sarcoplasmic reticulum.

A number of ligand binding assays had suggested that NS1619 was specific to BK_{Ca} channels as it did not show interaction with 38 different binding sites of neurotransmitters and autocoids (Olesen *et al* 1994b). Further, to confer specificity, application of an array of potent ion channel inhibitors such as glibenclamide (K_{ATP}) and apamin (SK) did not affect the hyperpolarisation induced by NS1619 (Edwards *et al* 1994; Olesen *et al* 1994b).

NS004 was equally effective at activating BK_{Ca} channels when applied to either aspect of the channel and has an effective concentration of 0.1-0.4μM (McKay *et al* 1994; Hu *et al* 1995). NS004 caused an increase in whole cell BK_{Ca} channel currents in porcine coronary artery cells by 3-4 fold (Hu *et al* 1995) and by 2-5 times in *Xenopus oocytes* expressing BK_{Ca} channel subunits (McKay *et al* 1994). NS004 (30μM) has shown to induce an 24-fold increase in P_o in cerebellar granule cells and 7-8 fold in channels reconstituted lipid bilayers (McKay *et al* 1994; Olesen *et al* 1994b).

The first problems with the use of NS1619 as a selective BK_{Ca} channel opener were reported by Edwards *et al* (1994) who showed that NS1619 could inhibit K_v channels. This was subsequently reported by Holland *et al* (1996) where under conditions that minimised BK_{Ca} channel activation and optimised K_v channel opening NS1619 was able to produce a concentration-dependent reduction in K_v current. In addition, Edwards *et al* (1994) and Holland *et al* (1996) determined that NS1619 was able to block Ca^{2+} currents from rat portal vein and basilar artery cells respectively. A similar effect has been observed with NS004 (Sargent *et al* 1993).

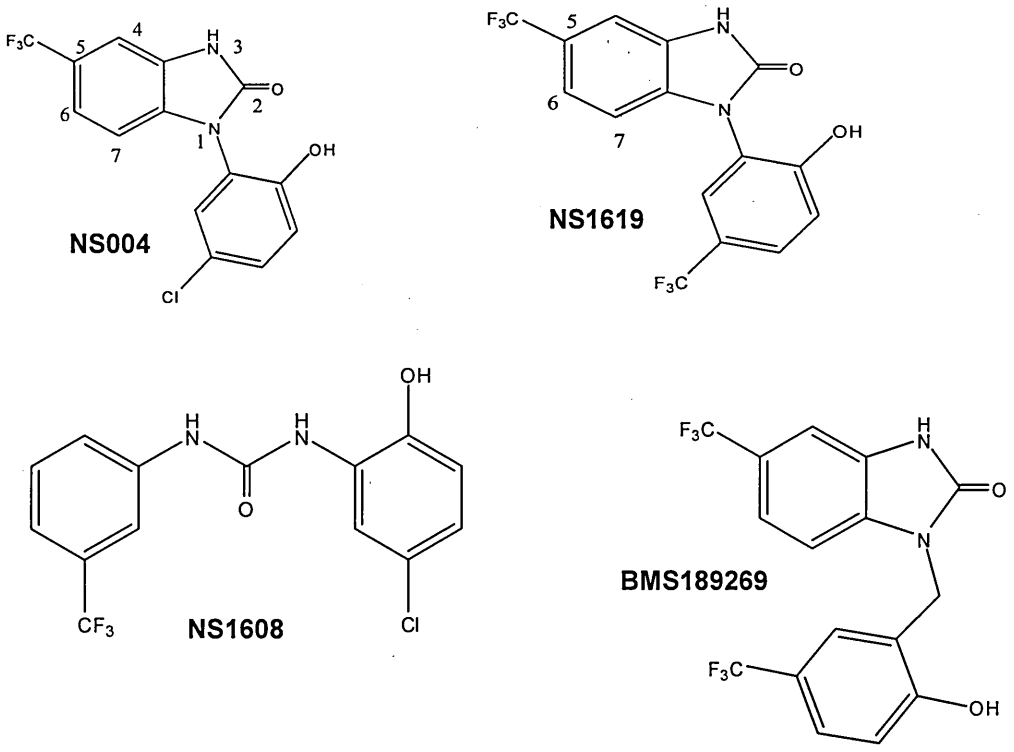


Figure 1.11: Benzimidazolone compounds

Further, the prolongation of action potentials observed in dorsal root ganglion upon application of NS1619 could be due the ability of NS1619 to inhibit delayed rectifier channels and through mobilisation of intracellular Ca^{2+} release rather than through direct BK_{Ca} channel activation (Yamamura *et al* 2001; Zhange *et al* 2003).

Furthermore, NS004 and NS1619 have shown ancillary pharmacology with the cystic fibrosis transmembrane conductance regulator (CFTR) whereby after pre-treatment with forskolin NS1619 and NS004 activated both the wild-type and the most common mutation observed in cystic fibrosis; a deletion of a phenyalanine residue (△F508) (Al Nakkash *et al* 2001). These compounds could be used in the development of therapeutics targeting the CFTR-Cl^- channel in cystic fibrosis (Gribkiff *et al* 1994; Al Nakkash *et al* 2001).

1.10.2 NS1608

The first modification to the benzimidazolone structure was the introduction of a biaryl urea linker opening up the heterocycle of NS1619. This compound termed NS1608 (Figure 1.11) has been characterised for its $BK_{Ca}CO$ properties. NS1608 effects were additive with increases in intracellular calcium, in that

responses in the presence of increasing calcium resulted in a 200mV shift in voltage required for channel activation. Where as in a calcium free system NS1608 alone produced a shift of 70mV in the hyperpolarising direction, a similar shift as that observed with NS1619. NS1608 was more potent than NS004 and NS1619 (Strobaek *et al* 1996; Siemer *et al* 2000).

1.11 Benzimidazolone derived BK_{Ca} channel openers

The first examination of the pharmacophore properties of benzimidazolone type compounds was performed by Meanwell and colleagues at Bristol-Myers Squibb (BMS). This has subsequently led to the development of a number of classes of novel BK_{Ca}CO compounds.

1.11.1 Benzyl-benzimidazol-2-ones

The first such structures were a series of benzyl-benzimidazol-2-ones based on the insertion of either a methyl or ethyl spacer separating the phenolic and the imidazolone ring system of NS004 by 1 or 2 carbon atoms and is represented by BMS-189269 (Figure 1.11). Different substitutions of the benzene rings surrounding the imidazolone nucleus were used to determine the aspects of these compounds that impart BK_{Ca} channel opener properties. Compounds were assessed using two electrode voltage-clamp on *Xenopus oocytes* injected with *mSlo*. The potency of compounds was determined as the maximum percentage increase in iberiotoxin-sensitive outward currents (above control) across a +20 to +140mV voltage range.

A phenolic ring appeared be a definite requirement for compounds to retain BK_{Ca}CO properties and that chlorination in the *para (p-)* position to the hydroxyl (OH) was important for activating properties (this was supported by Hewawasam *et al* 1997 in that des-chloro analogues were less efficacious), (Meanwell *et al* 1996). Another important feature is the trifluoro-methylated benzene ring, substitutions at position 5 with other halogens (Cl, Br, I) and NO_2 promoted BK_{Ca} channel opener properties quite considerably compared to substitution with hydroxyl, methyl or the un-substituted derivative. Interestingly, if chlorine or bromine is substituted at position 6 only weak BK_{Ca} channel opening properties are displayed. However, di-chlorination in positions 5 and 7

results in potency similar to that achieved in position 6 but di-chlorination in positions 5 and 6 resulted in BK_{Ca} channel activation at levels similar to that observed with chlorination at position 5 alone (Meanwell *et al* 1996). The importance of the trifluro-methylated (CF_3) benzene ring was further confirmed by substitution with an electron deficient pyridine ring system which was in effective. Therefore electron withdrawing groups (EWG), particularly halogens, appear to be important requirements in this molecules ability to activate BK_{Ca} channels, and that slight modifications in the positioning of substituents in particular chlorine, can have marked effects on potency and efficacy. This could provide clues on how the compound could potentially interact with the BK_{Ca} channel protein (Meanwell *et al* 1996). Hewawasam *et al* (1997) also showed that NS004 adopted more of a planar network as a mimic of this compound in which the phenol and the benzimidazolone rings adopted an orthogonal relationship was unable to activate BK_{Ca} channel currents.

It had been suggested that the amide NH of benzimidazolones acts as a weak acid and that this acidity is increased by the attachment of EWG to the heterocyclic nucleus. Furthermore, the amide NH together with the amide carbonyl oxygen is critical for activity and could act as a surrogate carboxylic acid (Li *et al* 1997). In addition, the phenolic OH and carbonyl oxygen were thought to act as a mimic of carboxylic acid as well (Meanwell *et al* 1996).

For a series of PDE inhibitors (heterocycle-phenyl-imidazole) molecular modelling has suggested that the imidazolone of the inhibitor could act/mimic a phosphate for the enzymes active site. Therefore, this ring system of NS1619 could act at a complementary site to the phosphate on the channel (Li *et al* 1997).

1.11.2 Identification of flavanoids as BK_{Ca}CO's

The similarity in NS004 and benzylated benzimidazol-2-one at activating BK_{Ca} channels indicated flexibility between these two bioisosteres. A three dimensional database search using the essential pharmacophore properties of NS004 was performed by Li *et al* (1997) at BMS. A pharmacophore was constructed involving the presence of a carbonyl O as a hydrogen bond (H-bond) acceptor, a H-bond donor site and an aromatic group located within a 3-

5Å distance of the amide carbonyl oxygen. Furthermore, the torsional angle of the H-bond donor was further constrained (HX...C=O ±30°). These criteria were queried against the 3-D CSDS database of 120,000 crystal structures, from which 300 hits were obtained (Li *et al* 1997). A particular chemotype identified was KAMJUD01 similar to the flavone quercetin that contains a hydroxyl and ketone phenol moiety that has been shown to mimic carboxylic acid of L-DOPA (Figure 1.12). Therefore, similar flavanoid compounds were studied, cyclic flavanoids were found to be more efficacious than acyclic; of those studied apigenine and kaempferol produced the greatest BK_{Ca} channel current. These compounds had hydroxyl at carbon 5 (C5) position located adjacent to the ketone and demonstrated greater activation than NS004; this difference in potency was thought to involve the OH at the C5 position providing a more acidic function than the equivalent amide NH in NS004 (Figure 1.12).

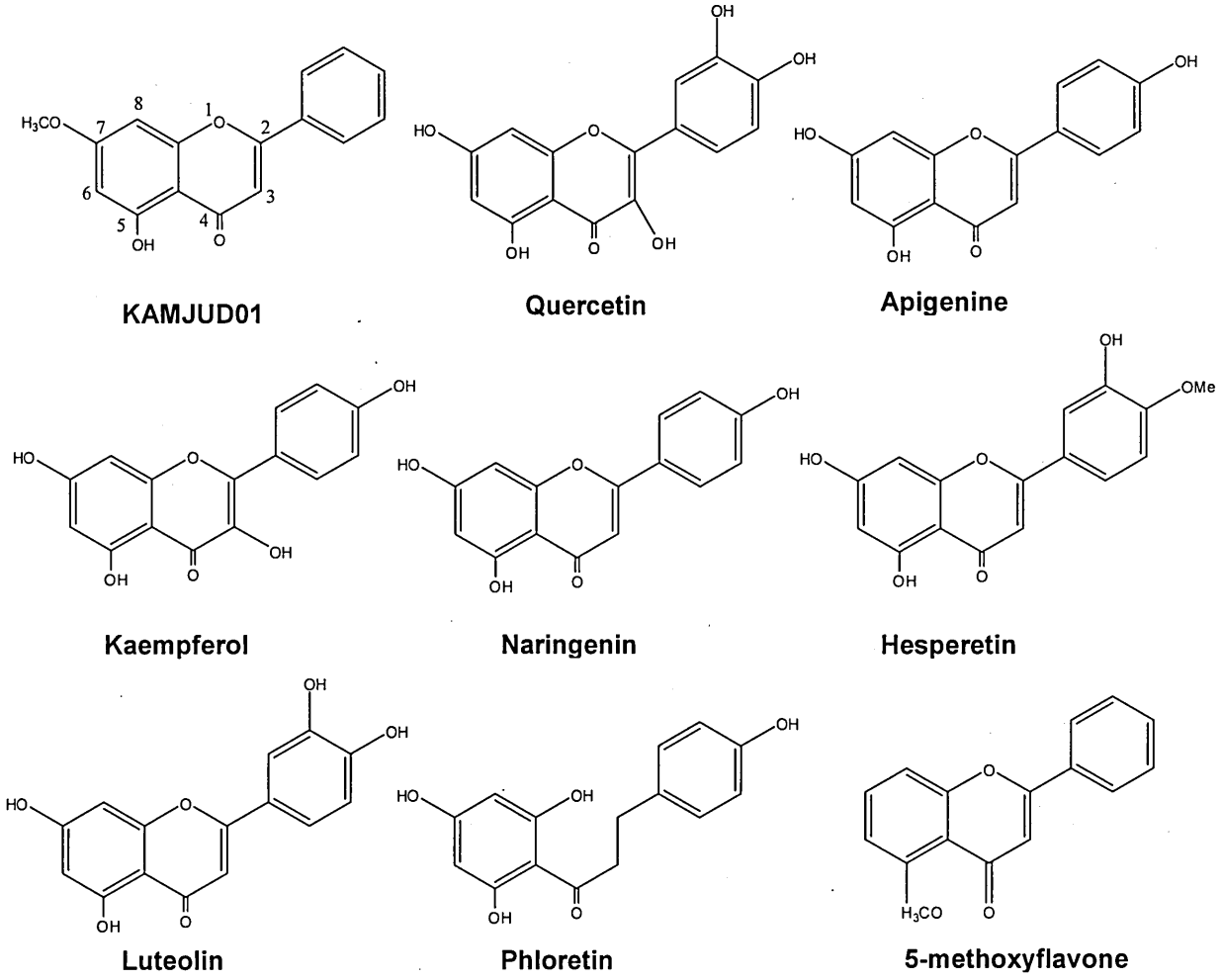


Figure 1.12: Flavonoids

Further, *meta*-OH substitution in the R2 group at position C7 of the same ring enhanced the activation properties but substitution with methoxy groups were less active (Li *et al* 1997). The work of Calderone *et al* (2004) revealed other flavanoids as potent $BK_{Ca}CO$'s including naringenine, hesperetin and luteolin (Figure 1.12). The hydroxyl group in carbon position 5 of these compounds was essential for flavonoids to display specific BK_{Ca} channel mediated vasorelaxant responses as 5-methoxyflavone could not induce IbTX sensitive vasorelaxation (Nardi *et al* 2003; Calderone *et al* 2004). In addition, a wider study of vascular relaxation of porcine coronary artery by flavanoids confirmed that the OH function in C5 and also in C7 position was important and that the presence of a double bond between C2 and C3 were important for vasorelaxant responses. Hydroxylation of the phenyl ring weakened the potency, although whether relaxation was specific to any type of K^+ channel including BK_{Ca} channels was not tested (Xu *et al* 2007).

This data supports the idea that the ketone oxygen and hydroxyl group at position C5 could act as a surrogate carboxylic acid imparting specificity to BK_{Ca} channels, this feature is also important to benzimidazolones.

1.11.2.1 Quercetin

Cogolludo *et al* (2007) demonstrated that quercetin, the most commonly eaten dietary flavanoid, found in most plant and vegetables could activate BK_{Ca} channels and that its mechanism of activation was dependent upon the production of the reactive oxygen species hydrogen peroxide (H_2O_2). The production of H_2O_2 led to an increase in STOC frequency mediated by BK_{Ca} channel activation. These effects were inhibited by application of IbTX and the reactive oxygen species scavenger catalase. The effects were independent of the release of substances from the endothelium although Khulmann *et al* (2005a) have demonstrated that quercetin can activate BK_{Ca} channels in endothelial cells through the activation of NO/cGMP pathway. These actions may explain the beneficial effects of quercetin in rat hypertensive models (Cogolludo *et al* 2007).

1.11.2.2 Phloreitin

Another flavanoid, phloretin had been independently studied for its $BK_{Ca}CO$ activity on peroneal nerve fibres of the clawed toad *Xenopus laevis* (Figure 1.12), (Koh *et al* 1994). Phloretin produced a 68mV hyperpolarising shift in activation curve and was equally effective when applied to either aspect of the channel, however, it had slow onset effects and slow wash-out effects. The mechanism of action of phloretin was proposed to occur via its absorption in neutral form into the membrane where it changes the gating properties of the channel. A decrease in the steepness of the activation curve (P_o Vs Voltage) accompanied by a shift to more hyperpolarised voltage was observed maybe due to dipole potentiation on the membrane and consequently affecting the gating charge and voltage sensitivity of the channel (Koh *et al* 1994).

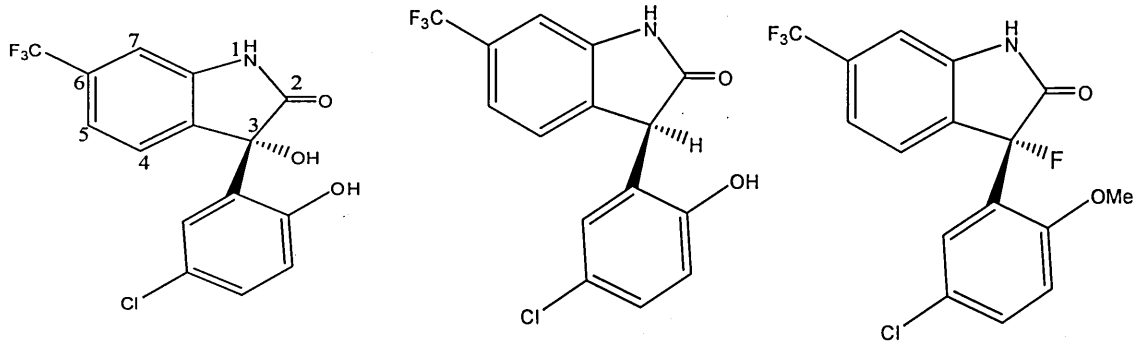
1.11.2.3 Naringenin

Racemate (±) Naringenin (Figure 1.12) has been studied in further detail using electrophysiology in isolated rat tail artery myocytes. Using whole cell electrophysiology it induced a 22mV shift in V_{50} from control; interestingly potency was significantly reduced when the intracellular K^+ concentration was reduced (from 90 to 14mM). Its effects were independent of the concentration of intracellular Ca^{2+} whereby naringenin activated BK_{Ca} channels by affecting the voltage sensitivity of the channel as demonstrated by a change in slope of activation curve and by increasing the rate of channel activation (Saponara *et al* 2006).

1.11.3 3-aryloxindoles

Further insights into the BK_{Ca} channel opener characteristics of NS004 were addressed by replacing the nitrogen atom of the imidazolone bearing the phenyl ring with a carbon introducing a stereogenic centre at the 3'-position of the indole nucleus, this allowed for assessment of the 3D effects and stereochemistry (Hewawasam *et al* 1997) and the first example studied had the addition of a hydroxyl group at the carbon atom (Figure 1.13). Enantiomers of compounds were generated and tested either as racemate, optically active or inactive compounds. The trifluoro group was found to be essential (similar to NS004 and NS1619) and by substituting different groups on the C-phenyl ring it

was shown that like NS004, chlorine in the *para* position to a hydroxyl group was essential for BK_{Ca} channel activity although replacement of the hydroxyl with a methoxy group was tolerated. With regards to the chirality of the methoxy-chloro compound no particular enantiomeric composition displayed any significant advantage (Hewawasam *et al* 1997). However, 3-aryl-3-hydroxyindolones, displaying the same functional groups in the identical positions as NS004, the (-)-isomer was significantly more effective than the (±) or the (+)-isomer when compared to NS004 the eutomer was significantly more potent at 100μM, but not at 30μM, when tested on BK_{Ca} channels expressed in *Xenopus* Oocytes (Hewawasam *et al* 1997; Hewawasam *et al* 2002a).



(-) 3-aryl-3-hydroxyindole **3-aryl-3-des-hydroxyindole** **BMS-204352/MaxiPost**

Figure 1.13: Oxindoles

Further analysis of the structure-activity-relationships (SAR) of 3-aryloxindoles revealed that a des-hydroxy alternative was just as effective as the presence of 3-hydroxy group (Figure 1.13) and that an EWG (6'-CF_3) was not essential for a compound to display $BK_{Ca}CO$ properties. However its introduction greatly enhanced $BK_{Ca}CO$ properties and its positioning on the ring affected potency (Hewawasam *et al* 2002a). Exploring the effect of different functional groups and chirality of the 3-aryl-3-hydroxyindolones could lead to the development of molecules that may show tissue specificity for neurons. Administration in the *in-vivo* MCAO rat model of stroke showed that the des-hydroxyl compound was able to reduce infarct size and block the release of the excitatory neurotransmitter glutamate, whereas the hydroxyindole was not able to reduce infarct size. This difference maybe due to the lipophilicity of the compounds and their relative abilities to penetrate the blood brain barrier. However the des-

hydroxyindole compound was considered not suitable for advancement to clinical trials de to its poor pharmacokinetic properties and metabolic instability (Hewawasam *et.al* 2002a).

1.11.4 BMS-204352 and 3-Fluorooxindoles

Due to the poor pharmacokinetic and metabolic properties of the aryloxindoles a similar series of related 3-fluorooxindoles was further investigated by BMS (Figure 1.13). A fluorine atom substituted at the 3-hydroxyl site led to the development of a new template for $BK_{Ca}CO$ (Hewawasam *et al* BMS 1996; Hewawasam *et al* 2002b). From this set a compound bearing a 5-chloro-2-methoxy moiety at C3 position and a 6-substituted trifluoromethyl oxindole group was the most potent and termed BMS-204352 (Gribkoff *et al.* BMS, 2002). Again the positioning of CF_3 groups affected potency, also the solubility of BMS-204352 was low (<10μM). Examination of the chirality of this compound revealed that the (+) isomer produced the most robust and consistent increase in BK_{Ca} channel currents in cloned cell lines and demonstrated significant IbTX sensitive increase in current at 0.01 and 0.1μM with an EC_{50} determined as 0.35μM (Hewawasam *et al* 2002b; Gribkoff *et al* 2001b). Further it was found to be very brain permeable and the authors described a method for the stereospecific production of the (+) isomer. The compound was used as the lead in clinical trials for post stroke neuroprotection (MaxiPost) section 1.6 (Gribkoff *et al* 2001b; Gribkoff *et al.* BMS, 2002).

The presence of the methyl ether moiety in place of the usual more polar hydroxyl function could account for the high prevalence of MaxiPost in the brain. It was proposed that it could be converted to a more active phenolic compound by metabolic O-demethylation by enzymes in the blood brain barrier and that this may account for its lack of side effect in vascular smooth muscle (Kiesewetter *et al* 2002). Recently researchers at Neurosearch A/S have shown that racemate BMS-204352 can activate KCNQ/K_v7 channels expressed in HEK293 cells (Schroder *et al* 2003) and that different enantiomers of BMS-204352 had differential anxiolytic effects in animal models of anxiety and stress via activation of K_v7 channels (Korsgaard *et al* 2005).

1.11.5 Substituted Quinolinones

Retaining the aryl elements of the indole compounds and enlarging the 5-membered imidazolone ring into a 6-membered heterocycle led to the reporting of quinolinones as BK_{Ca} channel openers. These were investigated by altering the substituents in the 3' position and led to the reporting of several classes of quinolinone molecules by researchers at BMS (Figure 1.14).

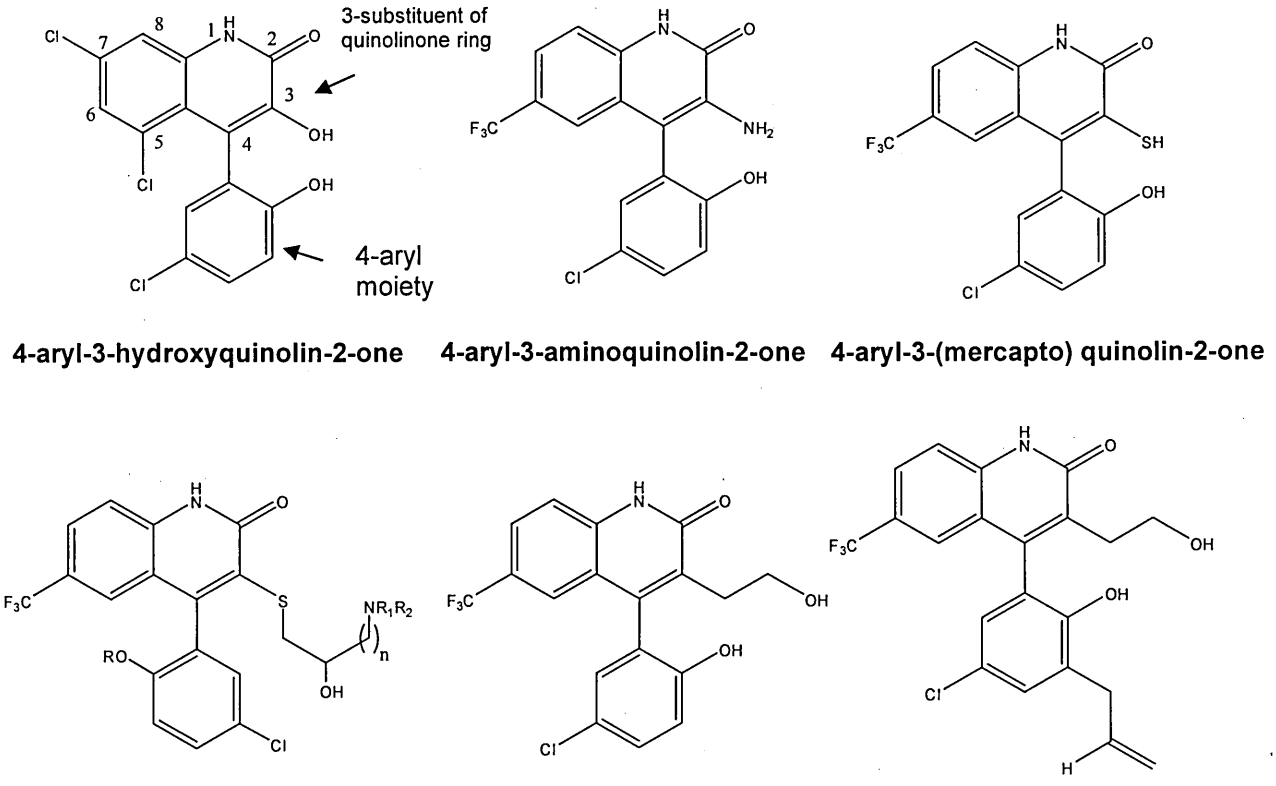


Figure 1.14: Substituted quinolin-2-ones

1.11.5.1 Amino and hydroxyl quinolin-2-ones

4-aryl-3-hydroxyquinolin-2-ones (Figure 1.14) were the first of this series to be evaluated and patented as BK_{Ca}CO's for treatment in post stroke neuroprotection (Sit and Meanwell, BMS, 1998). Interestingly they also showed potent antibiotic activity (Sit and Meanwell, BMS, 1999). However, the optimised compounds did not display the ability to penetrate the blood brain barrier this was thought to be a result of the presence of an ionisable 3-hydroxyl group (Hewawasam *et al* 2002c). As a result replacement of the 3-hydroxyl moiety of

quinolinone with a less ionisable amine group generated 4-aryl-3-aminoquinolin-2-one derivatives (Figure 1.14) that were tested (Hewawasam *et al* 2002c).

The substitution of the quinine nucleus demonstrated that the presence of 5-CF_3 and 6-CF_3 had the most pronounced effect on channel activation than in other positions of the ring and that removal of hydroxyl or chlorine from the 4-aryl moiety decreased potency. This is further evidence of the requirement of $BK_{Ca}CO$'s to contain a hydroxyl function with a concurrent halogen in *p*-position, and that the presence and positioning of EWG was essential for optimisation of $BK_{Ca}CO$ structural properties. Also, compounds that contained (trifluoromethyl) sulphonamide in replacement of the 3-amine moiety produced even greater activation. However, unlike the 4-aryl-3-aminoquinolin-2-one it failed to demonstrate brain penetrability. Therefore, the effects of 4-aryl-3-aminoquinolin-2-one were tested in MCAO stroke model where application of the compound reduced infarct size by around 10% (Hewawasam *et al* 2002c).

1.11.5.2 Mercapto quinolin-2-ones

Furthermore, quinolin-2-one compounds bearing 3-mercapto (SH) groups were also investigated (Figure 1.14). Although these compounds showed significant increases in BK_{Ca} channel current relative to NS004 they demonstrated poor aqueous solubility (<0.005mg ml^{-1}). From this series a hydroxyethyl derivative of the mercapto group (S-$(CH_2)_2$)OH) produced significant potency (3 times more potentiation of current than NS004) but still lacked sufficient solubility for potential therapeutic and *in-vivo* testing (Hewawasam *et al* 2004). In an attempt to increase solubility derivatives were added to the SH group. The addition of an amino alcohol retained equivalent BK_{Ca} channel activation while improving solubility 100 times. The amino alcohol compound demonstrated potent vasodilator response in pre-contracted corpus cavernosum tissue. Further, in a rat model of erectile dysfunction (ED), it demonstrated comparable *in-vivo* efficacy to phentolamine increasing intra-cavernosum pressure without adversely affecting blood pressure as demonstrated with phentolamine. Therefore, these structures may provide therapeutic tools in erectile dysfunction (Hewawasam *et al* 2004).

Following the work on 4-aryl-3-(mercapto) quinolin-2-ones Boy *et al* (2004) investigated attaching various alky subsituents to the 3-thiol group to characterise further compounds for the treatment of ED (Figure 1.14). This involved the generation of over 60 compounds of four major classes, of which the most potent class of compounds were typified by the presence of a 3-amino-2-hydroxy thiol side chain. A variable length of the tether (n) could be tolerated and phenols were generally more active than methylesters. However, those that showed the most potent activating potential had a H-bond acceptor with a remote amino group on the side chain of compounds.

1.11.5.3 4-aryl-3-(hydroxyalkyl)quinolin-2-ones (BMS-223131)

Further probing of the quinolin-2-one nucleus was performed to determine the optimal hydroxyl-alkyl group by varying the number of bonds between the hydroxyl and C3 position (Hewawasam *et al* 2003a). These compounds had either a 4-aryl moiety of *p-chlorophenol* or *p-chloroainsole* both previously shown to have beneficial effects to derivatives of hydroxyquinolin-2-ones and aminoquinolin-2-one compounds. Here *p-chlorophenol* derivatives were more effective than *p-chloroainsole* derivative at relaxing pre-contacted corporal tissue and activating whole cell BK_{Ca} channel currents. Compounds with variable length (1-3 carbons) all demonstrated $BK_{Ca}CO$ properties with a trend for increasing potency with increasing bond separation. However, the most potent hydroxyalkyl termed BMS-223131 (Figure 1.14) was examined in rat model of ED. Interestingly the order of potency of these compounds determined using electrophysiology did not correlate well in relation to the order of potency determined by testing their vasorelaxant effects in rabbit corporeal tissue. This indicated possible contribution of tissue specific BK_{Ca} channel expression, other biochemical mediators and differences in experimental setup (Hewawasam *et al* 2003a). A similar discrepancy was observed with 3-thioquinoline compounds (Boy *et al* 2004).

In a novel application for $BK_{Ca}CO$'s, BMS-223131 demonstrated dramatic reduction of stress induced colonic motility and visceral noiception in rat *in-vivo* models, implicating a role for this compound in the treatment of diseases such as irritable bowl syndrome (Sivarao *et al* 2005). Whether the effects of BMS-

224131 in the animal models used was specific to BK_{Ca} channel activation was not determined. This was because antagonising the effects with BK_{Ca} channel blockers could not be tested due to their high *in-vivo* toxicity. In addition, this series of quinolin-2-ones demonstrated potent cytochrome P450 (2C9 isoform) inhibition within a therapeutic range. This has resulted in the design of new synthetic routes generating new compounds that demonstrate a decreased cytochrome P450 profile while retaining $BK_{Ca}CO$ properties (Vrudhula *et al* 2005). As the phenolic OH group is an essential element for $BK_{Ca}CO$ properties Vrudhula *et al* (2007) investigated the addition of hydrophilic and hydrophobic subsituents *ortho* to the phenolic OH group of C ring of BMS-223131 with the most potent compound displaying decreased cytochrome P450 interation was the olefin derivative (Figure 1.14) although these derivatives were only half as potent as the parent BMS-223131 compound.

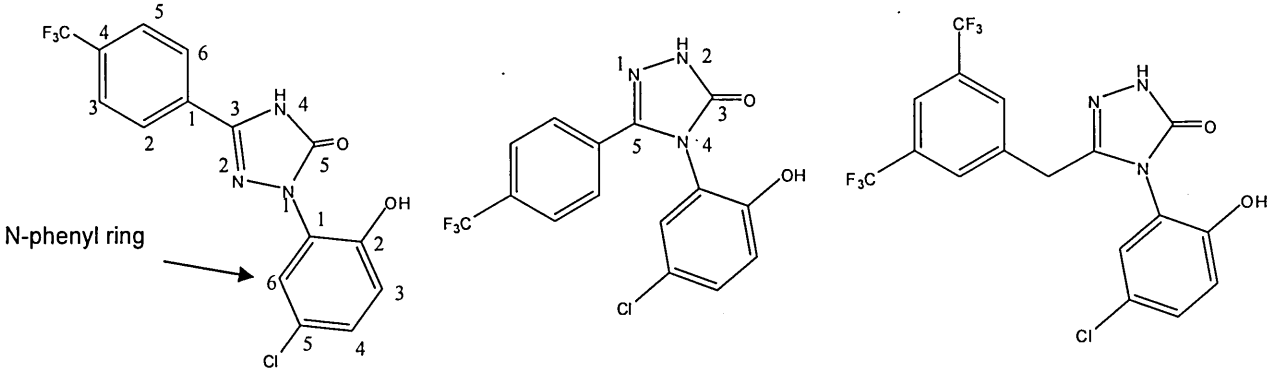
Further investigation of BMS-223131 included analysing the atropisomers of the compound, these were obtained by chiral separation of the molecule using high performance liquid chromatography. The atropisomers were not previously studied as it was assumed that the hydroxyethyl in the C3 position did not impose a strong rotational barrier between the phenolic and quinolinone rings and that under physiological conditions this would not have any consequences. However, the (-) isomer in all cases had the greater activating effect compared to the (+) isomer, suggesting stereoselective activation of BK_{Ca} channels by these compounds (Vrudhula *et al* 2007). Therefore the atropisomeric effects should be considered when dealing with the development of chiral drugs; and that stereospecific drugs may also provide clues into sites of action on the channel.

1.11.6 Triazolones

Further exploration of the benzimidazolone derivatives included simplifying the structure by deanulation of the bicyclic benzimidazolone ring through the introduction of a heterocycle linker between the phenolic and electron deficient rings. The first example of these compounds including maintaining the *p-chlorophenol* ring and separating it from the other phenyl ring with a 1,2,4 triazol-5-one heterocycle (Figure 1.15). These diaryl 1,2,4 triazol-5-ones were

pioneered as a template structure for the treatment of urinary incontinence (Hewawasam *et al* 2002d).

In this molecule the un-substituted phenyl analogue was ineffective but the addition of EWG to it induced activation properties similar to that observed for benzyl-benzimidazol-2-ones, aryloxindoles and quinolinones. Further, the positioning of the CF_3 was important and increasing the electron deficiency by addition of 7,4 and 3,5 bisCF_3 maintained $BK_{Ca}CO$ ability. The effects of these compounds were studied *in-vitro* using pre-contracted bladder tissue strips and like with the quniolin-2-ones and 3-thioquinolinones the rank order of potency of inhibition of contraction did not correlate with electrophysiology. For example the phenyl analogue (no EWG attached) produced ~90% inhibition in contraction but did not activate BK_{Ca} channels in electrophysiology. This highlights the need for representative functional *in-vitro* and *in-vivo* tests to assess the physiological effect of compounds (Hewawasam *et al* 2002d).



1,2,4 triazol-5-ones **4,5 diphenyltriazol-3-ones** **Methyl linker insertion of triazol-3-ones**

Figure 1.15: Triazolones

A more in-depth SAR analysis of triazolone structure was examined by Romine *et al* (2002) via examination of a series of 4,5 diphenyltriazol-3-ones (Figure 1.15). While retaining a 4-CF_3 the positioning of the hydroxyl and chloro function of the N-phenyl ring was manipulated. Maintaining a 2-OH and 5-Cl position was important as a 4-OH, 3-Cl or 5-OH, 2-Cl configuration resulted in inactive compounds. This highlighted the importance of the phenol ring and the positioning of a 2'-hydroxyl function as demonstrated for other benzimidazolone derived $BK_{Ca}CO$'s. Further studies maintaining the 2-OH and 5-chloro groups and altering the positioning of the CF_3 group demonstrated that it was preferred in either the *para* or *meta* position increasing the electron deficiency of the ring.

Further bisCF_3 groups potentiated BK_{Ca} channels but replacement with the less electronegative fluorine led to a reduction in channel activation and the absence of a EWG altogether led to a loss of $BK_{Ca}CO$ ability.

Introduction of flexibility into this template structure was not tolerated as addition of a methylene spacer between the triazol-3-one and the N-phenolic ring had deleterious effects. Insertion of a methylene linker between the phenolic ring and imidazolone nucleus of benzyl-benzimidazol-2-ones had previously shown to retain BK_{Ca} channel activation (Meanwell *et al* 1996). Interestingly compounds that had a methylene spacer inserted between the electron deficient ring and the heterocycle triazol-3-ones retained activity (Figure 1.15). Romine *et al* (2002) suggested that the electron deficiency of the ring is not important for binding to the channel but rather the lipophilic nature of the aromatic ring imparted by the trifluoromethylated groups.

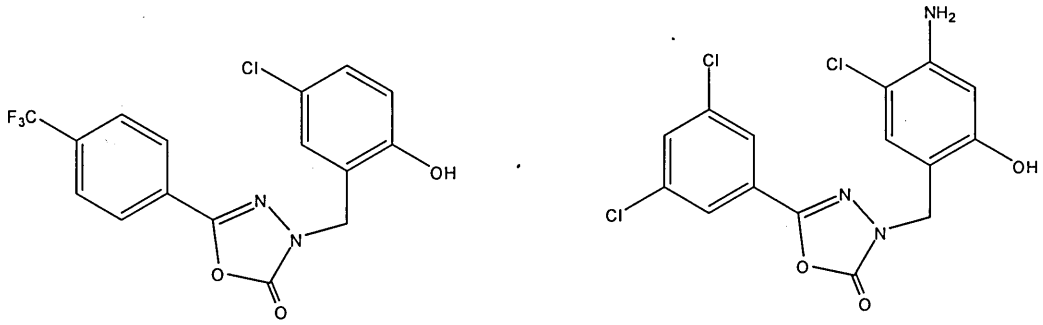
The carbonyl oxygen and its relation to phenol ring has been proposed to be important for quinolin-2-ones and benzimidazolone based compounds. However, for the deannulated triazoles the positioning of the carbonyl oxygen in the heterocycle ring and the different regioisomer forms did not have any effect on the overall $BK_{Ca}CO$ ability of these compounds. Modelling studies revealed that the relationship between carbonyl group and the phenol was not important but the relationship and distance between the oxygen of phenol and the hydrogen of NH of the triazole ring important for compounds that activated the channel. Further removal of NH while retaining the carbonyl group led to inactive compounds, therefore an H-bond donor or protonated N in the heterocycle was thought to be essential for these compounds (Romine *et al* 2002). An amide NH group was also thought to be important for benzimidazolones and benzyl-benzimidazol-2-one derivatives (Meanwell *et al* 1996; Hewawasam *et al* 1997; Li *et al* 1997).

1.11.7 Oxadiazolones/BMS-191011

Replacement of the triazolone heterocycle with oxadiazolone revealed a series of new compound typified by BMS-191011 (Figure 1.16). This compound was able to increase BK_{Ca} channel currents by 26% at 1μM and has been proposed as another target for the treatment of post stroke neuroprotection having shown

efficacy in rat stroke models. However, like other $BK_{Ca}CO$ structures its therapeutic potential is limited by poor solubility in aqueous buffer. This led the authors to guide the design and synthesis of pro-drug versions of BMS-191011 resulting in the addition of deoxycarnitine to the phenolic hydroxyl. The pro-drug did not display BK_{Ca} channel activity but was readily converted (>80%) to the active compound upon intravenous administration, by undetermined esterases in blood plasma. The converted form was able to potently reduce the effects of MCAO stroke in rats and was submitted for potential clinical development (Hewawasam *et al* 2003b).

More recently there have been reports of attempts to increase solubility of BMS-191011 through the appendage of different heterocycles such as piperizines and piperidines to the phenol or electron deficient rings. However, the simple addition of aniline yeilded sufficient solubility and increased BK_{Ca} channel potentiation (Figure 1.16). The aniline derivative showed enhanced brain to plasma partitioning relative to BMS-191011, but had no significant effect in the rat MCAO stroke model (Romine *et al* 2007).



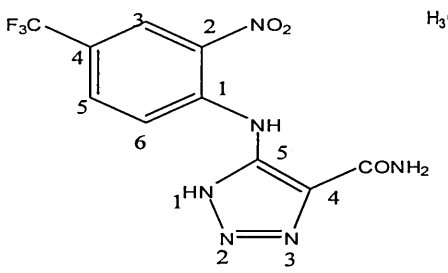
BMS-191011 **Aniline derivative**

Figure 1.16: Oxadiazolones

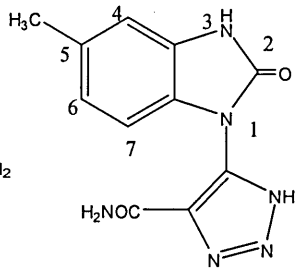
1.11.8 Triazoles

An extensive research program by V.Calderone, G.Biagi and I.Giorgi in Italy has led to the development of detailed SAR relationships for novel $BK_{Ca}CO$'s. Using triazole compounds as a starting point the 5-(4'-substututed-2'-nitroanilino)-4-carboxyamido-1,2,3-triazoles were examined (Figure 1.17). The addition of halogens or methoxy and methyl groups to the 4' position of the nitroanilino ring gave compounds that retained similar activity to NS1619 but the presence of a

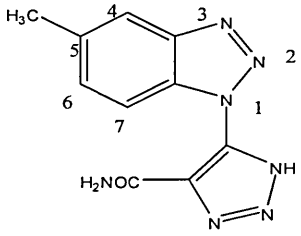
sec-butyl imparted the highest potency. Overlapping of the theoretical tri-dimensional structure of NS1619 or NS004 with triazoles revealed that the nitro group was at a position mimicking the carbonyl oxygen of the benzimidazolones. Replacement of the nitro group resulted in inactive compounds no matter what groups were added to the 4-position, this implied a role for this oxygen in $BK_{Ca}CO$ properties of these triazoles potentially acting as a H-bond acceptor (Biagi *et al* 2000).



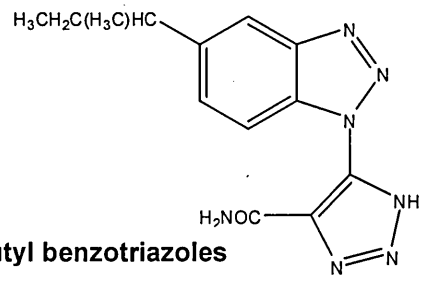
Nitro anilino carboxyamido -1,2,3-triazoles



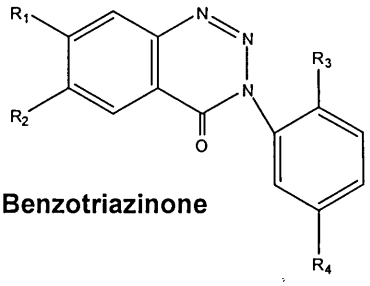
Triazolyl-benzimidazolone



Triazolyl-benzotriazoles



Sec-butyl benzotriazoles



Benzotriazinone

Figure 1.17: Triazoles I

Further exploration of triazoles included using them as intermediates in the synthesis of new compounds. These included 5-substituted triazolyl-benzimidazolones and 5-substituted triazolyl-benzotriazoles (Figure 1.17). Interestingly the former bearing the most structural resemblance to NS1619 were not activators (except for a structure with a CH_3 in 5' position). Conversely the benzotriazoles, although not quite as potent as NS1619, showed activating properties that were dependent, like carboxyamido-1,2,3-triazoles on substitution with Cl, F and CF_3. In contrast the methoxy group was deleterious but addition of methyl function resulted in a compound with greatest potency (Baragatti *et al* 2000). Therefore, as addition of methyl to both triazolyl – benzotriazoles or -benzimidazolones was advantageous. These structures were

further examined by increasing steric hindrance and lipophilicity by addition of sec-butyl (Figure 1.17) or phenyl groups. These additions to the benzotriazole skeleton were tolerated and led to full relaxations of pre-contracted aortic rings, however, additions to the triazolyl-benzimidazolone were less potent (Biagi *et al* 2001a) and opening up the benzimidazolone ring by replacement with an urea linker led to a decrease in activity. In addition, a series of benzotriazinone compounds (Figure 1.17) were found to be in effective $BK_{Ca}CO$'s suggesting that the molecular composition of the triazole ring was important to its pharmacophore (Biagi *et al* 2001a).

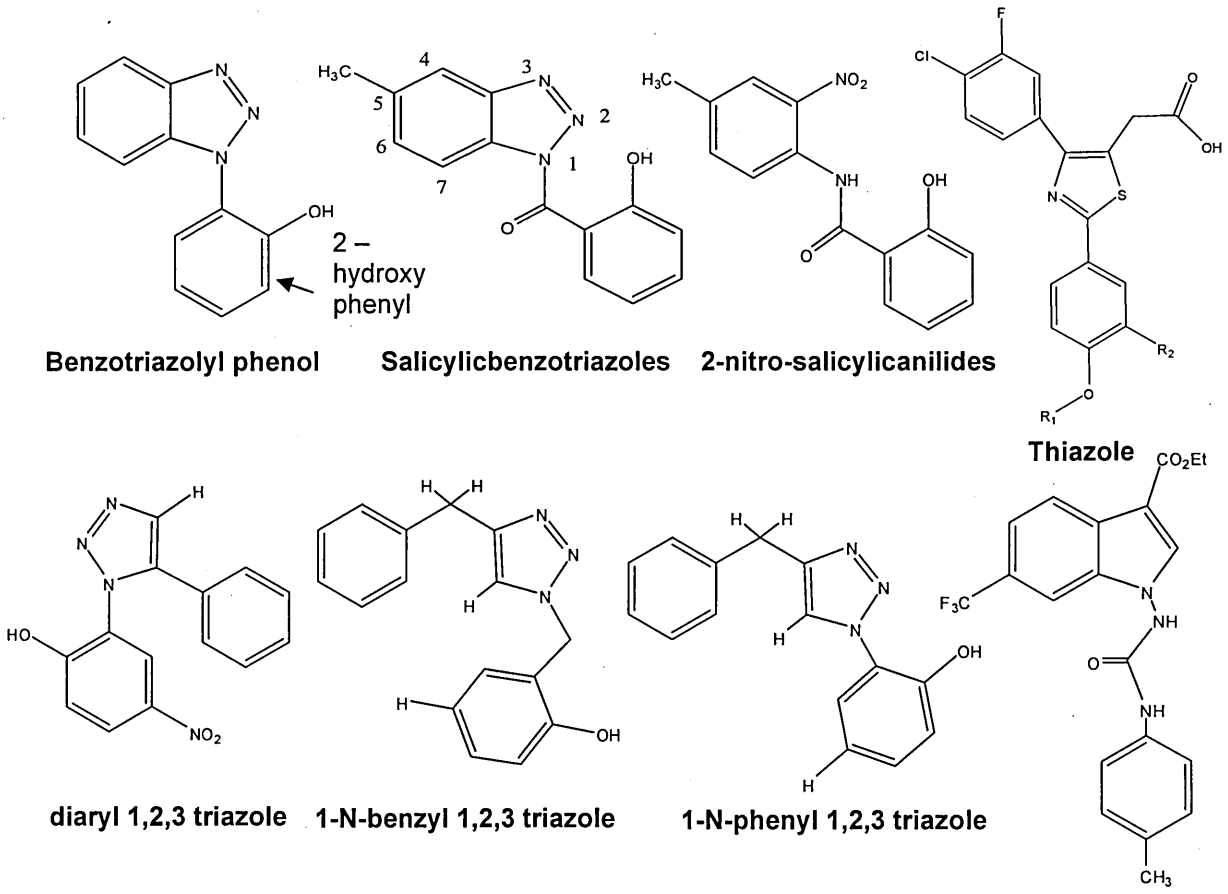


Figure 1.18: Triazoles II and related compounds

Interestingly replacement of the 1,2,3 triazole ring with a 2-hydroxyphenyl group resulted in a benzotriazolyl phenol compound (Figure 1.18) that was 10-fold more effective than triazolyl-benzotriazoles (Biagi *et al* 2001a) providing further evidence to the importance of a 2-hydroxyphenyl ring for potent $BK_{Ca}CO$'s. Further to this work, salicylicbenzotriazoles were generated by introduction of a carbonyl group, found present on the imidazolone ring of benzimidazolones, as a linker between the 2-hydroxyphenyl ring and that of the benzotriazole. This

introduced flexibility in the molecule of which salicylicbenzotriazoles with a methyl group in 5' position (Figure 1.18) were the most potent and was preferred to phenyl or halogenated groups in this position. In addition, the carbonyl function was an essential feature as removal led to a decrease in potency (Biagi *et al* 2001b). The salicylicbenzotriazoles were the first of the triazoles to be tested *in-vivo* where they demonstrated cardio-protective properties against ischaemic injury. Worth mentioning are 2-nitro-salicylicanilides (Figure 1.18) a more open structured version of 5-(2'-nitroanilino)-1,2,3 carboxyamido triazoles (Figure 1.17) also with an amide linker. This demonstrated comparable potency to NS1619 and again the addition of methyl group in 5'positon of 2-nitro-salicylicanilides produced the most potent compound (Figure 1.18), (Biagi *et al* 2001b).

A series of 1,5-diarylsubstituted 1,2,3 triazoles were selected for investigation by Calderone and researchers (Figure 1.18). These compounds were derived from the bioisoteric replacement of the 1,2,4 triazol-3-ones heterocycle studied by researchers at BMS with a monocylic 1,2,3 triazole ring. The diaryl substituted 1,2,3 triazoles demonstrated similar potency to NS1619 but did not display full vasorelaxant properties, on the whole the 1,2,3 triazole cyclic linker was not as profitable as triazol-3-ones. This led to the conclusion that the presence of a hydrogen-bond donor site such as the NH group of the 1,2,4 trizol-3-one heterocycle was important for activation. However, addition of a H-bond donor in the form of a secondary or tertiary alcohol near the 4-position of the 1,2,3 triazole ring caused a loss of activity (Biagi *et al* 2004a; Calderone *et al* 2005a). Another difference is the presence of a carbonyl group in triazol-3/5-ones, however, the lack of activity can not be fully attributed to lack of this group as triazoles tested demonstrated $BK_{Ca}CO$ without this function (Biagi *et al* 2004a; Calderone *et al* 2005a). In addition, the inclusion of methylene spacer was well tolerated but addition of a carbonyl linker was a deleterious requirement (Calderone *et al* 2005b).

Further SAR work with this series highlighted the importance of hydroxyl group in 2-position, as compounds lacking this function or substituted with nitro or methoxy were inactive (Calderone *et al* 2005a). Further, addition of Cl in *para* position to hydroxyl group increased potency. Whether the Cl atom has electronic or steric effects upon triazoles remains undetermined (Calderone *et al* 2005b).

Flexibility of these molecules was investigated and it was identified that 1-N-benzyl substituted 1,2,3 triazoles (Figure 1.18) were much more potent than NS1619 and induced a near complete relaxation of pre-contracted aortic rings, and that the less flexible 1-N phenyl substituted analogue was ineffective. This demonstrated that flexibility with a benzyl group is more favourable than the phenyl and may effect the conformation and interaction with channel as phenyl groups bear a more co-planar conformation (Calderone *et al* 2005b).

These data support that the pharmacophore of these deannulated $BK_{Ca}CO$'s (triazoles and triazol-3/5-ones) requires two H-bond sites. In addition the pre-requisites of $BK_{Ca}CO$'s having two phenyl rings one a hydroxy-*para*-chlorophenyl and the other containing an EWG is an important factor in the the design of deannulated $BK_{Ca}CO$'s. However, the composition of the linker/spacer region in particular placement of H-bond donating group in the series of triazol-3/5-ones and triazoles imparts a significant degree of biological activity.

Researchers at GlaxoSmithKline have recently reported in patents that a series of substituted thiazoles as $BK_{Ca}CO$ for the treatment of urinary bladder conditions (Figure 1.18). However, compounds described also demonstrated COX -1 inhibition. In an attempt to develop further compounds with lower EC_{50} for BK_{Ca} channel activation than that required for COX inhibition various substitutions have been described that fit into pharmacophore patterns described for other activators and include the presence of EWG and H-bond donors (Marino and Haifeng, GSK, 2007).

1.11.9 CGS-7181

The compounds CGS-7181 and CGS-7184 have been developed by researchers at Novartis Pharmaceuticals as $BK_{Ca}CO$'s. Structurally they are esters of indole-3-carboxylic acids and have shown to be far more potent than structurally related NS004 and NS1619 with similar potency to DHS-1 (<0.1μM). It was hypothesised that the acidic NH of the urea moiety could act as a bioisoteric replacement for the 2-hydroxy function of NS004 and NS1619 (Hu *et al* 1997) which has been shown to be critical for BK_{Ca} channel activation in these and related compounds (Meanwell *et al* 1996; Hewawasam *et al* 1997). CGS compounds were thought to act on a site located intracellular on the α-subunit of the channel; although when the compound was applied to either aspect of the channel it was able to activate BK_{Ca} channels by increasing open pore probability. Specificity was investigated via the effect of CGS-7181 to inhibit binding assays for 26 other enzymes and receptors. The effects of CGS-7181 on BK_{Ca} channels from cells isolated from vascular and non-vascular tissue systems across different animal species (dog and rat) was tested, differences in sensitivity to different compounds within the series were found yet further SAR work and *in-vivo* testing has not been reported (Hu *et al* 1997; Coughlan *et al* 2001).

1.11.10 Symmetrical bisphenols

The concept of a symmetrical pharmacophore of BK_{Ca} channel openers was explored by bioisoteric replacement of the amide of NS004 with phenolic hydroxyl groups to create symmetrical bisphenols. These compounds were assessed by substitutions with various halogens and separation of the two ring structures by a one or two bond linker (L) of either methylene or thiol bridges and by a four bond urea linker. Compounds of this description were obtained from BMS compound library and all compounds displayed C_2 symmetrisation (Figure 1.19), (Li *et al* 2003). Different bond lengths (1, 2 and 4) were tolerated and introduced flexibility in the molecule and multiple substitutions of the ring systems with EWGs were essential for compounds to display enhanced BK_{Ca} channel opener activity. It was shown that EWGs, in particular halogenations with chlorine, fluorine and bromine, increased the acidity of the phenolic OH (Figure 1.19).

The pKa (logarithm of the acid dissociation constant) for the series of compounds was determined and those with a value of less than 8 produced significant increases of IbTX sensitive current. Selected compounds with chlorine or bromine R' group substitution (with one bond methylene separation) produced the greatest increase in activation of BK_{Ca} channels (up-to 3-fold) as demonstrated by bromo bisphenols and chloro bisphenols, (Li *et al* 2003). NS1608, an un-symmetrical BK_{Ca} channel opener with a urea linker was less potent than the symmetrical bisphenols with a urea linker (Li *et al* 2003). The structure of magnolol can fit into the description of a symmetrical BK_{Ca} channel modulator.

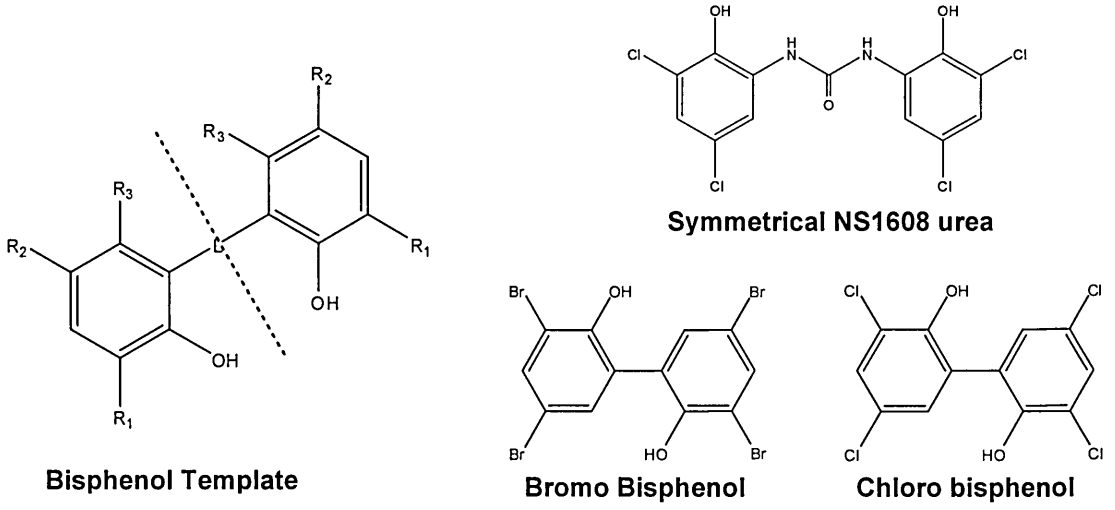


Figure 1.19: Symmetrical bisphenols

1.12 Non-benzimidazolone derived $BK_{Ca}CO$

A number of non-benzimidazolone derived compounds have been studied for $BK_{Ca}CO$ properties. A pharmacophore approach has been used for some of these classes of molecules to understand the chemical features pertaining to those compounds bearing $BK_{Ca}CO$ properties.

1.12.1 Pimaric acid derivatives

Further $BK_{Ca}CO$ compounds based on terpenoids (section 1.8.1) were discovered by the work of the research groups of Imaziumi and of Ohwada in Japan. Investigating compounds bearing similar structure to maxiKdiol they identified a series of pimarane compounds and their isomers. These compounds were first isolated from pine tree gum resins and used as the basis

to identify pharmacophore patterns using a membrane potential $DiBAC_4$ fluorescent assay and whole cell electrophysiology. From which pimaric acid (PiMA) was further characterised for its $BK_{Ca}CO$ activity (Figure 1.20). PiMA increased the calcium and voltage sensitivity of the channel and activated BK_{Ca} channels when applied to either side of the membrane unlike that observed with maxikdiol. However, the effect of PiMA was not dependent upon the presence of β-subunits similar to that observed with maxiKdiol (Imaizumi *et al* 2002). Interestingly abetic acid, a structural isomer of PiMA, could not activate BK_{Ca} channels (Figure 1.20).

Structural alignment between PiMA, maxiKdiol and abetic acid revealed that the small difference in the extension and direction of the hydrophobic residues at C13 was similar in maxiKdiol and PiMA but different in abetic acid, and it was concluded that these alkyl groups could bind to a similar hydrophobic site of the α-subunit. Another requirement of activation was the presence of a strong hydrogen bonding around the C4 region provided by carboxylic acid. PiMA was slightly more potent than maxiKdiol whose H-bond site was around position C5 (Imaizumi *et al* 2002).

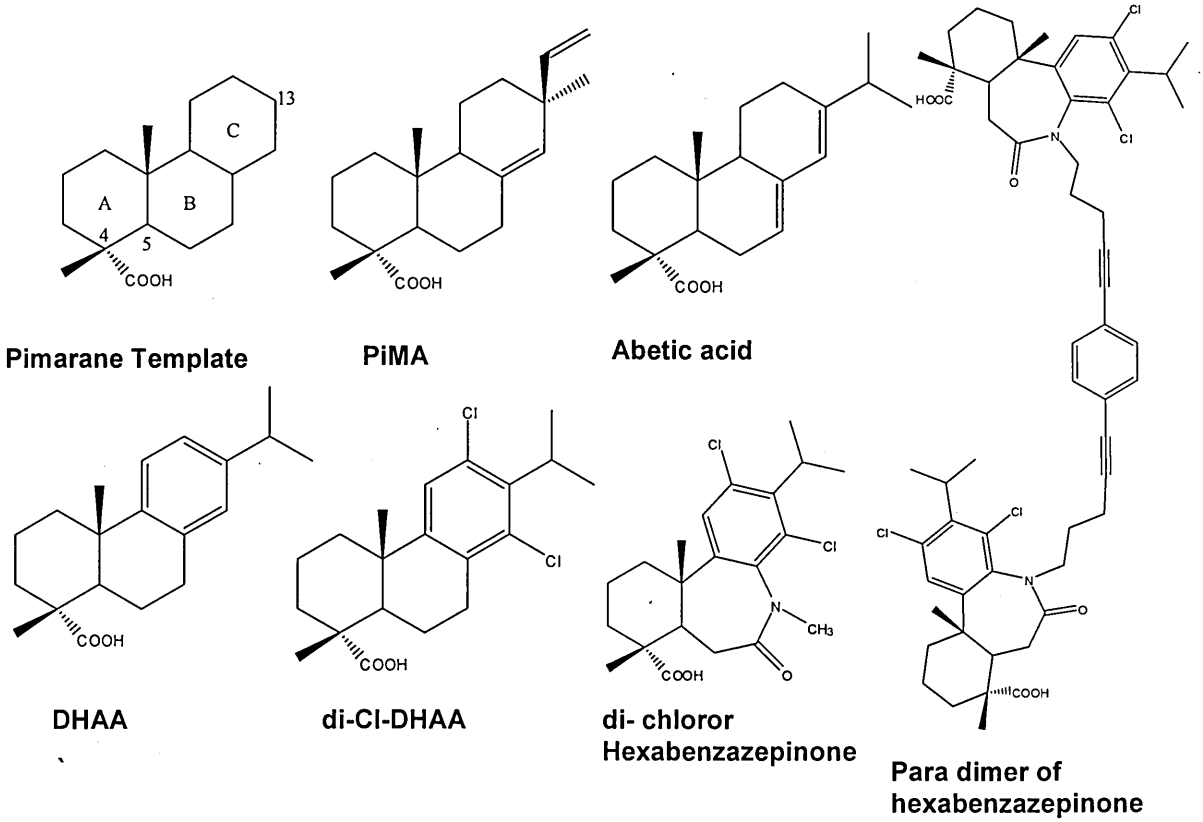


Figure 1.20: Pimaric acid (PiMA) and related compounds

Further structure activity studies revealed that dehydroabetic acid (DHAA), an aromatic form of abetic acid, showed $BK_{Ca}CO$ properties similar to that of PiMA and that superimposition of these compounds revealed a close match in ring 'A' and 'B' but the aromatic ring 'C' of DHAA was different (Ohwada *et al* 2003). Therefore, a series of mono and di halogenations with Br or Cl of the 'C' ring were performed and it was shown that di-chlorination provided a compound, 12-14-dichlorodehydroabietic acid (di-Cl-DHAA) that demonstrated marked increase in BK_{Ca} channel current (Figure 1.20). The study also confirmed the importance of a carboxylic acid moiety in the C4 position as replacement with hydroxymethyl was deleterious, and adding an oxygen functionality to the 'B' ring was negated (Ohwada *et al* 2003). Therefore, an OH provided by the carboxylic acid moiety at this position was important for activity, a common feature among $BK_{Ca}CO$'s.

The properties of di-Cl-DHAA were further assessed using electrophysiology and like PiMA and maxiKdiol it activated BK_{Ca} channels equi-effectively when applied to either aspect of the channel. Moreover, its effects were not dependent on or affected by the presence of the β-subunit and it did not modify single channel conductance. However, unlike PiMA and maxiKdiol, di-Cl-DHAA displayed marked potency with significant increases in BK_{Ca} channel current at concentrations <0.1μM and is one of the most potent $BK_{Ca}CO$'s published to date (Sakamoto *et al* 2006). Furthermore, di-Cl-DHAA was different to a number of other $BK_{Ca}CO$ such as BMS-204352 in that its potentiation of BK_{Ca} channel currents (relative to control) was much greater at more negative voltages and became diminished at more positive voltages. The mechanism of action was thought to decrease the time the channel remains in the closed state. Specificity was examined and at higher concentrations di-Cl-DHAA suppressed VDCC currents (Sakamoto *et al* 2006).

A new scaffold based on modifications of the dehydroabetic acid (DHAA) molecule has been proposed that involves linking two pharmacophore centres together forming a dimer. It was hypothesised that this would enhance the binding probability of a compound to the tetrameric structure of the α-subunit. Through the introduction of a hetero atom and ketone into ring 'B' of DHAA, hexabenzazepinone derivatives were synthesised, from which di-chloro-

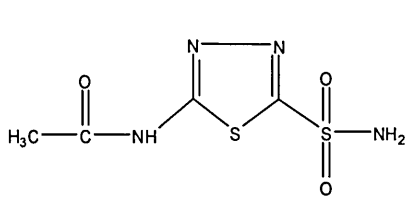
substituted N-methy-hexahydrodibenzazepinone (Figure 1.20) formed the basis of a novel BK_{Ca} channel pharmacophore.

Dimers were tethered to each other via a rigid diacetylenebenzene unit that linked each hexabenzaxepinone via n-propyl chains bonded to the N atom of hexabenzazepinone (Figure 1.20.) These propyl chains introduced molecular flexibility within the dimer. Furthermore, *meta*, *para* and *ortho* substitution of the benzene ring of the rigid diacetylenebenzene unit allowed to change the distance between the pharmacophore centres (Tashima *et al* 2006). This was a novel approach and was performed to try to probe particular binding sites and gain an understanding in terms of structure-fit relationships. There was no preference for a particular *meta, para* or *ortho* substitution pattern and Tashmia *et al* (2006) showed that some monomeric compounds particularly those possessing a terminal benzene were more potent than the dimers. The authors have recently obtained patent protection for the therapeutic use of PiMA derivatives as BK_{Ca}CO (Ohwada and Imaizumi, Tanabe Seiyaku, 2006).

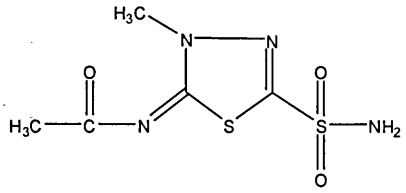
1.12.2 Carbonic anhydrase inhibitors

Recently Tricarico *et al* (2004) examined the pharmacophore of a series of carbonic anhydrase (CA) inhibitors identified as BK_{Ca} channel openers (Figure 1.21). This is typified by acetazolamide (ACTZ) used in the treatment of hyperkalemic periodic paralysis (hypoPP). ACTZ effects were dependent upon the level of intracellular Ca^{2+}. CA compounds that activated BK_{Ca} channels all showed an intramolecular hydrogen bond with inter-atomic distances between 1.82-3.01Å and contained a ring poor of electrons. The lack of one of these two properties led to inactive compounds with methazolamide lacking aromacity and hydrochlorthiazide demonstrating a large inter atomic hydrogen bond (>3.01Å). Further, analysis of spatial geometry and electrostatic potential profiles for ACTZ revealed three negative charges and one positive similar to that of NS004 and could represent a complementary binding site on the channel (Figure 1.21). Due to the Ca^{2+} dependent effect of ACTZ action Triacarico *et al* (2004) proposed that ACTZ could act to stabilize binding of Ca^{2+} ions to the Asp rich Ca^{2+} binding sites of the channel and at physiological intracellular pH Asp sequences are negatively charged. Further potency didn't correlate to logP or logD suggesting a cytosolic hydophillic site of action.

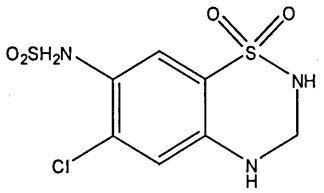
Carbonic anhydrase inhibitors



Acetazolamide

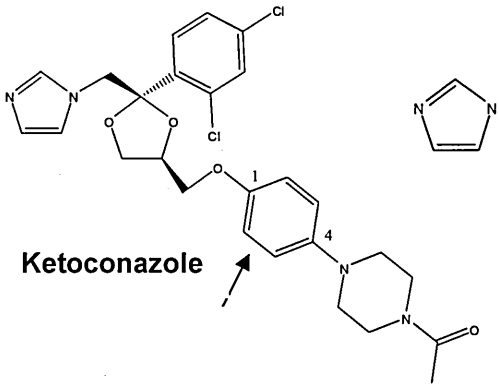


Methazolamide

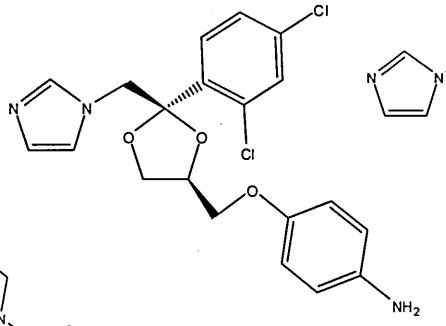


Hydrochlorthiazide

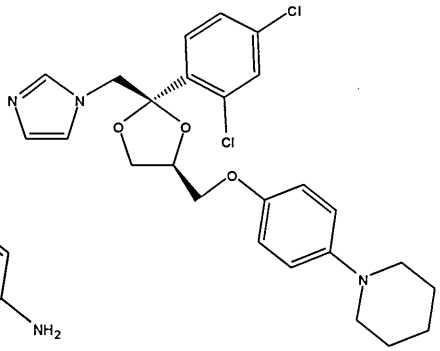
Ketoconazole derivatives



Ketoconazole



4-Aminophenoxy derivative



Piperidino derivative

Figure 1.21: Carbonic anhydrase inhibitors and Ketoconazole derivatives

1.12.3 Ketoconazole

Ketoconazole is a synthetic biaryl compound that is used as an antifungal agent in the treatment of fungal skin infection. Power *et al* (2006) determined that ketoconazole was an opener of BK_{Ca} channels in bovine pulmonary artery cells. The analysis of derivatives or partial structures of ketoconazole determined that the 2,4-dichlorophenyl group was essential for activity as its removal led to inactive compounds. In addition, the imidazole group of ketoconzaole was not important for activation. Removal of the acetyl moiety to leave piperizine as the moiety at position 4 of the phenoxy gave a compound that was similar in potency to ketoconazole. However, removal of the acetylpiperazine to leave a *p*-aminophenoxy group (Figure 1.21) gave a compound that was significantly more active than the ketoconazole. Interestingly, removal of the amino to leave a phenoxy group or substitution with bromine led to compounds that blocked BK_{Ca} channels, further replacement of the acetylpiperazine with piperidino (Figure 1.21) led to compounds that blocked BK_{Ca} channel currents (Power *et al*

2006). Therefore the aniline nitrogen atom is thought to confer $BK_{Ca}CO$ properties to these derivatives, such that it might act as a hydrogen bond acceptor or donor or increase the electron density in the phenoxy ring. Interestingly piperidino derivatives containing an anilino moiety was a BK_{Ca} channel blocker, the anilino compounds that activated the channel had hydrophilic subsituents whereas the piperidino compound was lipophillic. Therefore, phenyl groups seem to be important for binding and that the type of phenoxy moiety is crucial for ability of compounds to activate the channel.

1.12.4 Structural derivatives of tamoxifen

Sha *et al* (2005) investigated tamoxifen and other structurally related compounds using a $DiBAC_4$ fluorescent assay. It was determined that the N,N-dimethylaminoethyloxy group was not necessary for activity, and removal of this functionality to leave an acidic phenol was also not required for tamoxifen activity (Figure 1.22). Stilbene derivatives of tamoxifen were synthesised and it was shown that substitution of the ring (R1 group) with hydrophobic groups such as methoxy and trifluomethyl was crucial for activity where as halogens were not. Analysis of a structurally related synthetic non-steroidal derivative, diethylstilbestol (DES), was more potent than tamoxifen but a methoxybenzene derivative was the most potent compound examined (Figure 1.22). Structural differences resolved that the hydroxyl function of DES was not important and that the substitution with hydrophobic groups (methoxy) at the *para* position of the benzenes increased potency. In addition, substitutions of the dimethyloxystilbenes were more potent than the stilbene derivatives implicating the arrangement of benzene rings around the central double bond in determining the potency.

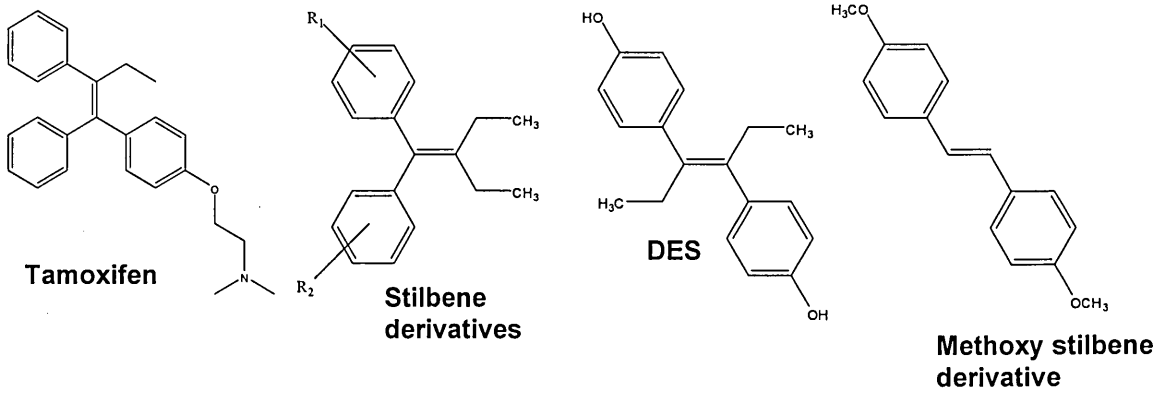


Figure 1.22: Xenoestrogens and their derivatives

1.13 Indole and acrylamide derived $BK_{Ca}CO$'s

1.13.1 NS-8 and 2-amino-4-azaindoles

The pyrrole compound NS-8 (2-amino-3-cyano-5-(2-fluorophenyl)-4-methylpyrrole) was identified by Tsuda at Nippon Shinyaku as a $BK_{Ca}CO$ that showed significant efficacy in the rat systometry and hyper-reflexia *in-vivo* models of overactive bladder (Tsuda *et al,* Nippon Shinyaku, 1999) however a phase-I clinical trial of NS-8 was recently discontinued (Japan Corporate News (2007), (Table 1.2).

Turner and colleagues at Abbott laboratories, using NS-8 as a starting template, have developed several derivatives of 2-amino-4-azaindoles (Figure 1.23). SAR studies were informed by analysing different 2-substituents of the azaindole nucleus. Compounds were assessed using a radioactive Rb^+ efflux assay with smaller acyclic aliphatics groups ($R'N(CH_3)_2$ and $R'N(CH_2CH_3)_2$ preferred to longer chains ($R'NHCH_2Ph$) and for cyclic aliphatics 6 and 7 member substituted rings were preferred to 5-membered rings. Compounds with either piperidine or morpholine ring subsituent were the most potent producing 7-fold higher increase in whole cell BK_{Ca} currents than NS8. In addition the position of the OH group of the morpholine and its steric conformation had great effect upon potency (Turner *et al* 2003; Turner *et al.* Abbott Lab, 2004). Further to this work a series of pyrrolopyridine compounds, in particular 2-amino-pyrrolpyridine (Figure 1.23), have been patented as novel BK_{Ca} channel openers although detailed information on SAR studies has yet to be published (Turner *et al.* Abbott Lab, 2004).

Further researchers as GSK are working on the basis of a similar template (Figure 1.23) to 2-amino-4-azaindole that has been disclosed in patents (Dennis *et al.* GSK. 2005).

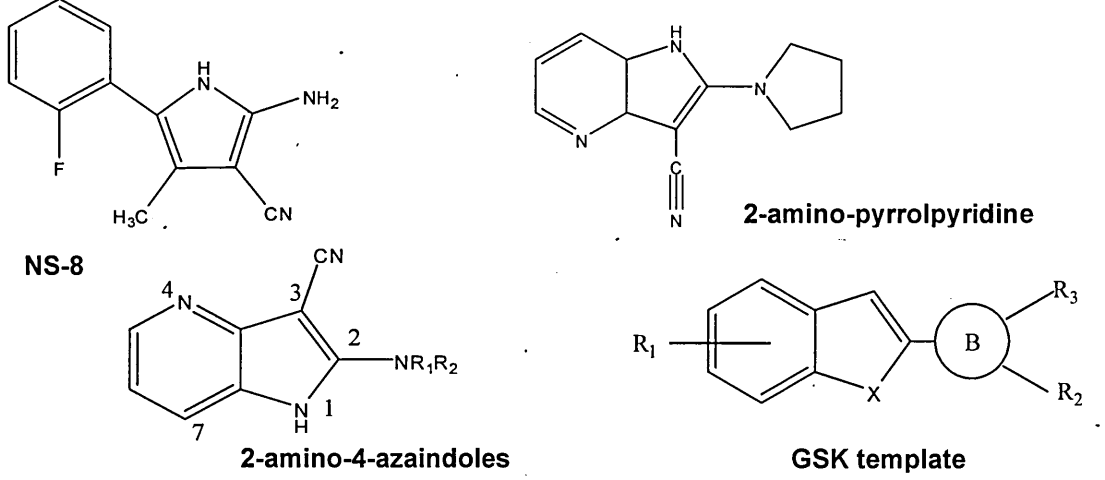


Figure 1.23: NS-8 and related compounds

1.13.2 Tetracyclic benzofuranindoles

Butera and colleagues at Wyeth have investigated tetracycle compounds as potential novel K_{ATP} channel openers based on structural modifications to Celikalim. Furanindole was the most potent of this series but examination of the vasorelaxant effect of these commpounds on pre-contracted bladder and aorta was not reversed by K_{ATP} channel blocker glibenclamide but could be reversed by application of IbTX (Figure 1.24). Interestingly these compounds showed selectivity in activation of BK_{Ca} channels to bladder than in aorta with an aorta to bladder potency ratio of 8 to 46-fold. From a SAR point of view removal of bromine from furanindole led to a 2-fold loss in potency but replacement with iodine, chlorine or nitro group led to a 2-fold increase in potency and that the indole NH and carboxylic acid (acting as a proton donor) was critical for activity. Although, replacement of the oxygen atom of benzofuran with carbon was also tolerated (Butera *et al* 2001).

At the same time the research group of Park and Kim in South Korea identified benzofuranindoles as $BK_{Ca}CO$'s. Based on the superimposition, using a MOPAC2002 protocol, of a benzofuroindole skeleton with that of BMS-204352 a novel pharmacophore was suggested that led to the generation of compounds with substitution at positions 1, 2, 7 and 8 with EWGs (Cl, Br and CF_3) or with a carboxylic acid. The most potent compounds, as tested in outside-out electrophysiology on cloned rat BK_{Ca} channels expressed in *Xenopus laevis*, had chloro and trifluoromethyl group substitution at position 4 and 7 and

carboxylic acid moiety at position 1 (Gorememis *et al* 2005). This fitted in with the pharmacophore of a benzofuroindole skeleton proposed by Butera and colleagues with negative charges at position 1 and electron withdrawing groups at position 4 and 7 (Butera *et al* 2001; Gorememis *et al* 2005).

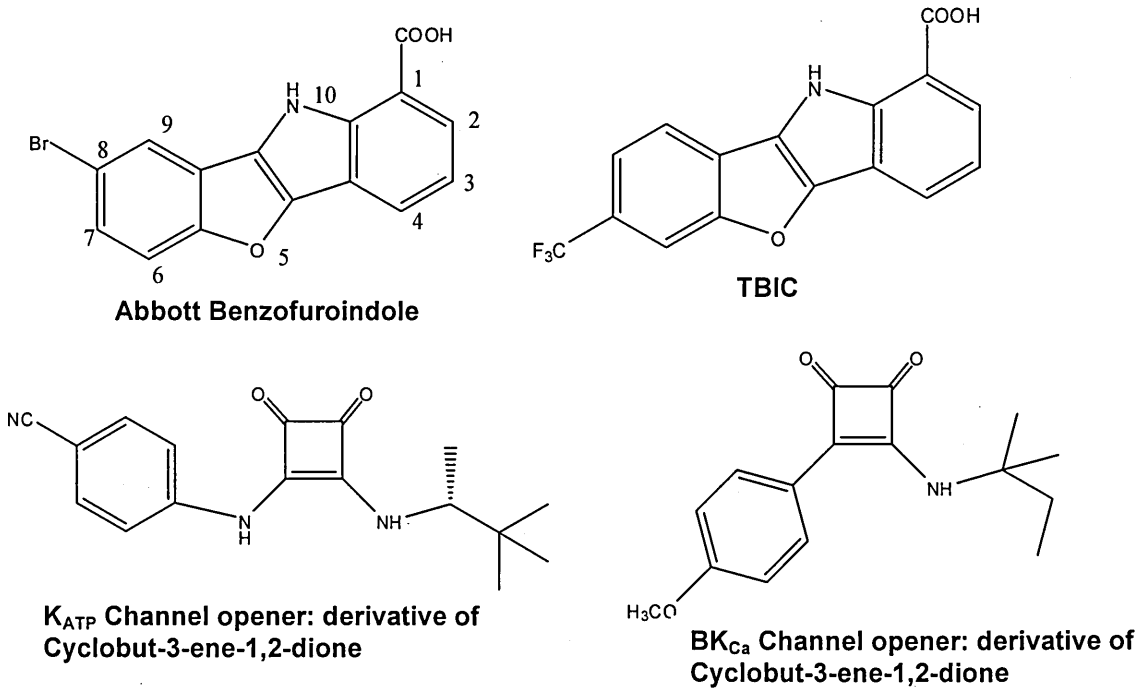


Figure 1.24: Benzofuroindoles and substituted cyclobut-3-enes

Further investigation of compound 8 of this series by Ha *et al* (2006) termed TBIC (Figure 1.24), activated BK_{Ca} channels in pyramidal neurons from the hippocampus with greater efficacy than those of cloned cell lines expressing rat BK_{Ca} channel α- and β1 subunits. Its action did not require the presence of β1 subunit and shifted conductance-voltage curve without affecting voltage sensitivity. Furthermore, it increased P_o and demonstrated fast onset of activation when applied and fast inactivation of BK_{Ca} channel currents when removed and a more pronounced effect was observed when it was applied to the extracellular side. Further investigation of the allosteric sites of interaction on the α-subunit in addition to investigating differential effects in different tissues needs to be investigated to identify tissue specificity (Ha *et al* 2006).

Cyclobut-3-ene-1,2-diones were developed as K_{ATP} channel openers by researchers at Wyeth (Figure 1.24). However slight modification to this structure revealed new BK_{Ca}CO's that did not demonstrate specificity to K_{ATP} channels

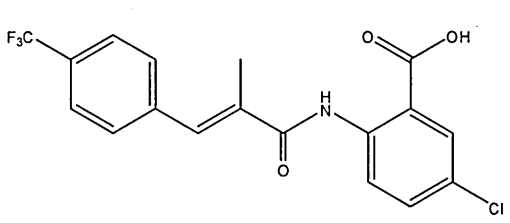
(could not be blocked by glibenclamide). This is typified by the removal of a N-atom of the cyclobut-3-ene-1,2-diones and replacing with aralkyl groups with the most potent derivatives achieving a bladder to aorta specificity ratio of >30 (Butera *et al* 2005).

1.13.3 Acrylamides

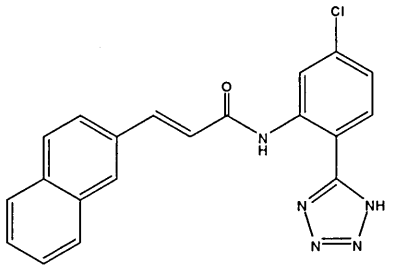
Another chemotype investigated by Wyeth were molecules composed of a cinnamic amyl group linked to an anthranilic acid group by an acrylamide linker (Figure 1.25) whose structures have been disclosed in patents (Lennox *et al*, Wyeth 1999; Lennox *et al,* Wyeth, 2000). These acrylamides share structural relationship to fenamates due to the presence of an anthranilic ring. These acrylamide compounds, as was demonstrated for cyclobut-3-ene-1,2-diones and benzofuroindoles, showed selectivity to bladder versus vascular tissue. Further SAR work revealed that EWG in both rings are important and that introduction of electron donor group such as methoxy was detrimental (Figure 1.25) to activity. Removal of Cl from right hand anthranilic acid ring led to a decrease of potency in smooth muscle relaxation but increased tissue specificity to bladder however on the left hand ring replacement of EWGs with other halogens was tolerated. The addition of a N atom into anthranilic acid ring or esterification of the carboxylic acid derivative was deleterious (Lennox *et al,* Wyeth, 1999, Lennox *et al*, Wyeth, 2000; Nardi and Olesen 2007). This work suggested that one of the aromatic groups should be substituted with an acidic H-bond donor such as that provided by carboxylic function and that this group is prefered in the *ortho* position to the linker region. Therefore these compounds have properties that have been ascribed for other $BK_{Ca}CO$'s (Coughlan *et al* 2001; Calderone *et al* 2005a,b; Nardi *et al* 2006). The acrylamide in Figure 1.25 and associated compounds were tested in the Malgrem's rat hypertrophied bladder model and led to a 70% reduction in spontaneous contraction (Argentieri *et al* 2006).

Nardi and Olesen at Neurosearch A/S investigated the structural similarity between benzofuroindole and acrylamides. Overlapping of the two molecules revealed that the right hand side of the rings demonstrated good alignment with a common polar feature formed from the O and NH within the linker regions and this is placed in close proximity to a carboxylic acid and phenyl groups.

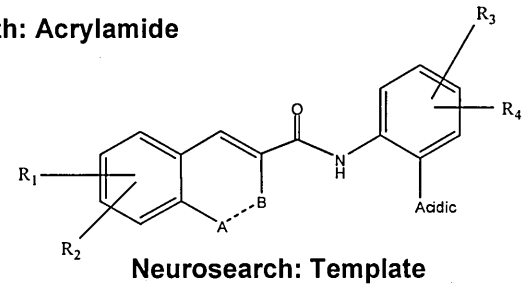
However the left hand side of the rings demonstrated poor overlap showing that these structures had tolerance to the different type of subsituents in this side of the molecule.



Wyeth: Acrylamide



Neurosearch: Tetrazole-acrylamide



Neurosearch: Template structure for generation of new acrylamides

Figure 1.25: Acrylamides

These compounds from Wyeth were limited in the patent protection to substituents in R4 or R5 as being either carboxylic acid or COOR, therefore due to the requirement of an acidic group in this position Neurosearch A/S investigated the optimal acidic moiety in this position (Nardi *et al* Neurosearch A/S, 2006; Nardi and Olesen 2007). They found that the optimal arrangement had an chlorine in position 5 and an acidic function in position 2, a similar optimal arrangement of a Cl in the *para* position to OH for a number of $BK_{Ca}CO$ (including NS1619, NS004 and benzyl-benzylated benzimidazolones etc) has been reported. The bioisoteric replacement of the carboxylic acid function of the anthranilic ring with tetrazole ring (Figure 1.25) increased BK_{Ca} channel current 10-fold at 1μM and substitution with a similar acidic sulphoxide moiety was also tolerated (Nardi *et al* Neurosearch A/S, 2006; Nardi and Olesen 2007). Nardi and Olesen (2007) propose a new template for the design of $BK_{Ca}CO$'s (Figure 1.25) as flexibility in the cinnamic amide/acrylamide linker retains $BK_{Ca}CO$ properties. In addition, the acrylamide linker is a known Michael acceptor (common in a number of different drugs) and prone to exhibit metabolic or toxicological side effects. Therefore, modification of this linker may provide more suitable therapeutics. It should be mentioned that similar cinnamic amides

based on modifications of the template (Figure 1.25) have been developed as potential KCNQ agonists (Nardi and Olesen 2007).

1.14 A generalised pharmacophore for BK_{Ca} channel openers

The current unavailability of a three dimensional crystal structure of BK_{Ca} channels has prevented the generation of 3D pharmacophores that would enable the evaluation of potential drug-channel interaction/binding sites for BK_{Ca}CO's. The diverse range of structures shown to be BK_{Ca} channel openers limits the generation of a unified pharmacophore model that encompasses all compounds. This is highlighted by the structural differences of compounds isolated from natural sources such as DHS-1 and synthetic molecules such as NS1619.

However, as reviewed here BK_{Ca}CO's have shown to share similar structural features or requirements. This has led to a hypothesis of a generalised pharmacophore pattern that could form the basis to guide the design of new molecules demonstrating BK_{Ca}CO properties (Figure 1.26), (Coughlan *et al* 2001; Calderone *et al* 2005a,b; Nardi *et al* 2007).

This pattern is represented by two phenyl rings, that can be symmetrical and structurally attached by a linker region. One of the phenyl rings (A ring) is often represented by a 2-hydroxyphenol as with benzimidazolones, benzyl-benzimidazol-2-ones/BMS-189269 (Meanwell *et al* 1996; Hewawasam *et al* 1997; Li *et al* 1997), quinolin-2-ones/BMS-223131 (Vrudhula *et al* 2007), oxadiazoles/BMS-191011 (Romine *et al* 2007). The replacement of the hydroxyl group or the presence of other acidic moieties is tolerated such as a carboxylic acid in benzofuroindole compounds (Butera *et al* 2001; Ha *et al* 2006), acrylamides (Lennox *et al*, Wyeth 1999; Lennox *et al,* Wyeth, 2000) fenamates (Ottolia and Toro 1994), PiMA (Imaizumi *et al* 2002) and di-C-DHAA (Ohwada *et al* 2003; Sakamoto *et al* 2006). Whether it is the carboxylic acid or the hydroxyl group of this moiety that is important remains unknown, Although, Meanwell *et al* (1996) and Hewawawsam *et al* (1997) suggested that the phenolic OH and carbonyl oxygen of benzimidaolones (NS1619 and NS004) and benzyl-benzimidazol-2-ones could mimic a carboxylic acid. Other classes of

$BK_{Ca}CO$ demonstrate the presence of hydroxyl group as an essential requirement and include phenolic compounds (Wu *et al* 2001; Nardi *et al* 2003), symmetrical bisphenols (Li *et al* 2003) and flavanoids such as phloretin (Koh *et al* 1994; Li *et al* 1997).

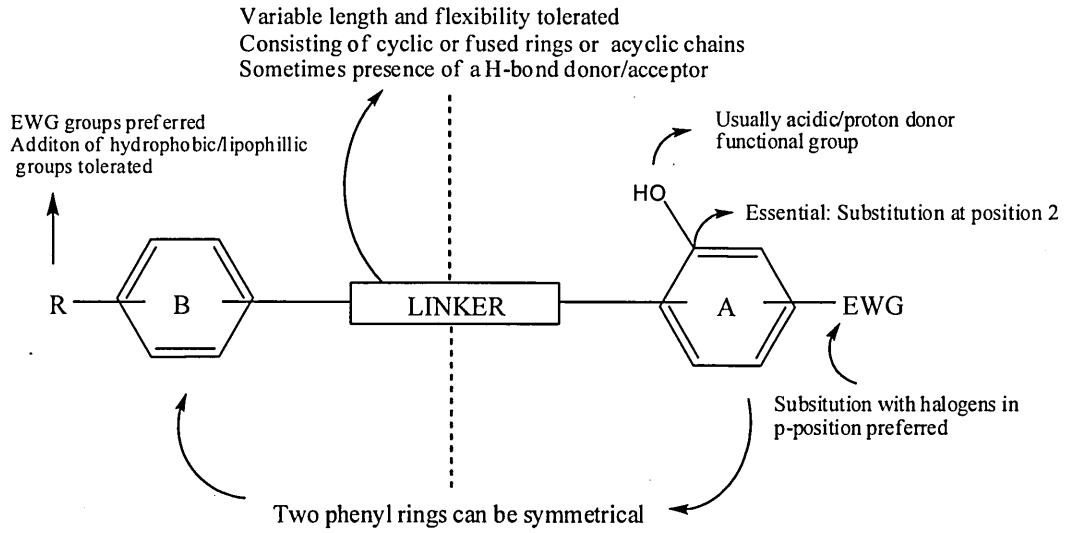


Figure 1.26: A generalised pharmacophore pattern for the development of novel synthetic $BK_{Ca}CO$

Further bioisoteric replacement of the acidic/proton donor group with a tetrazole or sulphoxide is tolerated as demonstrated by certain cinnamic-amide/acrylamide compounds (Nardi *et al.*, Neurosearch A/S, 2006; Nardi and Olesen 2007). Furthermore, the 2' subsituent has been shown to be required in the *ortho* position to the linker region for certain benzimidazolone derived compounds and it has been shown that swapping of the 2' acidic group in particular a hydroxyl group into other positions such as 5' of the ring has shown detrimental effects. This data suggested that the proximity of the 2' subsituent with the linker region maybe important for activity as demonstrated for acrylamides, NS1608, triazol-3/5-ones (Romine *et al* 2002) and benzimidazolone compounds (Meanwell *et al* 1996; Hewawasam *et al* 1997).

The 2' acidic function is thought to impart an H-bond donor site with the channel. In addition, the ionic form of this group such as with the hydroxyl subsituent (O^-) could promote a dipole interaction with the channel. Replacement of an acidic

moiety with a methoxy group is also tolerated but in certain compounds resulted in less active openers. Whether these discrepancies are due to either the steric 'bulk' effect on the compound or the type of interaction with the linker region or the affinity of the compound to the channel remain un-resolved. Another structural requirement in this ring is the presence of a halogen in the *para* position to the 2' subsituent and this has been best represented by a Cl atom. Further replacement of EWG groups in this *para* position with electron donating groups was detrimental to $BK_{Ca}CO$ activity in NS1619 and NS004. However appendage of bulkier heterocyles is tolerated as demonstrated with oxadiazolones/BMS-191011 (Romine *et al* 2007) and quinolin-2-ones/BMS-223131 (Vrudhula *et al* 2005; Vrudhula *et al* 2007). Whether the EWG has electronic or steric effects on the molecule and its ability to be a $BK_{Ca}CO$ remains unknown.

Interestingly the addition of EWG to the rings of bisphenols increased the acidity (pKa) of the phenolic OH and was critical for activity. Further, the positioning and type of halogenation greatly enhances the potency of pimaric acid derivatives (Imaizumi *et al* 2002; Ohwada *et al* 2003; Sakamoto *et al* 2006).

The constituents of the left sided 'B' phenyl ring of the molecule are less stringent. Although a number of $BK_{Ca}CO$ usually have the presence of an EWG typified by a CF_3 group whose positioning on the ring can affect potency. Interestingly the addition of hydrophobic groups such as alkyl chains or benzene rings can increase potency of compounds presumably through its effects on lipopholicity or steric hindrance of the compound. Further, the addition of a methyl group on the B ring has showed to increase potency for a number of compounds including triazoles and triazolones. Therefore, a combination of steric, lipophilic and electronic properties of the B ring can contribute to activating properties.

A linker region, when present, can be a five or six member cyclic ring typified by the triazole or triazolone compounds or a ring fused to one of the phenyl rings such as with the benzimidazolones. In addition, heterogeneous acyclic linkers are tolerated with separation of the phenyl rings by one bond as with magnolol and NS-8 and by two bond linkers such as with symmetrical bisphenols and

fenamates, four bond linkers provided in NS1608, phloretin and CGS-7181 and more recently acrylamides separated by 5-bond linkers have been reported. Therefore, flexibility in this linker region can be tolerated by the presence of different linker moiety.

Furthermore, as shown for cyclic or fused-cyclic linkers the insertion of a methylene spacer between the phenyl ring and the linker can be tolerated for a number of $BK_{Ca}CO$ such as 1,2,3 and 1,2,4 triazoles and benzyl-benzimidazolones also bioisoteric replacement of linker regions can be tolerated. In addition, introduction of stereo-centres into the linker region can open up the structure and induce chirality. Further, chirality and apoisoterism where it exits can have an effect on potency with one isomer being more effective, for example, the (-) isomer for BMS-223131 and (+) isomer for BMS204352/MaxiPost has shown to be preferable.

It has been proposed that at least two H-bonding sites on a molecule are required for $BK_{Ca}CO$ properties provided by an OH group (as part of the A ring) and an NH group. Interestingly an amino group has provided at least one potential site of H-bonding for a number of $BK_{Ca}CO$ and typically occurs within either an acyclic or cyclic ring within the linker region. This is exemplified by an amide moiety found in the linker region of benzimidazolones, BMS-189269, NS1608 (Strobaek *et al* 1996), fenamates (Ottolia and Toro 1994), CGS-7181 (Hu *et al* 1997), benzofuranindole (Butera *et al* 2001), TBIC (Goreméms *et al* 2005), cyclobut-3-ene-1,2-diones (Butera *et al* 2005), triazolones (Romine *et al* 2002) and acrylamides (Lennox *et al.*, Wyeth, 2000; Nardi and Olesen 2007).

However the addition of a hydroxyl function into the 'B' ring for a number of compounds does not increase potency of $BK_{Ca}CO$'s particularly if there are additional sites of H-bonding in the 'A' ring or linker region.

Therefore, a simple model consisting of two phenyl rings separated by a linker region can form the basis of a template structure for the generation of BK_{Ca} channel openers. Substitutions of these rings with various halogens, hydrophobic and hydroxyl groups in addition to chirality can have dramatic effects on potency and efficacy of BK_{Ca} channel openers.

1.15 Future perspectives in the identification of $BK_{Ca}CO$'s

At present compounds identified lack selectivity to a specific ion channel and display different degrees of ancillary pharmacology (Lawson 2000; Coghlan and Carroll 2001). As discussed, NS1619 and NS004 have shown interactions with a number other ion channels (Holland *et al* 1996; Patel *et al* 1998; Al Nakkash *et al* 2001). A lack of selectivity can induce unwanted side effects however the complexity of many pathological conditions including channelopathies where compounds displaying properties of multiple channel activation may be ideal (Lawson and Dunne 2001).

Interestingly subtle differences in template structures for K_{ATP} channel openers can have drastic effects switching specificity to BK_{Ca} channels. In addition cinammic acrylamide compounds also share common pharmacophore pattern to that being optimised for KCNQ/K_v7 channel modulators, this might reveal subtle differences in protein structures of KCNQ vs BK_{Ca} channels that could account for the differential effects of these compounds. In addition, derivatives of ketoconazole deficient of an aniline group were BK_{Ca} channel blockers, however, the presence of this function led to $BK_{Ca}CO$ properties with these derivatives. In addition, other compounds such as abetic acid and menthol do not activate BK_{Ca} channels but their respective structural isomers, PiMA and thymol, have demonstrated potent BK_{Ca} channel activation profiles.

Further investigation of these compounds could help in the elucidation of binding sites and guide the better synthesis of more selective compounds. Consequently, a goal for KCO is the identification of ligand binding sites, recently the development of mutated cell lines has been used in combination with ligand binding assays to identify binding sites of particular protein structures using peptides and structures (Van regenmortel 2001; Lawson 2001a,b).

Another issue with the identification of $BK_{Ca}CO$ is the termination of lead compounds or investigation of particular pharmacophores, this in part due to the industry led nature of $BK_{Ca}CO$ compound development. Furthermore, that the information obtained from in-depth SAR studies of these structures is hidden in

patents or not disclosed for competition reasons. Further, the level of detail and types of investigation vary with different cell line expression systems, tissue types, organ bath setups, electrophysiology configurations and different parameters being tested which hinders comparison between levels of potency and mechanisms of action. Furthermore, many investigations do not include reference $BK_{Ca}CO$'s such as NS1619 to compare the properties of novel compounds against. Therefore detailed comparative studies of newer $BK_{Ca}CO$ have not been reported. Gribkoff *et al* (1996) report the only multiple comparison of $BK_{Ca}CO$ with NS1619, niflumic acid and phloretin. These studies have been limited by a lack of commercially available $BK_{Ca}CO$ as pharmaceutical tools.

The assessment of $BK_{Ca}CO$ compounds is usually achieved by determining the ability of a single concentration of the compound to increase currents of cloned channels expressed in cell lines or oocytes, this limits comparisons of potencies and efficacies. Furthermore, other investigators assessment of compounds involves their functional ability to induce vasorelaxation of tissue following initiation of contraction with a partial depolarisation buffer. The controversy here is whether the compound itself results in relaxation through activation of BK_{Ca} channels or that activation is a result of the depolarisation stimulus (V. Calderone 2007 personal communication). Interestingly in some studies where NS1619 was used as a control it has been unable to induce a vasorelaxant response (Baragatti *et al* 2000). In addition, the effects of potassium channel modulators on vasodilatation of tissue strips can be affected by the presence of the endothelium and its associated vascular mediators (Calderone *et al* 2007).

Discrepancies in rank order of potency have been reported when determining the effect of a single compound concentration to potentiate BK_{Ca} current using whole cell electrophysiology Vs the vasorelaxant properties, as determined by the IC_{50} for relaxation of pre-contracted tissues. These differences could be attributed to the ancillary effect of these compounds on other channels and receptors in the tissue (Hewawasam *et al* 2003a; Hewawasam *et al* 2002d; Boy *et al* 2004). Whether this is due to the molecular composition of the channel, experimental set-up or ancillary pharmacology effects remain un-described

Whether compounds associate with the α or β subunit has been determined for certain compounds, for example, some differentially activate BK_{Ca} channels with specificity to β-subunits such as $DiBAC_4$, DHS-1 and tamoxifen and others activate through association with the α-subunit including benzimidazolones, PiMA and BMS-204352. However specific binding sites on the channel have not been fully elucidated for known BK_{Ca} channel openers. Although sulphatides have shown decreased potency in cell lines expressing STREX splice variant (Chi and Qi 2006). Tissue selectivity has been reported for bladder over aorta for certain compounds (Lennox *et al.*, Wyeth, 2000; Butera *et al* 2001; Butera *et al* 2005; Nardi and Olesen 2007) and again whether this is due to the molecular composition of the channel, experimental set-up or ancillary pharmacology effects remain un-reported.

Although the *in-vivo* effect of certain $BK_{Ca}CO$ has been reported in disease models of stroke, erectile dysfunction, urinary incontinence and hypertension the specificity of action of $BK_{Ca}CO$ is limited by the *in-vivo* toxicological effect of blockers. In addition good potentiation of BK_{Ca} channel currents observed in electrophysiology does not always translate to *in-vivo* efficacy.

Detailed analysis of mechanism of action of compounds using electrophysiology has mainly been described for $BK_{Ca}CO$ isolated from natural sources or ancillary agents such as enzyme activators and inhibitors. From these limited mechanistic studies most compounds activate BK_{Ca} channels by hyperpolarising cells moving the membrane potential closer to the equilibrium potential for K^+ (E_K) by increasing the open time kinetics of the channel with differential effects upon activation when applied to either aspect of the channel observed. Although NS1619 and NS004 have been extensively characterised and used as a pharmacological tool for a number of different studies there still remains a lack of consensus on the mechanism of action in the literature for many newer structural classes of $BK_{Ca}CO$'s. In addition older synthetic $BK_{Ca}CO$ compounds have shown different degrees of potency and have been relatively weak agonists (Kaczorowski and Garcia 1999; Coghlan and Carroll 2001) compared to those identified from natural sources.

Other disadvantages to $BK_{Ca}CO$'s are that some (including DHS-1 and maxiKdiol) only activate BK_{Ca} channels when applied to the intracellular aspect of the channel and display poor cell membrane permeability, this consequently limits their potential as therapeutic tools. The expansion in the development of novel synthetic BK_{Ca} channel openers has been hindered by solubility issues of compounds in aqueous buffers. This has led to a trade off between potency and solubility in the selection of $BK_{Ca}CO$ for further investigation, and to further SAR analysis to improve solubility or exploring the use of pro-drugs as with BMS-191011 (Hewawasam *et al* 2003b; Romine *et al* 2007).

There also remains little published molecular modelling data examining the molecular properties and energy minimised conformations of $BK_{Ca}CO$'s. However, over the past decade the field of identification of $BK_{Ca}CO$'s has made many advances in the identification of numerous heterogenous groups of compounds and elucidation of the properties of these compounds that impart BK_{Ca} channel opener properties.

1.16 Aims of the thesis

The work presented in this thesis aims to explore a series of compounds based on a benzanilide template for $BK_{Ca}CO$ properties and to further probe the pharmacophore of $BK_{Ca}CO$'s. The initial phase involved the development, validation, and optimisation of a medium through-put, non-radioactive, Rb^+ efflux assay as a technique to investigate the pharmacological properties of BK_{Ca} channel modulators **(Chapter 2)**. The Rb^+ efflux assay was used to test compounds on HEK293 cell lines expressing BK_{Ca} channel α subunit alone or co-expressed with the $\beta 1$ subunit for their efficacy, potency, and selectivity by testing against standard pharmacological tools as positive controls **(Chapter 3)**.

The pharmacological profile established with Rb^+ efflux was then assessed using whole cell patch clamp electrophysiology **(Chapter 4)**. The candidate molecule, BKOEt1, identified from the Rb^+ efflux and electrophysiological screen was further characterised in respect to its BK_{Ca} channel activation properties using single channel patch-clamp electrophysiology recording. In addition, the specificity of BKOEt1 to BK_{Ca} channels was assessed by determining the Rb^+ efflux from rat bladder myocytes in the presence of different potassium channel blockers **(Chapter 5)**. The data obtained from these studies at a cellular level using rubidium (Rb^+) efflux assay and patch clamp electrophysiology provides fundamental information regarding the nature of compounds with BK_{Ca} channel opener properties. The use of molecular modelling techniques has enabled the development of a putative pharmacophore model of these compounds **(Chapter 6)**.

Chapter 2

Optimisation of a non-radioactive Rubidium (Rb^+) efflux assay for the assessment of BK_{Ca} channel opener activity

2.1 Introduction

2.1.1 Ion channel screening assays

In the past decade advances in understanding the physiology and the pathophysiology of ion channel function has highlighted them as attractive therapeutic targets for numerous diseases (Bennet and Guthrie 2003; Ebreth 2002). However, until recently there was a lack of progress in ion channel drug discovery and this was in part due to the lack of high throughput screening technologies (HTS), (Bennett and Gutherie 2003; Gill *et al* 2003; Stankovich *et al* 2004).

The shift in direction was in part due to the phenomenon of drug induced long QT (LQT) syndrome and led to the introduction of new drug safety guidelines (Gill *et al* 2003; Stankovich *et al* 2004). LQT syndrome is a side effect of the action of many classes of drugs including, anti-arrythmics, antibiotics, anti-histamine and anti-psychotics. The LQT effect has shown to be mediated by inhibition of the hERG (human ether a *go-go*) channel in cardiac myocytes that can lead to fatal ventricular fibrillation (Sanguinetti *et al* 1995). In light of this the US Food and Drug Administration requires the activity of all new drugs to be assessed against the hERG channel. This has lead to the development of new HTS assay technologies (Zheng *et al* 2004).

In the analysis of ion channels electrophysiology remains the gold standard technique for measuring biophysical characteristics of ion channel function (Gill *et al* 2003; Birch *et al* 2004; Zheng *et al* 2004), but despite advances in automation it remains labour intensive and highly specialised. However, HTS provides a more robust, cost effective, reproducible and informative method of assessing ion channel activity (Zheng *et al* 2004). An ideal HTS assay is ameanable to scale-up, automation and miniaturisation (Bennett and Gutherie 2003; Gill *et al* 2003; Kiss *et al* 2003) and is a trade off between information content of the assay and throughput (Xu *et al* 2001; Bennett and Gutherie 2003).

Screening technologies that have been developed can be grouped into functional or non-functional assays that either provide a direct measure of ion channel activity such as with automated electrophysiology and ion flux assays;

or an in-direct measure such as in membrane potential, calcium fluorescent and radioligand binding assays (Xu *et al* 2001; Bennett and Gutherie 2003).

Fluorescent based assays involve measuring membrane potential changes using voltage-sensitive membrane dyes (oxonal dyes, $DiBAC_4$ series) or with calcium fluorescent dyes (Fura-2, Flo-3, -4) to detect changes in intracellular calcium. The development of instrumentation such as the fluorometric image plate reader (FLIPR) has enabled high-through screening for these fluorescent techniques and consequently the cost per data point is relatively low (Wheng *et al* 2004; Worley and Main 2002). However, there are many disadvantages to fluorescent techniques in that they only offer an indirect measure of channel function and suffer from high background noise, low sensitivity, selectivity, and expensive consumables (Xu *et al* 2001; Gill *et al* 2003; Wheng *et al* 2004).

The lack of ion specific intracellular dyes has limited this technique to the analysis of Ca^{2+} channels and Ca^{2+} activated K^+ channels. Therefore, membrane potential assays have been the focus of further development particularly the use of fluorescent resonance energy transfer (FRET) based systems. However, limitations to this include a higher level of assay complexity, dye-compound interaction and slow response times (Birch *et al* 2004). Furthermore, recent studies of the membrane potential dye $DiBAC_4(3)$, commonly used for the assessment of BK_{Ca} channel modulators (Yamamura *et al* 2002; Yamada *et al* 2001), have demonstrated potent BK_{Ca} channel opener activity with selectively for channels composed the β1-subunit (Morimoto *et al* 2007).

Another indirect method includes radioligand binding assays, for example, the use of radiolabelled charybdotoxin (^{125}I-ChTX) as a ligand in binding assays for BK_{Ca} channel openers. These methods are limited and dependent upon the availability and affinity of different ligands. In addition, binding assays do not show those channel openers that work via an allosteric mechanism or via associated subunits (Kaczorowoski and Garcia 1999). However, this technique has allowed the identification of a number of BK_{Ca} channel openers including terpenes (McManus *et al* 1993; Singh *et al* 1994; Lee *et al* 1995).

2.1.2 Ion flux assays

Ion flux assays are preferred to the techniques described above as they offer a more direct assessment of channel activity. In addition, studies have shown better correlation of compound potency determined between patch clamp electrophysiology and flux studies than with fluorescent based techniques. Ion flux involves the use of a tracer ion for detection of flow through ion channels. Traditional techniques used radioactive tracers including ^{22}Na and ^{14}C guanidium for Na^+ channel, ^{86}Rb or ^{42}K for K^+ channels, ^{45}Ca for Ca^{2+} channels and ^{36}Cl or ^{125}I for Cl^- channels. These techniques used scintillation detection to asses the level of flux that occurs through the channels. For a number of different ion channels radio-active efflux experiments has shown to be comparable to electrophysiology in the determination of compound potency (Becq *et al* 2003; Gill *et al* 2003).

There are a number of disadvantages in the radioactive approach that centre around the safety hazards associated with the use and disposal of radio-labelled ions. In addition, ions such as ^{86}Rb have high emission energy (β_{max} 1.77MeV; γ_{max} 1.08MeV) and a short-half life (18.65 days), these properties do not confer radioactive tracer ions meanable to automation and therefore in HTS applications (Terstappen *et al* 1999; Terstappen *et al* 2004).

2.1.3 Non-radioactive Rb^+ efflux assay

To address the issues associated with the use of radioactive Rb^+ Terstappen (1999) introduced a non-radioactive method incorporating atomic absorption spectrometry (AAS) for qualitative and quantitative determination of ion fluxes.

AAS has long been used for the assessment of trace metals in environmental and biological samples due to its relative simplicity, precision and specificity in the analysis of a selected analyte (absence of matrix interference), (Apostoli *et al* 2002). In flame-AAS, a sample is atomised and sprayed into a flame, the thermal energy generates free ground state atoms that absorb light of a particular wavelength (Rb^+, λ=780.1nm). Applying the principles of Beer-Lamberts law, the amount of absorption by the sample is proportional to the concentration of the analyte, which is determined from standard curves of known amounts of analyte before each analysis (Morton and Roberts 2003).

Non-radioactive AAS flux assays have shown to offer a versatile technique that has been applied to a variety of ion channels (Table 2.1), including the use of Li^+ ions for the detection of Na^+ channels, and the use of Ag^+ ions to detect Cl^- ions measured as efflux through channels (Gill *et al* 2003; Stankovich *et al* 2004; Birch *et al* 2004).

Rb^+ efflux assays have shown to be cost effective and be a better tool than membrane potential assays and calcium fluorescence assays (Tang *et al* 2001) amenable to automation and comparable in terms of potency to patch clamp electrophysiology (Gill *et al* 2003).

The development of ion flux HTS assays has been led by the pharmaceutical industry for a number of different ion channels (Table 2.1). Although any AAS instrument could be used for detection, recent developments in novel high throughput instrumentation technologies such as the ICR8000 and ICR12000 developed by Aurora Biomed enable the analysis of up to 60,000 wells/day (Aurora Biomed 2007).

The rubidium (Rb^+) efflux assay has provided a useful method for the screening of compounds that have modulating effects on efflux through potassium (K^+) channels (Terstappen 1999). Here, Rb^+ is used as a tracer ion for detecting ion flow through K^+ channels due to it having a similar atomic radius to K^+ and its absence in cellular systems (Terstappen 2004). The method is relatively easy to perform but requires a number of assay validation experiments. A number of channels (usually in recombinant cell lines) have been optimised with respect to the assay (Table 2.1) and continues to be proven as a reliable technique for the assessment of K^+ channel function (Scott *et al* 2003; Terstappen *et al* 2004; Chaudry *et al* 2006; Jow *et al* 2006; Wang *et al* 2006; Gill *et al* 2007).

Voltage-gated K^+ channels	Ca^{2+}-activated K^+ channels	Ligand-gated non-selective cation channels	Transporters and Pumps
Kv1.1 Kv1.3 Kv1.4 Kv1.5 Kv7.1 (KCNQ1) Kv7.2 (KCNQ2) Kv7.2/3 (KCNQ2/3) Kv11 (hERG)	BK SK	nAChR P2X	Na^+ K^+ ATPase K^+ Cl^- Co-transporter

Table 2.1: Current examples of channels that non-radioactive Rb^+ efflux has been developed and optimised

2.2 Chapter Aims

The aim was to optimise and validate a medium through-put, Rb^+ efflux assay to quantify BK_{Ca} channel activity for the assessment of candidate molecules as potential BK_{Ca} channel openers.

- Validity of HEK293 cells expressing BK_{Ca} channels as a cell model to use in Rb^+ efflux assay.

- Optimise the individual assay parameters: Instrument validation: Rb^+ loading, extracellular washes, basal efflux and stimulating efflux.

- Determine the ability of the assay to discriminate known BK_{Ca} channel modulators on the basis of efficacy and potency.

- Test the specificity of these openers to BK_{Ca} channels in the cell line used.

2.3 Methods

2.3.1 Materials

Cell culture reagents and equipment

BD Biosciences, Oxford, U.K: T75 cell culture flasks, serological pipettes (10ml), 96-well microplates and Falcon tubes (15 and 50ml).

Invitrogen (GIBCO), Paisley, U.K: Dulbecco's modified eagles medium (DMEM), Dulbecco's phosphate buffered saline (D-PBS), fetal calf serum (FCS) and trypsin-EDTA (0.05%).

Merck Biosciences (Calbiochem), Nottingham, UK: G418 sulphate solution
Sigma Aldrich, Gillingham, UK: Brightline Haemocytometer

General Purpose Reagents

Sigma Aldrich, Gillingham, U.K: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), phosphate buffered saline (PBS) tablets, rubidium chloride (RbCl) and, triton X-100.

VWR International Ltd: BDH, Lutterworth, U.K: Calcium chloride ($CaCl_2$), colour Key pH buffer solutions, dimethylsulfoxide (DMSO), glucose, magnesium chloride ($MgCl_2$), potassium chloride (KCl), sodium chloride (NaCl), sodium dihydrogen phosphate (NaH_2PO_4).

Ion channel modulators

Sigma Aldrich, Gillingham, U.K: 1-(2'-hydroxy-5'-trifluoromethyl phenyl)-5 trifluoromethyl-2(3H)benzimidazolone (NS1619), 4-aminopyridine (4-AP), calcium ionophore A23187, glibenclamide, niflumic acid, ouabain, phloretin and tetraethylammonium (TEA).

Axxora Ltd, Nottingham, U.K: paxilline (pax)

2.3.2 HEK293 cells

The parental Human Embryonic Kidney (HEK293) cell line was generated by Graham *et al* in 1977 through the transformation of HEK cells with adenovirus 5 DNA. HEK293 cells have epithelial cell morphology and grow as an adherent monolayer (Graham *et al* 1977). This cell line has previously been generated (Ahring *et al* 1997) and characterised as stably expressing the zero variant of the human BK_{Ca} α–subunit (h*Slo, KCMNA*), (Hartness *et al* 2003).

2.3.3 Cell culture and preparation of cells for Rb^+ efflux assay

Cell culture was performed under sterile conditions using high efficiency particulate air filters (HEPA) in a class II lamina flow-hood (Heraeus, Germany). All plastics used were sterile and appropriate aseptic techniques were used. Culture media was warmed in a 37°C water bath prior to its use. All cells were grown by incubation at 37°C in a 5% CO_2 humidified incubator (Heraeus, Germany).

HEK293 cells were grown in T75 vented cell culture flasks with Dulbecco's modified eagles media (DMEM with 1.0 g l^{-1} glucose and sodium pyruvate) supplemented with fetal calf serum (10%) and G418 sulphate (0.5μg/μl).

Cells were initially grown from frozen stocks (DMSO, 10%) and then routinely passaged throughout the experimentation period (passage number 10 to 35).

Prior to cell passage the flasks were checked for the degree of confluence and morphological state, in addition the flasks were checked for the absence of microbial contamination.

Confluent cells were passaged by decanting the media, washing adherent cells once with calcium free Dulbecco's phosphate buffered saline (D-PBS, 10mls) and incubating with trypsin-EDTA (3mls, 0.05%) for 3-5 minutes in the incubator. Cells were then detached from the surface of flasks by gentle agitation and re-suspended by dilution with DMEM (10mls). The cell suspension was then split into various densities (1:5 - 1:40) in DMEM (total volume 20mls) in a new T75 flask to acquire confluent cells when needed.

Detached and re-suspended cells were counted using a Brightline hemocytometer and seeded in 96-well tissue culture plates at a density of 20,000 cells per well in a total volume of 200µl of media. Plates were incubated for 48-hours as above until cell growth in the wells reached confluence.

2.3.4 General method for Rubidium (Rb^+) efflux assay

To prepare the cells, media was removed and the cell monolayer was washed once with PBS (200µl). Following this cells were incubated (200µl/well) with rubidium chloride (RbCl) buffer (mM: RbCl 5.4, NaCl 150, $MgCl_2$ 1.0, NaH_2PO_4 0.8, $CaCl_2$ 2.0, HEPES 25.0 and Glucose 5.0) for 0.5-5.0 hours and then washed rapidly 4 times (PBS, 200µl/well), in certain experiments these washes were collected for analysis of Rb^+ content.

Following the washes cells were then exposed to drug treatment or vehicle (DMSO) in KCl buffer (mM: KCl 5.4, NaCl 150, $MgCl_2$ 1.0, NaH_2PO_4 0.8, $CaCl_2$ 2.0, HEPES 25.0 and Glucose 5.0). At specified time intervals the supernatant (Extracellular effluxed Rb^+) was removed from individual wells and collected for analysis, by transferring to a 96-well plate. The cells were immediately lysed to obtain intracellular Rb^+ by the addition of Triton X-100 (0.1%, 200µl/well) in KCl buffer for at least 30-minutes before analysis.

For depolarisation experiments, a high extracellular, isosmotically adjusted, KCl (50mM) buffer was prepared (mM: KCl 50, NaCl 105.4, $MgCl_2$ 1.0, NaH_2PO_4 0.8, $CaCl_2$ 2.0, HEPES 25.0 and Glucose 5.0).

In competition experiments, ion channel blockers were added to the RbCl buffer for the final 20 minutes of the loading stage and to the KCl buffer containing the test compounds or vehicle. Variations to this general method are described where appropriate.

If sample analysis was not performed immediately following an experiment then 96-well plates would be stored in a damp environment (at 4°C) without detriment to the result.

2.3.5 Atomic Absorption Spectrometry (AAS) analysis

The supernatant, lysate and wash samples were analysed, for Rb^+ content, using an S-series Atomic Absorption Spectrometer (Thermo Scientific, Hemel Hempsted, U.K) coupled to a Gilson 222XL auto-sampler (Thermo Scientific, Hemel Hempsted, U.K). The absorbance of Rb^+ was determined at a wavelength of 780.1nm from the samples following atomisation in a stoichometric air-acetylene flame.

Prior to analysis the instrument was cleaned and allowed to warm up for 20-minutes with the Rb^+ hollow cathode lamp and flame on. The instrument was validated, to ensure optimal and consistent performance, by the addition (300μl) of RbCl standards (0-100μM prepared in KCl buffer) into a microtitre plate and comparing the absorption values obtained to ensure values did not exceed 5% error of each other. These values were recorded as a quality control measure to ensure consistent instrument performance.

The samples were diluted with KCl buffer (100μl) prior to analysis. This provided the minimum volume required to ensure confidence and consistency in obtaining a steady state Rb^+ absorbance reading. In addition, dilution ensured that absorption readings did not exceed the maximum limit of detection for the instrument.

The sample in each well was nebulised for one second by the instrument and then following this the absorption was measured continually for one second, from which the average absorbance value from this period was recorded. An inter-well wash time (10 seconds in distilled water) of the needle of the auto-sampler was sufficient to clean the instrument.

Standard curves were generated from the measurement of 1–100μM RbCl standard solutions prepared in KCl buffer prior to each 96-well plate analysis. Standard curves were constructed before and after each analysis to ensure reliability in analysis.

2.3.6 Data Analysis

Rb^+ efflux (%) was calculated as follows:

$$\% \text{ efflux} = \frac{[Rb^+]_{(Extracellular)}}{[Rb^+]_{(Extracellular)} + [Rb^+]_{(Intracellular)}} \times 100$$

Efflux values were exported to Microsoft Excel for data analysis in preparation for graph and statistical analysis in GraphPad PRISM software (version 4). BK_{Ca} channel opener data was normalised as the percentage (%) increase in Rb^+ efflux above control (KCl buffer with appropriate vehicle).

Sigmoidal-concentration response curves were constructed using the four parameter logistic equation as follows:

Y=Bottom + (Top-Bottom)/(1+10^((LogEC_{50}-X)*HillSlope))

X is the logarithm of concentration. Y is the % activated Rb^+ efflux.

E_{max} (maximal % increase in Rb^+ efflux) and $EC_{40\%}$ (the concentration of compound that results in a 40% increase in Rb^+ efflux above control) were calculated.

Results are presented as means±SEM for n determinations. Data was analysed using either Student's un-paired t-test or for multiple comparisons one-way ANOVA followed by Dunnett's or Bonferroni's multiple comparison test (Wallenstein *et al* 1980). The accepted level of significance was $p \leq 0.05$.

2.4 Results

2.4.1 Instrument validation and performance

The optimised AAS instrument was able to detect to a sensitivity of 1.0μM RbCl in KCl buffer (0.005 Absorbance units), (Figure 2.1). The precision of the instrument was tested by application of standard (300μl of 60μM Rb^+) to each well of a 96-well. The average absorbance (0.483±0.001, n=96) and concurrent concentration value (60.5±0.1μM, n=96) showed good reproducibility in analysis. This is further supported by the precision in measuring RbCl standards for 40 separate determinations (Figure 2.1).

Rb^+ measurements were not affected by the presence of other ionic components of the buffer, cell lysates or Triton X-100.

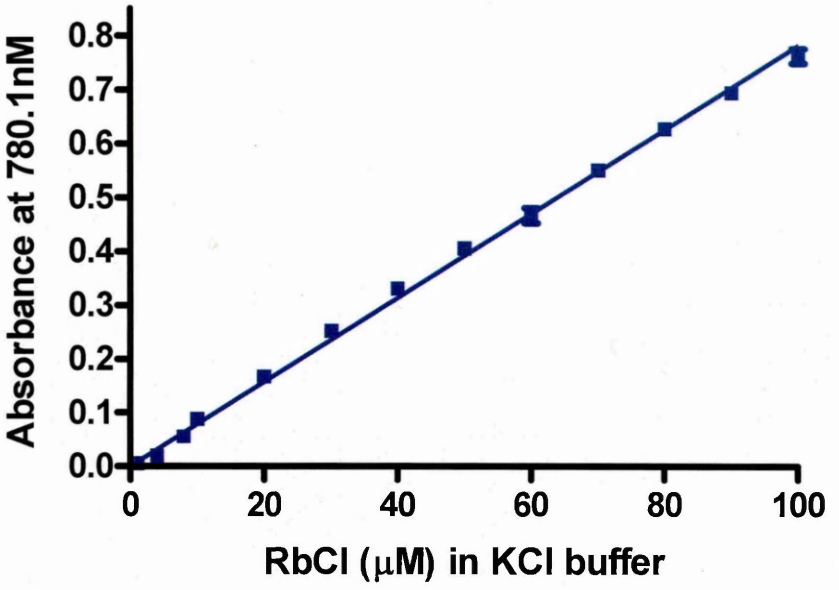


Figure 2.1: Standard curves of RbCl

Absorbance values determined by the AAS from RbCl standards (0-100μM) prepared in KCl buffer. Data represents the average absorbance value taken from 40 determinations (mean±SEM). Data points were fitted by linear regression (y= mx + C) analysis, r^2=0.998.

2.4.2 Measurement of Rb^+ uptake by HEK293 cells

HEK293 cells were exposed to RbCl buffer (0.5-5.0 hours) and immediately lysed following removal of extracellular buffer by washing. The Rb^+ content of the lysate of the HEK293 cells progressively increased with time of incubation in Rb^+ buffer (Figure 2.2). The Rb^+ content appeared to become saturated from two hours incubation and a four hour incubation period was sufficient to reach maximal loading (92.4±4.1μM, n=24; Figure 2.2).

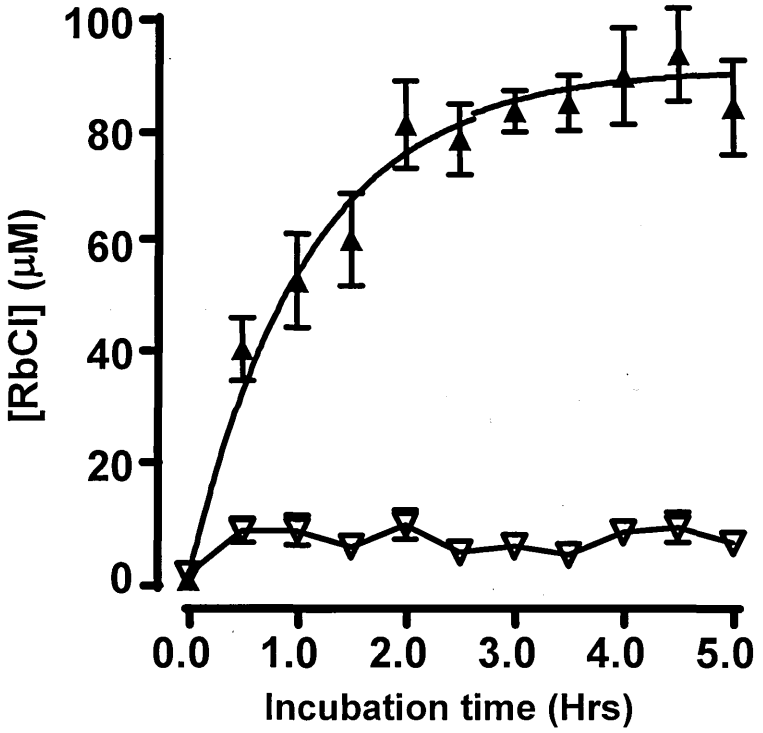


Figure 2.2: Time course of Rb^+ loading in HEK293 cells expressing BK_{Ca} channels

Cells were incubated with RbCl buffer. At timed intervals up-to 5-hours the extracellular buffer was removed and cells were washed 4 times with PBS before lysis with Triton-X100. The Rb^+ content of lysate (▲) and fourth wash (▽) samples are represented as means ± SEM (n=24).

2.4.3 Extracellular washing optimisation

The optimum number of washes was determined to have the confidence that all intracellular Rb^+ measured following the loading time period reflected that which was inside the cell and not a consequence of extracellular Rb^+ associated with the cell surface. Following incubation in RbCl buffer (4-hours during the loading stage) the cells were washed (D-PBS, 200μl/well) a number of times and samples were collected and analysed to determine Rb^+ content (Figure 2.3). Following three sequential washes a low extracellular Rb^+ concentration was obtained (Rb^+=10.2±2.0μM) that did not significantly (Student's un-paired t-test,>0.05) vary with further washes (4 washes= 4.2±0.81 μM; 5 washes 3.5±0.98 μM, p>0.05, n=24). These findings support the Rb^+ content of the lysate reflects the intracellular concentration of the ion and not that as a consequence of insufficient washing.

The effect of insufficient washing on Rb^+ efflux was determined by measuring the Rb^+ efflux from cells (10-minutes exposure to KCl buffer) after different numbers of PBS washes (1-4). Insufficient washing leads to artificially high efflux values (Figure 2.4), these values significantly decreased ($p<0.05$) with subsequent washes (1 to 4 washes). The fifth wash did not significantly affect the measured Rb^+ efflux (Four washes= 25.2±2.9%; Five washes= 21.6±2.6%, P=0.41, n=24). Therefore this value after four washes represents the Rb^+ efflux in response to exposure to the KCl buffer and not that which is associated from insufficient washing.

Although three washes removed extracellular Rb^+ from the cells, four washes was chosen to ensure confidence in removal of all cell surface Rb^+.

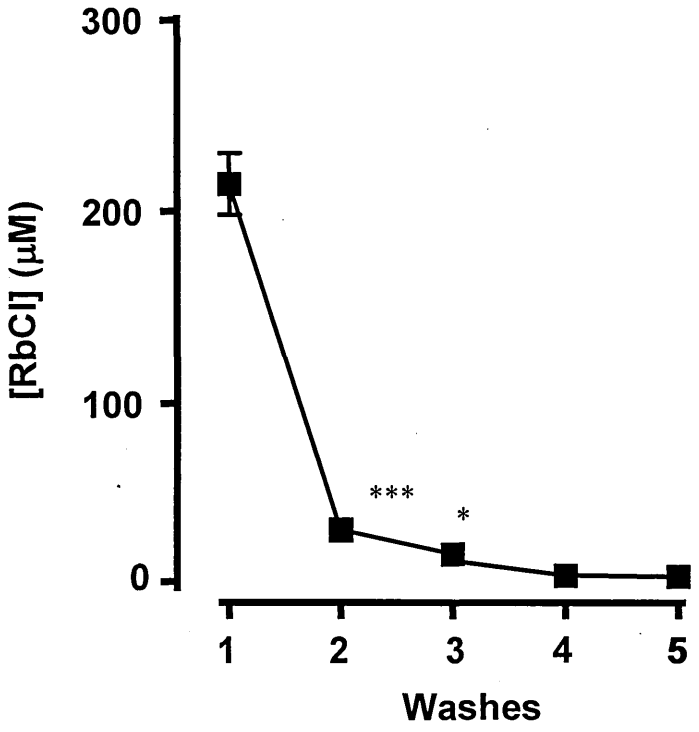


Figure 2.3: Rb^+ content of extracellular wash buffer

The effect of the number of sequential washes (PBS, 200μl) on the removal of Rb^+ from the extracellular compartment of HEK293 cells (■), Data presented as means±SEM (n=24). Student's un-paired t-test, *=$p<0.05$, ***$p<0.01$ indicates significance level relative to previous wash number

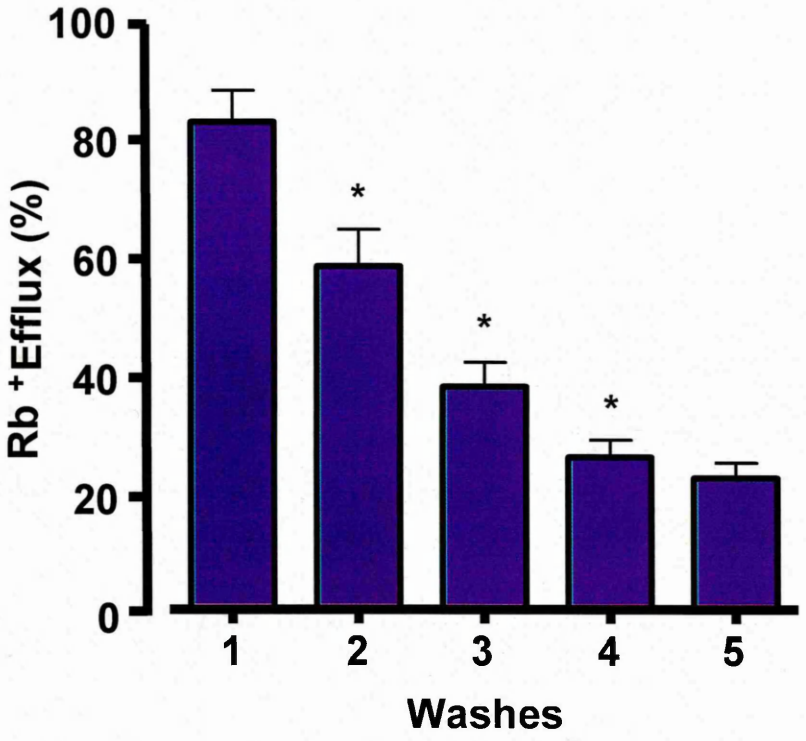


Figure 2.4: The effect of incomplete removal of Rb^+ on efflux values

Cells were exposed to KCl buffer for 10-minutes following sequential washes. Rb^+ content determined in KCl buffer and lysate were used to calculate % efflux. Data expressed as means±SEM (n=24) per group. Student's un-paired t-test, *=$p<0.05$, indicates significance level relative to previous wash number.

2.4.4 Determination of Basal Efflux

2.4.4.1 Effect of time on Rb^+ efflux following exposure to KCl buffer

The Rb^+ efflux response from Rb^+ loaded HEK293 cells following 2-30 minutes exposure to non-depolarising KCl (5.4 mM) buffer was assessed (Figure 2.5). A Rb^+ efflux of 27.5±1.6% (n =32) was observed after 2 minutes, this value did not differ with increased exposure time (Bonferroni's multiple comparison test $p>0.05$), (Figure 2.5). In the presence of the K^+ channel blockers, 4-AP (1mM) and TEA (10mM), the Rb^+ efflux following 2-30 minute exposure to KCl (5.4 mM) buffer did not differ from values obtained in the absence of treatment (Figure 2.5).

2.4.4.2 Effect of selective K^+ channel blockers and ouabain on basal Rb^+ efflux

The Rb^+ efflux during 10-minute exposure to KCl (5.4 mM) buffer was also not modified by the presence of the selective blockers iberiotoxin (0.1μM), (BK_{Ca} channels, test values = 29.7±2.6% and control = 30.1±3.7% (n=8)) and paxilline (10μM), (BK_{Ca} channels, test =30.8±2.2%, control =33.3±3.1%) or glibenclamide (K_{ATP} channels, test = 27.7±0.7%, control = 28.3±0.9% (n=16), (Figure 2.6). In addition, ouabain (100μM), a Na^+/K^+ ATPase inhibitor, failed to modify the Rb^+ efflux (10-minutes) from cells in KCl (5.4 mM) buffer (ouabain: 24.1±2.9%, control: 27.0±4.6%, n=8), (Figure 2.6).

These finding are consistent with the Rb^+ efflux from un-stimulated cells (basal efflux) not involving K^+ channel and is not dependent on the activity of Na^+/K^+ ATPase.

2.4.4.3 DMSO effects on Rb^+ efflux

Dimethylsulphoxide (DMSO) was used as the solvent for the preparation of stock concentrations for the majority of pharmacological tools used including novel BK_{Ca} channel agents. The effect of DMSO on basal efflux in response to KCl buffer was examined. DMSO (0.3-20% v/v in KCl buffer) had no significant effect on Rb^+ efflux values compared to KCl buffer alone (Figure 2.7).

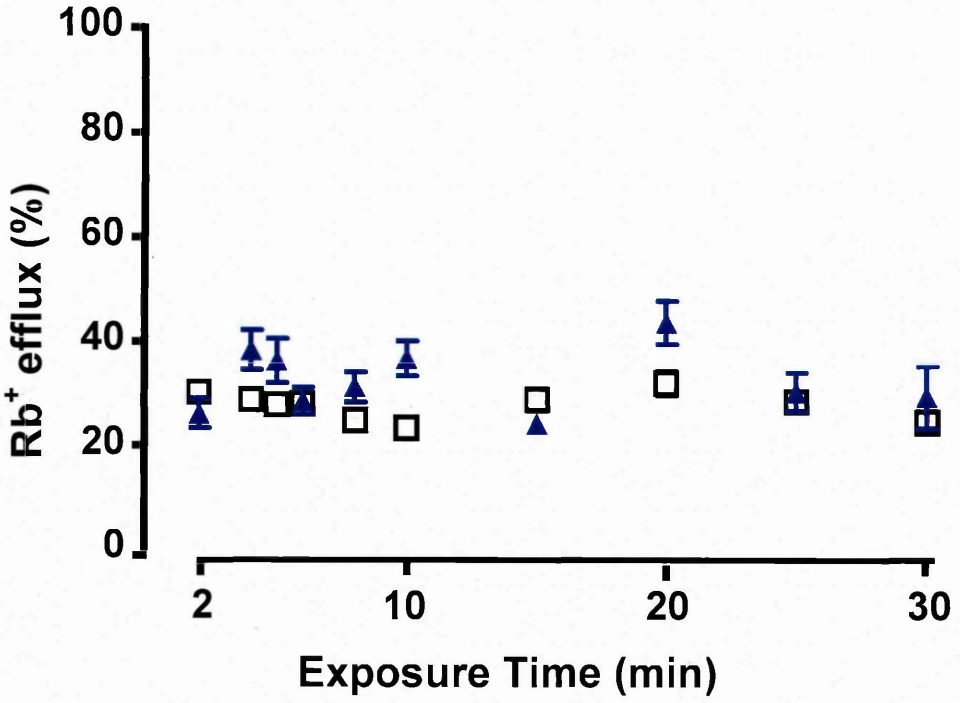


Figure 2.5: Effect of time on Rb^+ efflux following exposure to KCl buffer

Cells were exposed for varying times to KCl buffer in the absence (□) or in the presence of 4-AP (1.0mM) and TEA (10mM), (▲). Data presented as means±SEM (n=32/group).

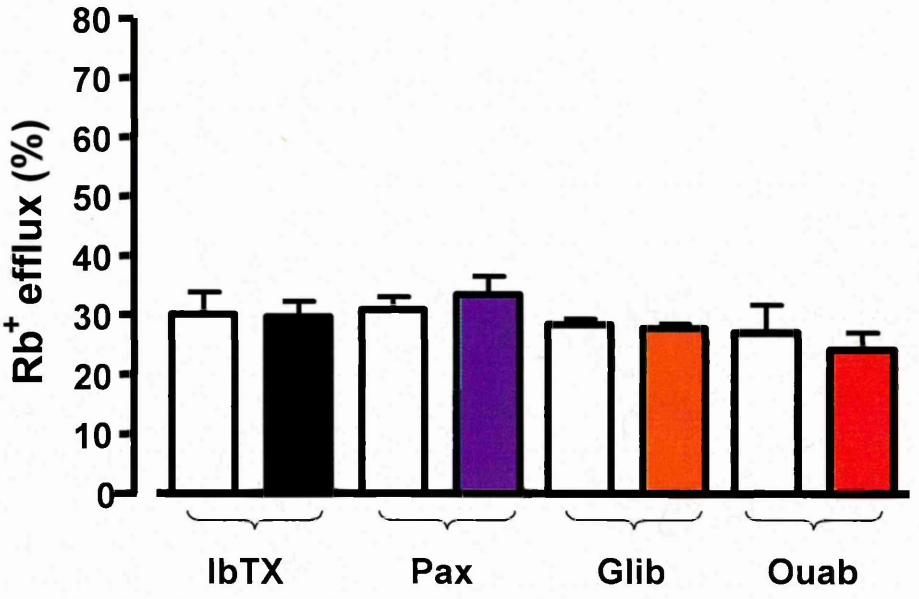


Figure 2.6: The effect of selective K^+ channel blockers and ouabain on basal Rb^+ efflux

Rb^+ efflux during 10 min exposure to KCl buffer in the absence (clear bars), and presence (filled bars) of the following treatments, glibenclamide (Glib, 10μM), iberiotoxin (IbTX, 0.1μM), paxilline (pax, 10μM) and ouabain (Ouab, 100μM). Each treatment group was paired to corresponding KCl buffer (clear bars) control. Data presented as means±SEM (n=8-16).

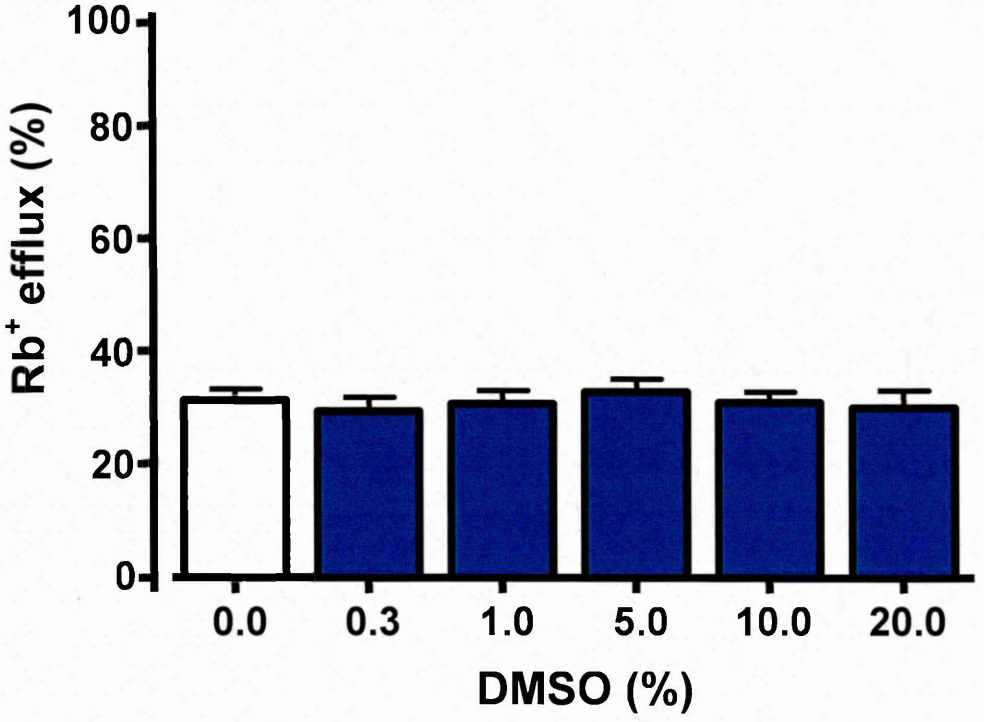


Figure 2.7: DMSO effects on Rb^+ efflux

Rb^+ efflux from cells exposed to KCl buffer (10-min) alone (Clear bar) or containing DMSO (0-20%) (Blue bars). Data presented as means±SEM (n=16-20).

2.4.5 Stimulation of cells by depolarisation and calcium ionophore

Exposure of HEK293 cells to a depolarising KCl (50 mM) buffer for 2-minutes produced an efflux (43.8±5.1%, n=16) that did not significantly (Bonferroni's, $p>0.05$) change with further incubation time. In addition at all time points high K^+ KCl buffer produced significantly greater efflux than that observed with exposure to low (5.4mM) KCl buffer (Figure 2.8).

Exposure of cells to calcium ionophore A23187 (1-100μM) produced significant (Dunnett's test $p<0.05$) increases in efflux relative to vehicle control (Figure 2.9).

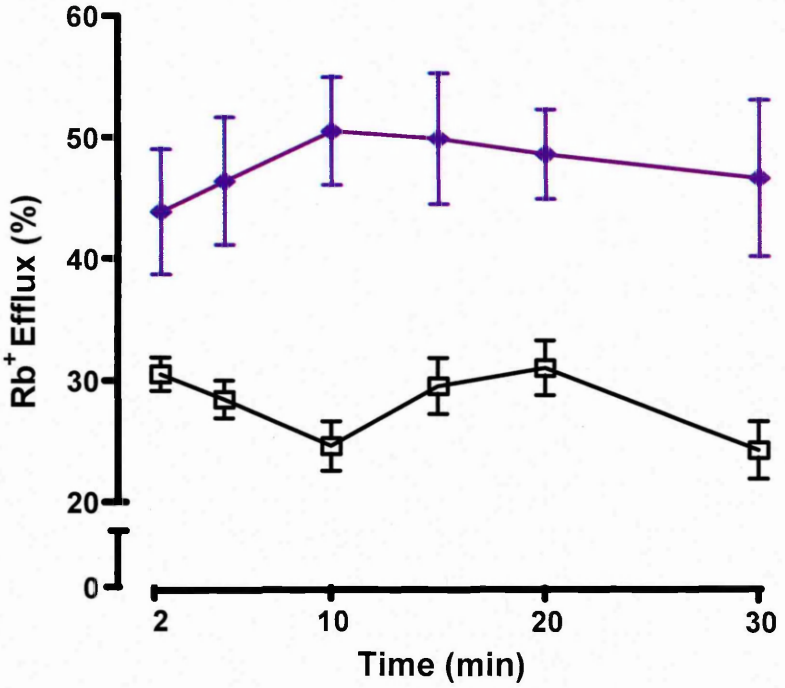


Figure 2.8: The effect of high extracellular K^+ (depolarisation) on Rb^+ efflux

Cells were exposed, for varying time points (2-30min), to high K^+ (50mM) KCl buffer (◆, n=16) or 5.4mM KCl buffer (□, n=32). Data presented as means ± SEM.

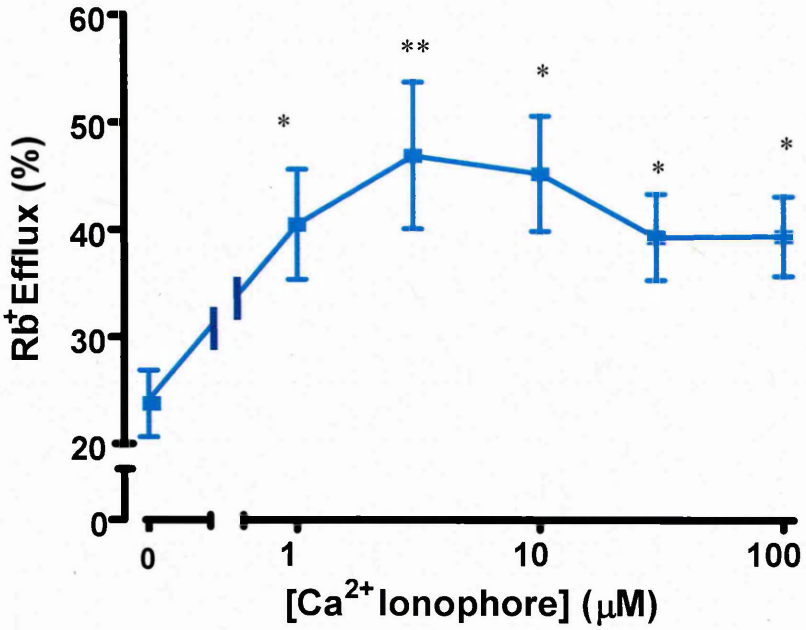


Figure 2.9: The effect of calcium ionophore A23187 on Rb^+ efflux

Cells were exposed to calcium ionophore (1-100μM) for 10 minutes in KCl buffer. One-way ANOVA with Dunnett's test *=p<0.05, **=p<0.01, indicates significance compared to control (KCl buffer including vehicle). Data presented as means±SEM, (■, n=16).

2.4.6 Time dependent effects of NS1619 on stimulating Rb^+ efflux

The Rb^+ efflux response to the BK_{Ca} channel opener NS1619 (30μM) was assessed following differing timed exposure (2-30mins). NS1619 produced a Rb^+ efflux response that was significantly different from control (un-stimulated efflux with KCl buffer for 10-minutes) that was not time dependent and the full response to NS1619 was observed within 2 minutes (57.1±3.9% n=16), (Figure 2.10).

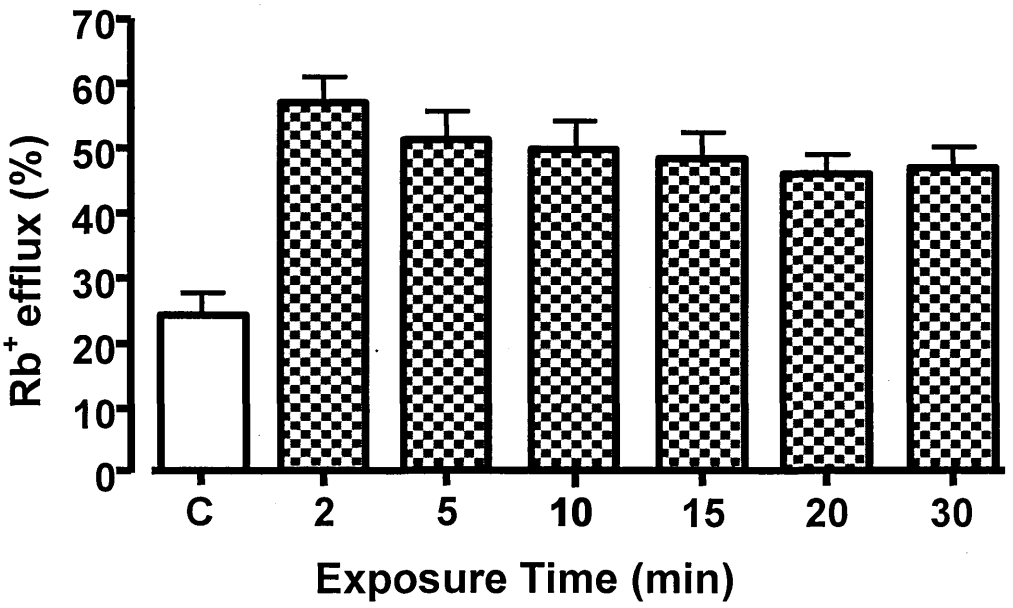


Figure 2.10: Time-dependent effect of NS1619 on stimulating Rb^+ efflux
Cells were exposed to NS1619 (30μM) in KCl buffer for varying timed intervals (2-30 minutes). C= un-stimulated efflux (KCl buffer with DMSO (0.3%) for 10-minutes exposure only). Data presented as means±SEM (n=16).

2.4.7 Characterisation of BK_{Ca} channel openers

The effects of BK_{Ca} channel openers NS1619, niflumic acid and phloretin (0.001-100μM) on Rb^+ efflux were assessed (Figure 2.11).

NS1619 and niflumic acid evoked significant concentration related increases in Rb^+ efflux above baseline producing significant differing E_{max} and $EC_{40\%}$ values. phloretin produced only significant increases in Rb^+ efflux above control at higher concentrations (100μM), (Figure 2.11; Table 2.2).

Niflumic acid (105.7±11.4% n=24) produced a significantly greater (Bonferroni's $p<0.05$) E_{max} compared to NS1619 (79.0±9.8% n=32) and phloretin (40.3±7.4% n=24). In addition the maximum response to NS1619 was significantly greater ($p<0.001$) than that achieved by phloretin (Figure 2.11, Table 2.2).

The potency of the BK_{Ca} channel openers was measured as the $EC_{40\%}$ (concentration of compound required to stimulate an increase in efflux above background by 40%). Niflumic acid (0.13±0.10μM, n=24) and NS1619 (0.32±0.19μM, n=32) had achieved similar levels of potency where as phloretin was unable to stimulate Rb^+ above 40% over the concentration range tested (Table 2.2).

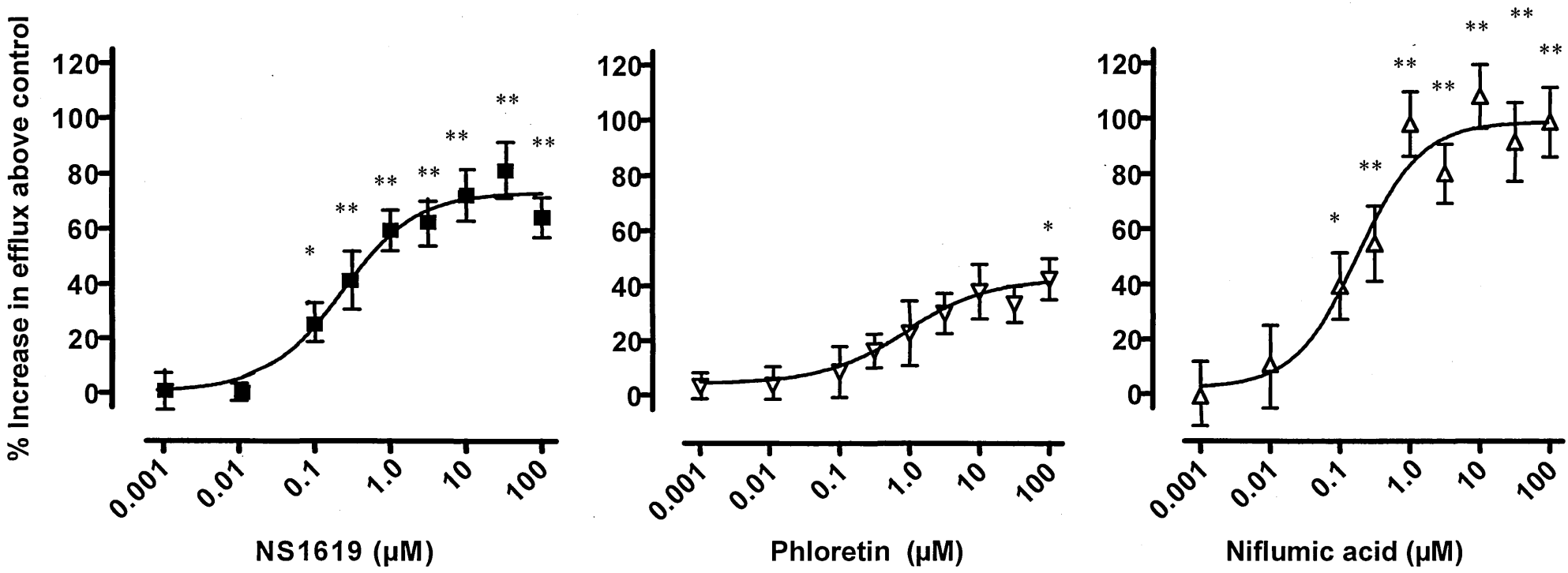


Figure 2.11: The Rb^+ efflux activation profile of BK_{Ca} channel openers

Cells were exposed (0.001-100µM) to either NS1619 (■), phloretin (▽) and niflumic acid (△) for 10-minutes. The data was normalised as the percentage increase in Rb^+ efflux above control for each concentration (n=24-32). One-way ANOVA with Dunnett's (*$p<0.05$, **$p<0.01$) indicate significance to relevant vehicle control.

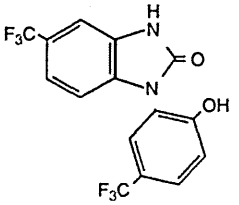
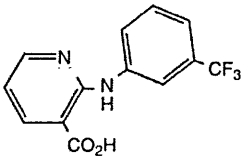
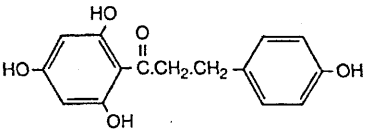
BK_{Ca} Opener		E_{max} (%)	$EC_{40\%}$ (μM)
NS1619		79.0±9.8	0.32±0.19
Niflumic Acid		105.7±11.4*	0.13±0.10
Phloretin		40.3±7.4**	n.d

Table 2.2: Pharmacological Properties of BK_{Ca} channel openers

Potency ($EC_{40\%}$) defined as the concentration of compound required to increase Rb^+ efflux above baseline by 40% and efficacy (E_{max}), defined as the maximum increase in activated efflux was determined for each BK_{Ca} channel opener tested. Data presented as means±SEM. Student's un-paired t-test, *$p<0.05$, **$p<0.01$ indicate differences in efficacy achieved between NS1619 and the other compounds tested. n.d = not determined.

2.4.8 The effect of Iberiotoxin (IbTX) on NS1619 activated Rb^+ efflux

In the presence of IbTX (0.1μM), NS1619 (3, 10 and 30μM) failed to significantly (Dunnet's $p>0.05$) modify the Rb^+ efflux response above control levels (5.4 mM KCl buffer alone) in contrast to NS1619 alone (Figure 2.12). These findings are consistent with the activation of the BK_{Ca} channel expressed in the HEK 293 cells.

Preliminary experiments were performed to determine whether iberiotoxin (IbTX) was needed to be included in the washes in addition to its inclusion in RbCl buffer (Figure 2.13). NS1619 (10μM in KCl buffer) significantly (Bonferroni's $p<0.001$) increased Rb^+ efflux (47.6±2.2%, n=8) relative to vehicle control (23.8±3.3%, n=8). NS1619 failed to significantly (Bonferroni's $P>0.05$) increase Rb^+ efflux above background (vehicle controls: DMSO 0.3% and BSA 0.1% in PBS) upon co-incubation with IbTX (0.1μM in KCl buffer). The degree of inhibition in efflux by IbTX was unaffected by the inclusion of IbTX in the PBS washes (absence = 23.2±3.4%; presence = 28.7±2.1% n=8, Bonferroni's $p>0.05$), (Figure 2.13).

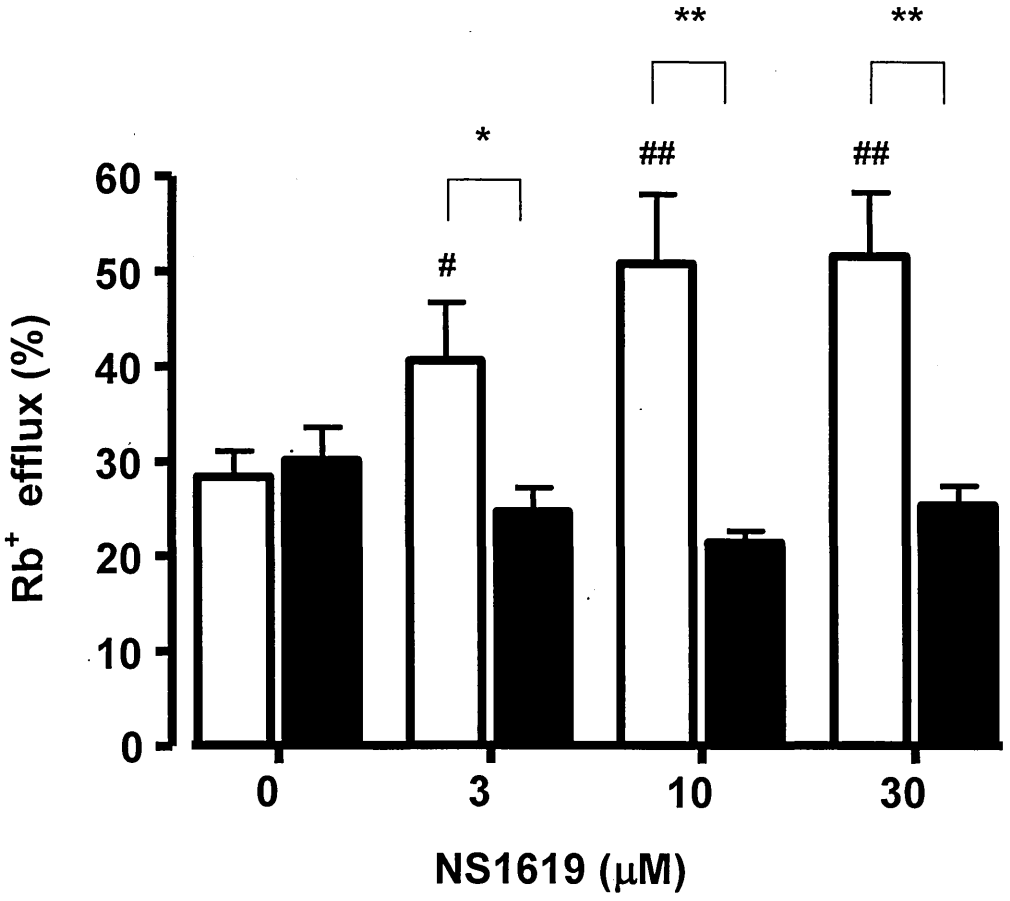


Figure 2.12: Effect of Iberiotoxin on NS1619 induced Rb^+ efflux

Cells were exposed to NS1619 (3, 10, 30μM) or to vehicle control in the absence (clear bars) or presence (black bars) of iberiotoxin (0.1μM). Data presented as means±SEM (n=16). Student's un-paired t-test *$p<0.05$, **$p<0.01$ indicate significant differences in Rb^+ efflux relative to the absence of IbTX. One-way ANOVA with Dunnett's post t-test, #=$p<0.05$, ##=$p<0.01$ indicates significant difference in efflux compared to control.

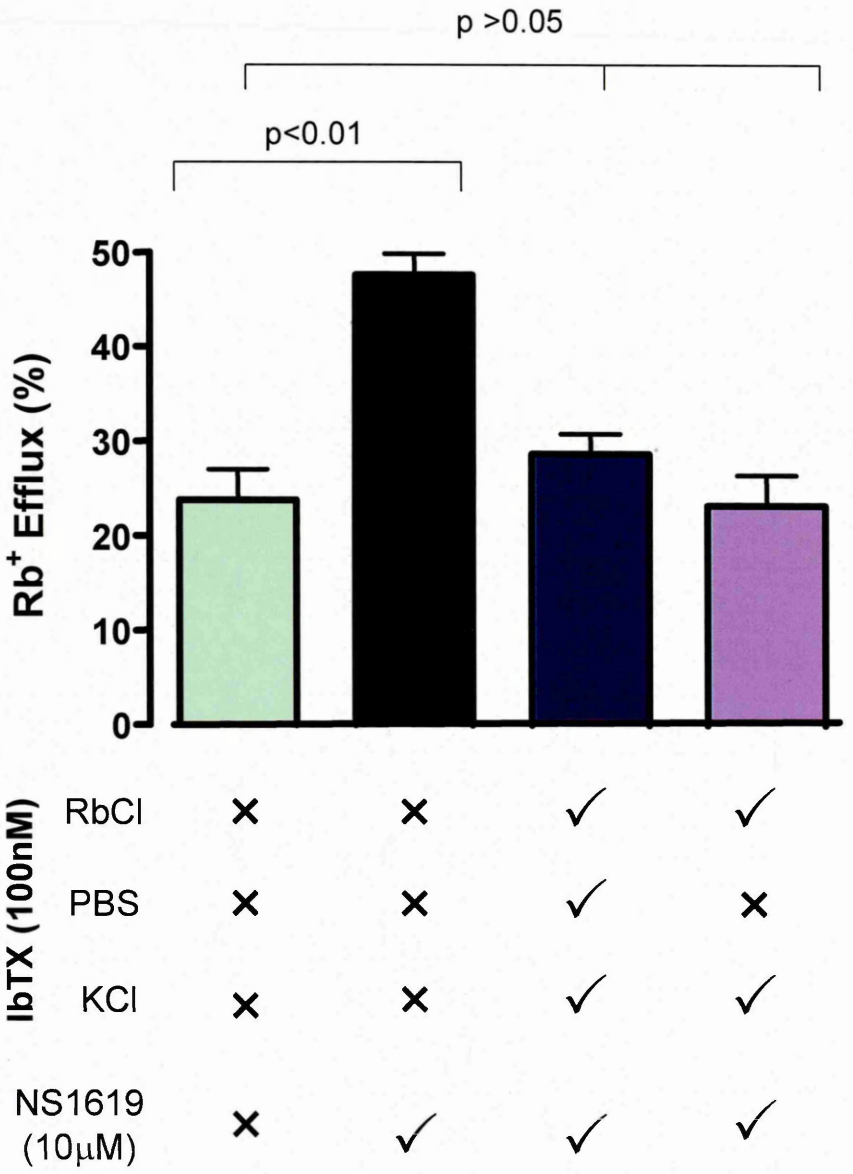


Figure 2.13: Optimising Iberiotoxin blocking experiment

Cells were exposed to KCl buffer with vehicle control (Green bar, DMSO 0.3%, 0.1% BSA in PBS) treated or with NS1619 alone (Black bar) or with co-incubation of IbTX (0.1μM) following either the inclusion absence (light purple) or presence (dark purple) IbTX during PBS washes. x = excluded in treatment, ✓ included in treatment. $p<0.01$ indicates significance relative to control (Bonferroni's) and $P>0.05$ indicated no difference between groups, (n=8).

2.4.9 Ouabain

To determine whether the response to NS1619 (30μM) was affected by Rb^+ re-uptake by the Na^+/K^+ ATPase, ouabain (100μM) a blocker of this pump was tested in the absence and presence of NS1619 (Figure 2.14). NS1619 (30μM) alone significantly raised efflux relative to vehicle control (KCl buffer=17.4±6.0%; NS1619=43.5±1.0%, n=16) and was not modified by the presence of ouabain (KCl buffer=23.1±2.9%; NS1619=43.1±2.9%, (n=16). Therefore, the effect of NS1619 on Rb^+ efflux represented the maximum effect and was not limited by Rb^+ recycling back into the cells (Figure 2.14).

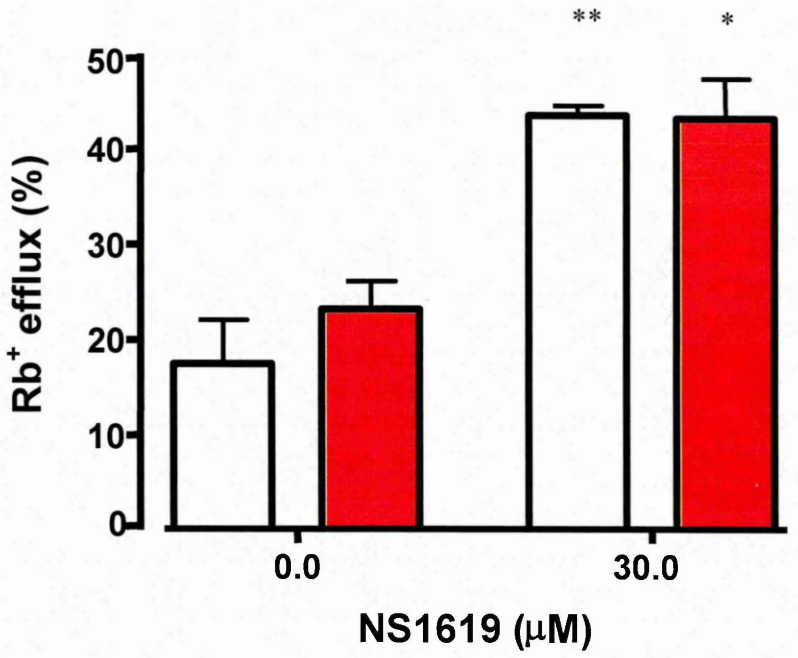


Figure 2.14: The effect of the Na^+/K^+ ATPase blocker ouabain on Rb^+ efflux

The effect of ouabain (100μM), (red bars) on the Rb^+ efflux response to KCl buffer alone or with the addition of NS1619 (30μM). Student's un-paired t-test (*$p<0.05$, **$p<0.01$) indicate significance to relevant control (NS1619, 0μM ± ouabain), (n=16).

2.5 Discussion

An optimised Rb^+ efflux assay can provide the basis for the assessment of novel compounds as K^+ channel modulators. A successful assay protocol depends upon the expression system used and the evaluation of each of the steps critical for assay performance (Bennett and Gutherie 2003; Terstappen 2004). These steps were important to optimise to gain understanding of the properties and the reliability of the assay. In addition to providing a framework to compare the results obtained by pharmacological stimulation of BK_{Ca} channels with novel openers.

2.5.1 HEK293 cells as suitable cell model for Rb^+ efflux assays

The most common cell types used for HTS screening are Chinese Hamster Ovary (CHO), COS and HEK293 cells. The choice of cell type is important because of the presence of endogenous ion channel expression. HEK293 cells have been shown, with electrophysiology to express various types of currents including five types of chloride, two types of potassium and an unknown cation current. Furthermore mRNA and protein has been detected for a number of different members of the K_v and VDCC families (Zhu *et al* 1998; Thomas and Smart 2005). Zhu *et al* (1998), suggest that HEK293 cells would make good vectors for the expression of K^+ and Na^+ channels but not Cl^- channels. HEK293 cells are preferred to Ooccytes as only transient expression can be achieved in Oocytes and HEK293 offer a human cell phenotype.

The HEK293 cells used in this study have been previously been modified and characterised as stably expressing BK_{Ca} channel subunits for use in electrophysiology (Hartness *et al* 2003). HEK293 cells contained a homogenous population of BK_{Ca} channels that was further confirmed by Rb^+ efflux studies.

Problems in the development of ion flux assays can be associated with the creation and choice of cell line to use. Ion channels are encoded by large cDNA's and are composed of multiple accessory subunits. Therefore, problems associated with cDNA stability and localisation can occur, in addition proteins can be toxic when over expressed. The use of inducible systems and the

inclusion of ion channel blockers to cell culture media can prevent toxicity (Worley and Main 2002).

Another factor to consider is the adherent strength of cells to the plastic of 96-well plates as the cells must be able to withstand multiple solution changes and washes. For example Wang *et al* (2004) used poly-l-lysine coated multi-well plates for the growth of CHO cells. Furthermore, HEK293 cells have been shown to be weaker adherent than other cell lines (Worley and Main 2002). However, during experimentation careful washing was used to ensure cells were not unnecessarily washed away.

Cell behaviour can be affected by seeding at high or low density formats, cell-cell interactions, cell cycle stage and length of exposure to trypsin can affect channel activity in cells. In addition, cells exhibit differential toxicity towards solvents (Worley and Main 2002). Therefore, the cell culture phase was kept as precise as possible in terms of seeding density and length of growth on plates. Cells were also checked for changes in morphology or sudden changes in cell growth rates. The cell passage number did not have an effect on the Rb^+ efflux response from HEK293 cells as demonstrated in the reliability of efflux values recorded under basal conditions (KCl buffer alone) and for the response to NS1619 over a number of different experiments and cell passages.

2.5.2 Rb^+ as a tracer ion

Rb^+ is found intracellular in various tissues with values ranging from 1300 - 1700 ng g^{-1} of tissue. The physiological role of Rb^+ is not well characterised, however, Rb^+ has shown to regulate synaptic neurotransmitter levels, K^+ homeostasis and leukocyte function (Canavese *et al* 2001). However, the use of cloned cell lines negates the presence of intracellular Rb^+. However studies have detected Rb^+ (7-10nM) in deionised water (Canavese *et al* 2001). Although Rb^+ could be present in water used in preparation of buffers the level is well below that of the sensitivity of the instrument, furthermore Rb^+ could not be detected in the PBS wash or KCl buffer.

The sensitivity in the detection of Rb^+ effluxes depends upon a number of factors including the requirement of a cell with a high level of channel expression (Worley and Main 2002; Parihar *et al* 2003) and secondly an optimised and sensitive detection system. Therefore, it was initially important to elucidate the instruments parameters for optimal analysis of Rb^+, including, determining nebulisation, measurement, and inter-well wash times In addition, to implement quality control measures to obtain reliability in function and maintain sensitivity of the instrument. The instrument was sensitive to 1μM and reproducibility was achieved in the determination of standard curves measured over a number of experiments. Rb^+ determination was performed using a Thermo Russell AAS which allowed medium-throughput analysis of samples.

2.5.3 Optimising the parameters

2.5.3.1 Rubidium Loading

The maximum amount of Rb^+ loaded is dependent upon the basal activity of the cell (Terstappen 1999; Terstappen 2004), studies have shown that Rb^+ uptake by cells is associated with Na^+/K^+ ATPase activity and that the application of the inhibitor ouabain depress Rb^+ uptake (Gill *et al* 2004).

Four hours was required to saturate cells with Rb^+, a loading phase time of 2 to 4 hours is a common time for recombinant cell lines. Wang *et al* (2004) for KCNQ/M channels expressed in CHO cells loaded cells for 3-hours, Scott *et al* (2003) determined 3 hours for KCNQ2/3 in HEK293 cells and Terstappen *et al* (1999) a 4-hour incubation time for pheochromocytoma PC-12 cells. In addition, analysis of the collected fourth wash revealed that the Rb^+ measured in the cells reflected that which had been loaded and not associated with the extracellular compartment this is in accordance with that observed previously.

2.5.3.2 Extracellular Washing

It is essential to determine the optimum washing conditions necessary to remove excess Rb^+ from the extracellular compartment of cells, following Rb^+ loading stage, as inefficient washing leads to artificially high efflux values and could account to false positive results.

A Rb^+ loading time was chosen (4-hours) and washes collected following four sequential washes. Four washes were required to gain confidence in the clearance of excess extracellular Rb^+. In addition, less than 3 washes produced artificially high efflux values. Therefore, four washes was chosen to ensure confidence in removal of cell surface Rb^+ and to save an 'economy in time' of performing excess washing. Variations to PBS used as a wash buffer include washing with KCl buffer without glucose (Wang *et al* 2004).

The frequency of washing is dependant upon a number of conditions including cell type, density and the system of washing used (Terstappen 2004) although in HTS flux assays three washes are typically used but whether three washes was the optimal number to use had not properly been defined in the literature (Scott *et al* 2003; Wang *et al* 2004; Gill *et al* 2004; Parihar *et al* 2003).

2.5.3.3 Determining basal Rb^+ efflux

Basal efflux values were examined to gauge the efflux generated in response to differing timed exposure to KCl buffer. This was because K^+ channel modulators were added to this and to establish whether basal efflux was affected by the duration of incubation with KCl buffer.

The average basal efflux observed in HEK293 cells was approximately 25-30%. Terstappen (1999) observed a similar level of efflux in response to KCl buffer (20-30%) in HEK293 cells expressing K_v channels and a lower 10-20% in CHO cells and PC-12 cells expressing BK_{Ca} and SK_{Ca} channels respectively, furthermore, this value was not different to wild-type HEK293 cells.

The basal level of Rb^+ efflux from the cells resulting from exposure to the KCl buffer is dependent upon a number of factors including the concentration of extracellular K^+ in the buffer and the native cell phenotype including the level of expression of endogenous pores, ATPases and ion channels.

This basal efflux response to KCl buffer was time independent and not modified by the presence of K^+ channel blockers (4-AP/TEA, Glib, IbTX and pax). The IC_{50} of ouabain for inhibiting Rb^+ uptake has been determined as 87μM for Na^+/K^+ ATPase's expressed in CHO cells and that its inhibition was unaffected

by K^+ concentration (Gill *et al* 2003). Application of ouabain could not inhibit the Rb^+ efflux response to KCl buffer, indicating that this efflux level observed was not maintained by Rb^+ re-uptake into cells via the Na^+/K^+ ATPase.

Therefore, K^+ channel blockade did not lower the average efflux value below that achieved in KCl buffer; this indicates that potassium channels are not being activated. Consequently this observed efflux value represents a basal level that may have resulted from leakage through other channels and pores including non-specific cation channels (Terstappen 2004).

In addition, DMSO (0-20% in KCl buffer) demonstrated no significant effect on basal efflux following 10-minutes exposure. No other studies have reported the effects of low concentrations of DMSO on Rb^+ efflux results. DMSO was used in the preparation of the majority of pharmaceutical tools used. For future experiments DMSO concentration did not exceed 0.3%.

Having established loading times, wash numbers and the basal level of efflux the next phase was to determine the maximum amount of efflux generated by stimulating the channel.

2.5.3.4 Stimulating Rb^+ efflux

Activating BK_{Ca} channel by mimicking endogenous conditions including partial depolarisation (by increasing extracellular K^+) or by increasing intracellular calcium through the application of calcium ionophore. Rb^+ efflux through BK_{Ca} channels was sensitive to changes in intracellular calcium and membrane voltage with both conditions stimulating a similar maximal increase in Rb^+ efflux. Previous studies by Terstappen (1999) examined the effect of calcium ionophore (25μM) on efflux from CHO cells expressing BK_{Ca} channel α and β1 subunits. An efflux of 70% was observed in CHO cells compared to 40% (at 30μM) observed here in HEK293 cells. An increased efflux value observed by Terstappen could be due to the presence of the β1 subunit and its effect upon calcium sensitivity of the BK_{Ca} channel. Other studies have used calcium ionophore to yield a maximum Rb^+ efflux response to use as a positive control to normalise data against (Parihar *et al* 2003). However, the concentration

response relationship to ionophore has not been fully characterised in these assay systems. High potassium buffers are used in certain efflux assays in the assessment of compounds as channel blockers. For example in hERG channel screening, cells are depolarised with high K^+ buffers (20-40mM) are used to activate the channel to test the effect of channel blockers (Rezazadeh *et al* 2004; Chaudry *et al* 2006; Wang *et al* 2007).

2.5.4 Pharmacological characterisation with BK_{Ca} channel openers

Pharmacological stimulation of BK_{Ca} channels with NS1619, niflumic acid and phloretin was used to characterise the maximum response that could be obtained from this system with known BK_{Ca} channel openers. In addition to determine whether the assay could discriminate accurately between differences in potency and efficacy values.

NS1619 (30μM) increased Rb^+ efflux above control and that this response was not modified by time over a 30-minute period. Therefore, the length of time to expose cells to KCl buffer containing ion channel modulators was chosen as 10-minutes. In addition, 10-minutes gave sufficient time to perform the liquid handling requirements of the assay and to physically do the experiment. Most HTS flux assays incorporate a 10-minute exposure time (Scott *et al* 2003).

Although not modified by time the effect of NS1619 on Rb^+ efflux was concentration dependent. Further, it was important to establish that the efflux observed in the presence of NS1619 was due to the activation of BK_{Ca} channels and not through its ancillary effects (activation of other channels and affecting membrane permeability). Blockers were incorporated into the final 20 minutes of Rb^+ loading stage, a similar pattern has been observed by other research groups (Parihar *et al* 2003; Scott *et al* 2003). IbTX prevented NS1619 meditated increases in efflux. Therefore, the maximum efflux determined is representative of efflux through BK_{Ca} channels.

Niflumic acid demonstrated concentration related increases in Rb^+ efflux producing a similar level of potency to NS1619. However phloretin was less potent only evoking significant increases in Rb^+ efflux at the highest

concentration tested. NS1619 bears closer structural similarity to niflumic acid than phloretin and this may explain the comparable potency observed between NS1619 and niflumic acid.

Different 'windows' of activation in Rb^+ efflux have been observed, Terstappen (2004) defined a 2-fold or greater increase in Rb^+ efflux upon activation above basal level for a good quality HTS assay. A 2-4 fold window was observed with NS1619, niflumic acid and phloretin. Parihar *et al* (2003) showed a 3-4 fold window for BK_{Ca} channels expressed in HEK293 cells.

The addition of ouabain to KCl buffer containing NS1619 failed to increase the maximum efflux obtained with NS1619 alone showing that the maximum efflux achieved was not affected by Rb^+ being recycled into the cell by the Na^+/K^+ ATPase, and represented the maximum level of Rb^+ efflux achieved with NS1619.

2.5.5 Considerations

AAS offers a sensitive technique that was able to measure Rb^+ concentrations in low micro-molar ranges. Furthermore, the recombinant HEK293 cells used demonstrated a high expression level of channels and a high cell density. These two factors are important to obtain a high signal to noise ratio. Therefore, common problems with optimisation of efflux assays are associated with lack of weak positive signals from cells, however, the system used afforded a 2-4 fold window upon stimulation.

Furthermore, some ion channels are only activated transiently (sub-millisecond) and so the limited time resolution of the assay means Rb^+ efflux can not be measured for certain ion channels (Gill *et al* 2003), this was not a factor for BK_{Ca} channels with some channels requiring manipulating to stay open (Gill *et al* 2003; Wang *et al* 2004). Furthermore, the assay, in comparison to electrophysiology, does not provide any kinetic measurements and does not allow for the real-time assessment of Rb^+ effluxes.

Another important variable in flux assays is the control of membrane potential, in that flux experiments are not performed under voltage clamp. Therefore, the membrane potential will be affected by buffer composition, cell size and the number of channels expressed, consequently changes in membrane potential can therefore alter ion channel conductance (Willumsen *et al* 2003). In addition, compounds with a voltage dependent mechanism of action could be lost in Rb^+ efflux screening.

It may have been favourable to compare more BK_{Ca} channel openers using the assay. This could be used to generate a compendium of knowledge comparing the potencies of a wide range of known BK_{Ca} channel openers that could provide clues into the pharmacophoric nature of BK_{Ca} channel openers. However, the lack of commercial availability limited an in-depth analysis.

2.6 Conclusions

Presented here is a fully optimised Rb^+ efflux assay for the assessment of potential compounds as BK_{Ca} channel openers. The assay comprised many components including the cell biology (growth of cells expressing BK_{Ca} α-subunit), the assay (optimal assay development) and validating the analysis of sample (atomic absorption spectrometry).

Xu *et al* (2001) define the requirements of an ideal HTS assay that include, sensitivity, specificity, through-put, robustness, information content, flexibility, cost and physiological relevance. Therefore to meet these requirements it was vital to optimise each step of the assay to gain an understanding of the assay limits and performance. The optimised assay presented here has good reliability and reproducibility over a number of different experimental conditions. In addition, the assay was able to discriminate between different levels of efficacy and potency for BK_{Ca} channel openers providing a significant amount of pharmacological data. Furthermore, it is important to characterise these reference opener responses, because in the testing of novel compounds as BK_{Ca} openers it is essential to have a point of reference to compare against

potency and efficacy and in addition to providing a positive control for assay performance.

Therefore, an optimised non-radioactive Rb^+ efflux assay can provide a relatively quick and sensitive tool that can differentiate novel compounds based on potency and efficacy in their ability to activate BK_{Ca} channels.

Chapter 3

Identification of novel benzanilides as BK_{Ca} channel openers using a non-radioactive Rb^+ efflux assay

3.1 Introduction

Novel $BK_{Ca}CO$ structures have been proposed based on the general pharmacophore template of two substituted phenyl rings linked by a spacer unit. Initial studies have shown that compounds composed of two multi-substituted rings separated by an amido function introducing a three bond linker (Figure 3.1) could offer a new template for $BK_{Ca}CO$'s (Biagi *et al* 2004). Using *in-vitro* tissue bath studies these benzanilides have showed, IbTX sensitive, vasorelaxant activity to pre-contracted (with 20mM K^+ Krebs buffer) rat aortic rings with comparable potency to NS1619 (Biagi *et al* 2004).

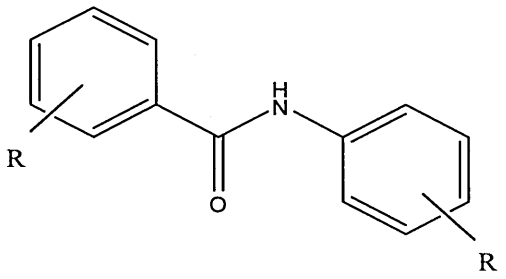


Figure 3.1: The template structure of benzanilides

Preliminary SAR work has revealed that the presence of a 2-OH function on N-phenyl ring is an important feature (Calderone *et al* 2006a,b). In addition replacement of the benzene ring with heterocycles such as thiopene, pyrrole, furan and indole is deleterious except if the ring is a pyridine then $BK_{Ca}CO$ properties are retained (Calderone *et al* 2006a,b). Insertion of a methylene bridge between the amido function and the benzene is not tolerated as all phenyl acetamides tested were deleterious. Further that inclusion of a second hydroxyl group to the other ring introducing a third site of hydrogen bonding resulted in a lowering of potency (Calderone *et al* 2006a,b). However, previous work has identified that compounds pertaining to the benzanilide structure have shown potency and efficacy similar to NS1619 in organ bath experiments (Calderone *et al* 2006a,b).

From this work seven novel benzanilides were selected to study the pharmacophore of this template structure. The benzanilides investigated have substitutions within the ring systems that have an N-phenyl ring that is either a pyridine with a N atom at the *ortho*, *meta* or *para* position or with an phenyl ring with alkyl chains. The other phenyl ring is either a hydroxyphenyl or methoxyphenyl ring with a Cl atom in the *para* position (Figure 3.2).

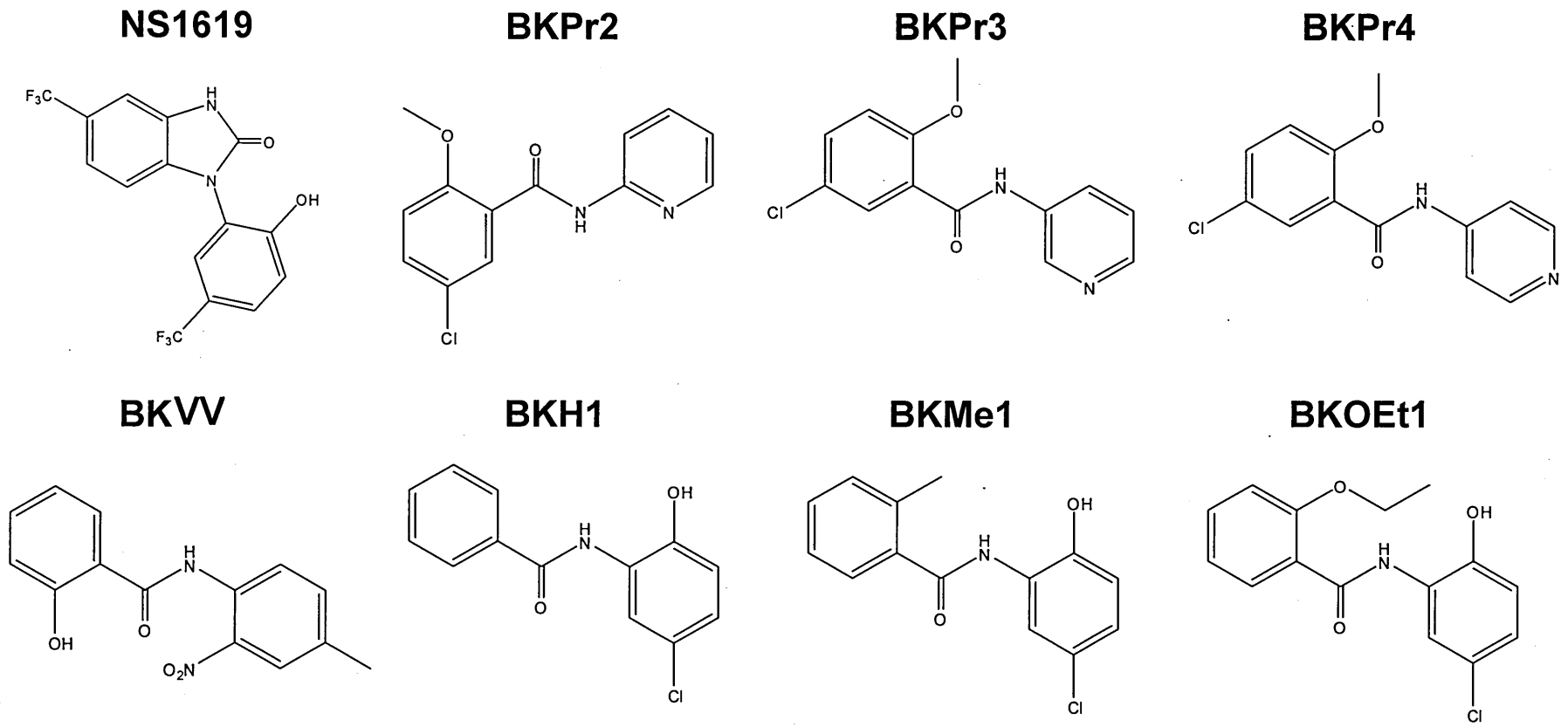


Figure 3.2: The structure of NS1619 and benzanilides under investigation

3.2 Chapter Aims

To investigate a series of novel benzanilides for BK_{Ca} channel opener properties using an optimised non-radioactive Rb^+ efflux assay.

- Characterise pharmacological properties of benzanilides.
- Examine the effect of the BK_{Ca} channel β1 subunit on BK_{Ca} channel opener properties of benzanilides.
- Determine the specificity of benzanilides to BK_{Ca} channels in the cell lines used.

3.3 Methods

3.3.1 Materials

3.3.1.1 Compound preparations

Novel compounds to be assessed were provided by Dr Vincenzo Calderone from the chemical library of the Departmento di Scienze Farmaceutiche, Universita di Pisa, Italy.

The synthetic route for the preparation of the compounds is described by Calderone *et al* (2006a) and Biagi *et al* (2004). Compounds were supplied as powder (~20mg) and prepared as required by dissolving in DMSO (100%) to a stock concentration of 10 or 100mM and aliquots (10-100μl) stored at -20°C.

3.3.2 Cell Culture

HEK293 cells previously characterised (Hartness *et al* 2003) and stably expressing either the human BK_{Ca} channel α-subunit alone (referred to as BK_{Ca} α) or with co-expression of the human BK_{Ca} channel β1 subunit (referred to as BK_{Ca} α + β1) were used. Cells were prepared for experimentation as described previously in Chapter 2, section 3.3.

3.3.3 Rb^+ efflux assay

The methodology is as that described in Chapter 2 section 3.4.
NS1619 was used as a positive control and appropriate vehicle controls were used. Each compound was tested in replicates of 6-8 wells per plate and was tested at least 3 times.

3.3.4 Data Analysis

Rb^+ efflux (%) was calculated and values normalised as described previously (Chapter 2.3.6) from which sigmoidal-concentration response curves were constructed using the four parameter logistic equation as follows:

$$\text{Activatedefflux} = \textit{Bottom} + (\textit{Top} - \textit{bottom}) / 1 + 10\text{^}[(\log EC_{50} - X) * \text{HillSlope}]$$

X is the logarithm of concentration. Y is the response. From which Hill slope, E_{max} and $EC_{40\%}$ were calculated.

Results are presented as mean (±SEM) for n determinations. Data was analysed using either Student's un-paired t-test or for multiple comparisons one-way ANOVA followed by Dunnett's or Bonferroni's multiple comparison test (Wallenstein *et al* 1980). The accepted level of significance was $p \leq 0.05$.

3.4 Results

3.4.1 BK_{Ca} channel opener properties of benzanilides and NS1619

The Rb^+ efflux response to varying concentrations of benzanilides or NS1619 was examined on cells expressing BK_{Ca} α alone or co-expressing BK_{Ca} α + β1 subunits (Figures 3.3, 3.4 and 3.5).

The concentration response curves (CRC) determined for the benzanilides demonstrated that certain compounds showed activation profiles above and below that achieved for NS1619, with the benzanilides producing varying degrees in efficacy and potency.

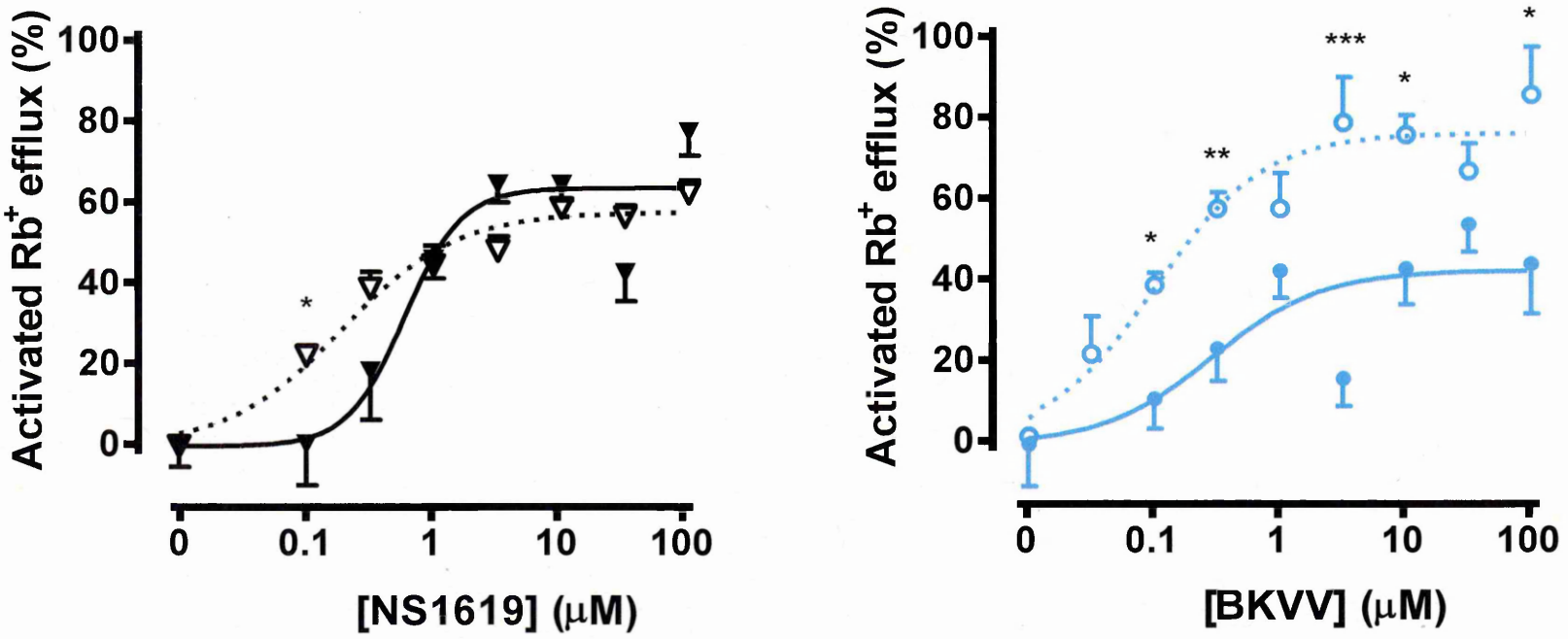


Figure 3.3: The concentration response effect of BKVV and NS1619 on stimulating Rb^+ efflux

BK_{Ca} α (dashed line, open symbols) or BK_{Ca} α + β1 cells (solid line, filled symbols) were exposed to either BKVV (●) or NS1619 (▼) for 10-minutes. The data was normalised as the percentage increase in Rb^+ efflux above control for each concentration. Data presented as means±SEM (n=24-32). Student's un-paired t-test, *p<0.05, **p<0.01 and ***p<0.001 indicate differences in efflux at each concentration tested between the two cell lines.

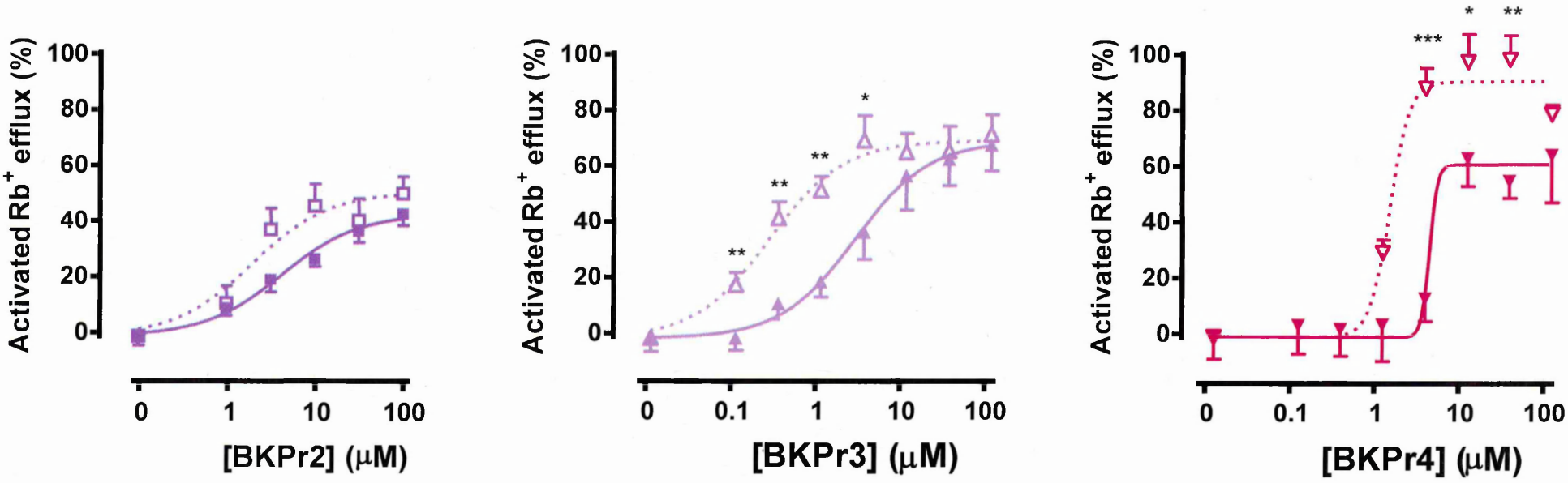


Figure 3.4: The concentration response effect of BKPr2, BKPr3 and BKPr4 on stimulating Rb^+ efflux

BK_{Ca} α (dashed line, open symbols) or BK_{Ca} α + β1 cells (solid line, filled symbols) were exposed to either BKPr2 (■), BKPr3 (▲) or BKPr4 (▼) for 10-minutes. The data was normalised as the percentage increase in Rb^+ efflux above control achieved at each concentration. Data presented as means±SEM (n=24-32). Student's un-paired t-test, *p<0.05, **p<0.01 and ***p<0.001 indicate differences in Rb^+ efflux at each concentration tested between the two cell lines.

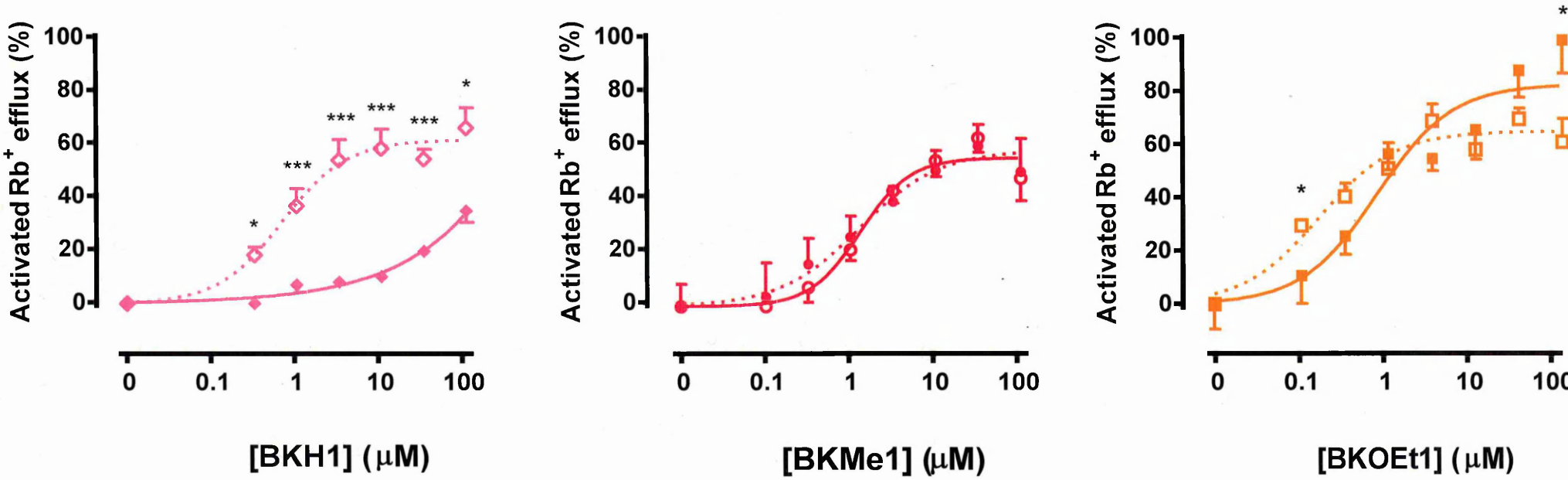


Figure 3.5: The concentration response effect of BKH1, BKMe1 and BKOEt1 on stimulating Rb^+ efflux

BK_{Ca} α (dashed line, open symbols) or BK_{Ca} α + β1 cells (solid line, filled symbols) were exposed to either BKH1 (◆), BKMe1 (●) or BKOEt1 (■) for 10-minutes. The data was normalised as the percentage increase in Rb^+ efflux above control for each concentration. Data presented as means±SEM (n=24-32). Student's un-paired t-test, *p<0.05, ***p<0.001 indicate differences in Rb^+ efflux at each concentration tested between the two cell lines.

3.4.1.1 Efficacy profile

Efficacy (E_{max}) defined as the maximum increase in activated Rb^+ efflux was determined for each compound and NS1619 tested. A range in efficacy (E_{max} = 49 - 99%, n=24-32) in BK_{Ca} α cells for the benzanilides was determined with BKH1 (62.5±7.2%), BKMe1 (59.7±5.2%), and BKOEt1 (69.9±3.9%) displaying E_{max} values comparable to that observed for NS1619 (61.7±3.1%), (Table 3.1). The benzanilides; BKPr3 (72.5±3.3%), BKPr4 (99.2±8.8%) and BKVV (86.4±11.1%) produced a significantly ($p<0.05$, One-way ANOVA with Bonferroni's) greater level of E_{max} than NS1619. Conversely BKPr2 (49.1±2.5%) had significantly less E_{max} than NS1619 (Table 3.1).

A range in efficacy (E_{max} = 32 - 99%, n=24-32) was determined for benzanilides tested on BK_{Ca} α + β1 cells. The majority of compounds including BKPr2 (42.0±3.8%), BKH1 (32.9±4.2%), BKMe1 (56.8±7.6%) and BKVV (54.4±6.9%) demonstrated a significant ($p<0.05$, One-way ANOVA with Bonferroni's) reduction in efficacy compared to NS1619 (76.6±5.8%). However, BKPr3 (68.8±9.3%) and BKPr4 (65.1±10.2%) demonstrated comparable efficacy to NS1619. The only compound to increase E_{max} above that achieved for NS1619 was BKOEt1 (99.5±12.6%), (Table 3.1).

3.4.1.2 Potency profile

The compounds demonstrated a 40-fold range in potency ($EC_{40\%}$ = 0.12-4.47μM) in BK_{Ca} α cells (Table 3.2). Compounds BKPr3 (0.31±0.3μM), BKPr4 (1.12±0.5μM), BKOEt1 (0.21±0.3μM) and BKVV (0.12±0.3μM) demonstrated potency comparable to that obtained with NS1619 (0.35±0.1μM). However, others including BKH1 (1.41±0.18μM), BKMe1 (2.95±0.24μM) and BKPr2 (4.47±0.18μM) were significantly ($p<0.05$, One-way ANOVA with Bonferroni's) less potent than NS1619 (Table 3.2).

Conversely, the benzanilides demonstrated an 80-fold range in potency ($EC_{40\%}$ = 0.56-46.8μM) in BK_{Ca} α + β1 cells with BKOEt1 (0.12±0.3μM) achieving potency levels comparable to NS1619 (0.79±0.4μM), (Table 3.2). However, BKPr2 (46.8±1.4μM), BKPr3 (3.47±0.2μM), BKPr4 (4.16±0.1μM) and BKMe1 (3.63±0.2μM) were significantly ($p<0.05$, One-way ANOVA with Bonferroni's) less potent than NS1619. BKH1 was unable to stimulate Rb^+ efflux above 40% in BK_{Ca} α + β1 cells (Table 3.2).

The order of potency ($EC_{40\%}$) of the compounds was:

BK_{Ca} α cells

NS1619 / BKOEt1 / BKVV / BKPr3 > BKPr4 / BKH1 > BKMe1 > BKPr2

BK_{Ca} α + β1 cells

NS1619 / BKOEt1 > BKVV > BKPr3 / BKMe1 / BKPr4 > BKPr2 > BKH1

E_{max} (%)	NS1619	BKPr2	BKPr3	BKPr4	BKH1	BKMe1	BKOEt1	BKVV
$BK_{Ca}\alpha$	61.7±3.1	49.1±2.5**	72.5±3.3*	99.2±8.8***	62.5±7.2	59.7±5.2	69.9±3.9	86.4±11.1*
$BK_{Ca}\alpha+\beta_1$	76.6±5.8	42.0±3.8**	68.8±9.3	65.1±10.2	32.9±4.2*	56.8±7.6*	99.5±12.6	54.4±6.9*

Table 3.1: Efficacy values determined for each compound

Efficacy (E_{max}), defined as the maximum increase in activated Rb^+ efflux, was determined for each novel compound or NS1619 tested on cells expressing either the BK_{Ca} α-subunit alone or cells co-expressing the β1 subunit. Data presented as means±SEM. One-way ANOVA with Bonferroni's, *p<0.05, **p<0.01 and ***p<0.001 indicate differences in E_{max} that was achieved for NS1619 and each novel compound (n=24-32).

$EC_{40\%}$ (μM)	NS1619	BKPr2	BKPr3	BKPr4	BKH1	BKMe1	BKOEt1	BKVV
$BK_{Ca}\alpha$	0.35±0.1	4.47±0.2***	0.31±0.3	1.12±0.5	1.41±0.2*	2.95±0.2***	0.21±0.3	0.56±0.2
$BK_{Ca}\alpha+\beta_1$	0.79±0.4	46.8±1.4***	3.47±0.2***	4.16±0.1***	n.d	3.63±0.2***	0.12±0.3	1.41±0.1*

Table 3.2: Potency values determined for each compounds

Potency ($EC_{40\%}$) defined as the concentration of compound required to increase Rb^+ efflux above control by 40% was determined for each novel compound or NS1619 tested on cells expressing either the BK_{Ca} α-subunit alone or cells co-expressing the β1 subunit. Data presented as means±SEM. One-way ANOVA with Bonferroni's, *p<0.05 and ***p<0.001 indicate differences in potency between that achieved for NS1619 and each novel compound (n=24-32). n.d could not be determined

3.4.1.3 Hill slopes

The Hill slopes were determined from the Rb^+ efflux CRC obtained for the benzanilides and NS1619 tested in both the BK_{Ca} α and BK_{Ca} α + β1 cells (Table 3.3). The benzanilides (except BKPr4) produced a range of slope values (benzanilides = 0.62 - 1.50, NS1619 = 0.97±0.05) and (benzanilides = 0.22 - 0.95, NS1619 = 1.99±0.22) around unity when tested on BK_{Ca} α cells and in BK_{Ca} α + β1 cells respectively. BKPr4 demonstrated a significant steeper Hill slope from unity in both cell lines (BK_{Ca} α = 3.79±0.86; BK_{Ca} α + β1 = 9.65±3.4), (Table 3.3).

For the benzanilides BKPr2, BKPr3 and BKPr4; the presence of the BK_{Ca} channel β1 subunit had no significant effect on the shape of the concentration response curve. In contrast, compounds BKH1, BKOEt1 and BKVV had different shapes in CRC in the presence of the β1 subunit.

Hill Slope	BK_{Ca} α	BK_{Ca} α + β1
NS1619	0.97±0.05	1.99±0.22**
BKPr2	1.19±0.03	0.74±0.03***
BKPr3	1.02±0.05	0.95±0.03
BKPr4	3.79±0.86**	9.65±3.4*
BKH1	1.50±0.08***	0.48±0.06*
BKMe1	1.47±0.06***	0.92±0.04
BKOEt1	1.08±0.14***	1.09±0.17
BKVV	0.62±0.08***	0.22±0.23***

Table 3.3: Hill slopes determination

Hill slopes determined from Rb^+ efflux CRC for each benzanilide and NS1619 tested on BK_{Ca} α and α + β1 cells. Data presented as means±SEM. Student's un-paired t-test *p<0.05, **p<0.01, ***p<0.001, indicates significant difference in Hill slope from unity (n=24-32).

3.4.2 The effect of the BK_{Ca} channel β1 subunit on potency and efficacy

The effect of BK_{Ca} channel composition on the potency of compounds was determined by comparing $EC_{40\%}$ values determined in the two cell lines (Figure 3.6). NS1619 and BKOEt1 were the only compounds to demonstrate similar potency. All other benzanilides showed a significant ($p<0.05$, Student's un-paired t-test) 2-10 fold reduction in potency in the BK_{Ca} α + β1 cell line compared to BK_{Ca} α cells.

Differences in efficacy were demonstrated by comparing E_{max} values determined in BK_{Ca} α and BK_{Ca} α + β1 cells (Figure 3.7). Compounds BKPr2, BKPr3, BKMe1 and NS1619 demonstrated apparent similar E_{max} whereas a 1.5-2.0 fold reduction in efficacy was demonstrated for BKPr4, BKH1 and BKVV in the BK_{Ca} α + β1 cells compared to BK_{Ca} α cells. However, BKOEt1 demonstrated an increase in E_{max} in BK_{Ca} α + β1 cells.

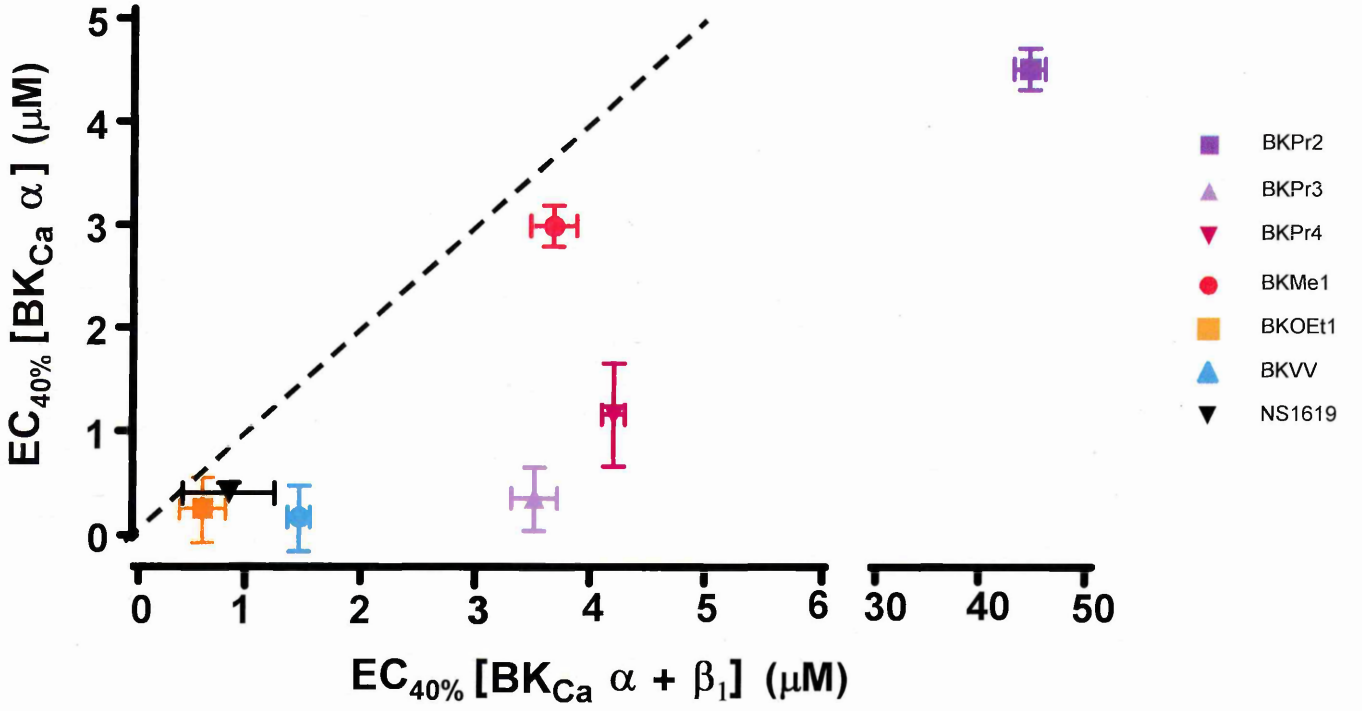


Figure 3.6: Effect of BK_{Ca} channel composition on the potency of compounds

A comparison of $EC_{40\%}$ values determined for compounds and NS1619 tested on BK_{Ca} α cells and BK_{Ca} α + β1 cells. Compounds demonstrating equal potency in each cell line occur close to the line of unity (−−). Data presented as means±SEM, (n=24-32). Nb, BKH1 did not evoke efflux above the 40% level in BK_{Ca} α + β1 cells and was not included in this analysis.

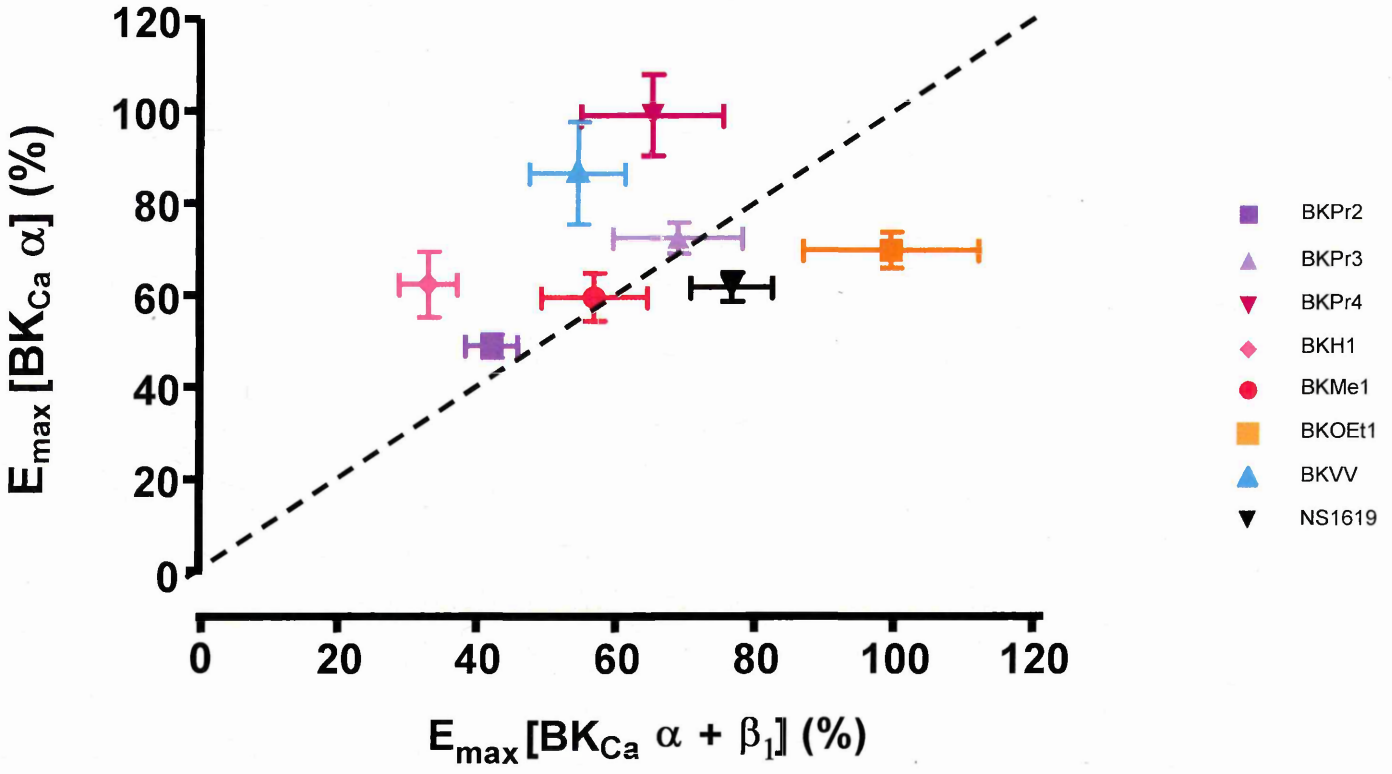


Figure 3.7: Effect of BK_{Ca} channel composition on the efficacy of compounds

A comparison of E_{max} values determined for compounds and NS1619 tested on BK_{Ca} α cells and BK_{Ca} α + β1 cells. Compounds demonstrating equal efficacy in each cell line occur close to the line of unity (−−). Data presented as means±SEM, (n=24-32).

3.4.3 Sensitivity of $BK_{Ca}CO$ induced Rb^+ efflux to selective BK_{Ca} channel blockers

To determine whether the increase in Rb^+ efflux stimulated by the benzanilide compounds was due to the specific activation of BK_{Ca} channels experiments using selective BK_{Ca} channel blockers were performed. The ability of benzanilides and NS1619 (100μM) to increase Rb^+ efflux was assessed in the presence and absence of IbTX (0.1μM).

For BK_{Ca} α cells all benzanilides (100μM for 10-minutes) evoked significant increases in Rb^+ efflux above control (KCl buffer alone) achieving levels of activated Rb^+ efflux (51 - 88%) in the range of that determined previously (51 - 88%, Table 3.1). The addition of IbTX significantly ($p<0.05$) inhibited the Rb^+ efflux to all benzanilides and NS1619 to a level that was not significantly different to vehicle control (KCl buffer, DMSO 0.3%, IbTX 0.1μM), (Figure 3.8).

The ability of IbTX to prevent benzanilide and NS1619 induced Rb^+ efflux on BK_{Ca} α + β1 cells was assessed (Figure 3.9). Initially the effect of IbTX on NS1619, BKH1, BKMe1 and BKOEt1 (100μM) mediated efflux was assessed. In the absence of IbTX these compounds were able to significantly increase Rb^+ efflux above basal control (KCl buffer alone) to a level (32 - 88%) similar to that achieved previously for these selected compounds (33-100%, Table 3.1). However, IbTX (0.1 μM) failed to modify the increase in Rb^+ efflux evoked by these compounds (Figure 3.9).

In contrast, in BK_{Ca} α + β1 cells paxilline (10μM) significantly reduced ($p<0.05$) benzanilide (except BKH1) and NS1619 mediated increases in Rb^+ efflux to a level that was not significantly different to vehicle control (KCl buffer, DMSO 0.3%, Pax 10μM), (Figure 3.10).

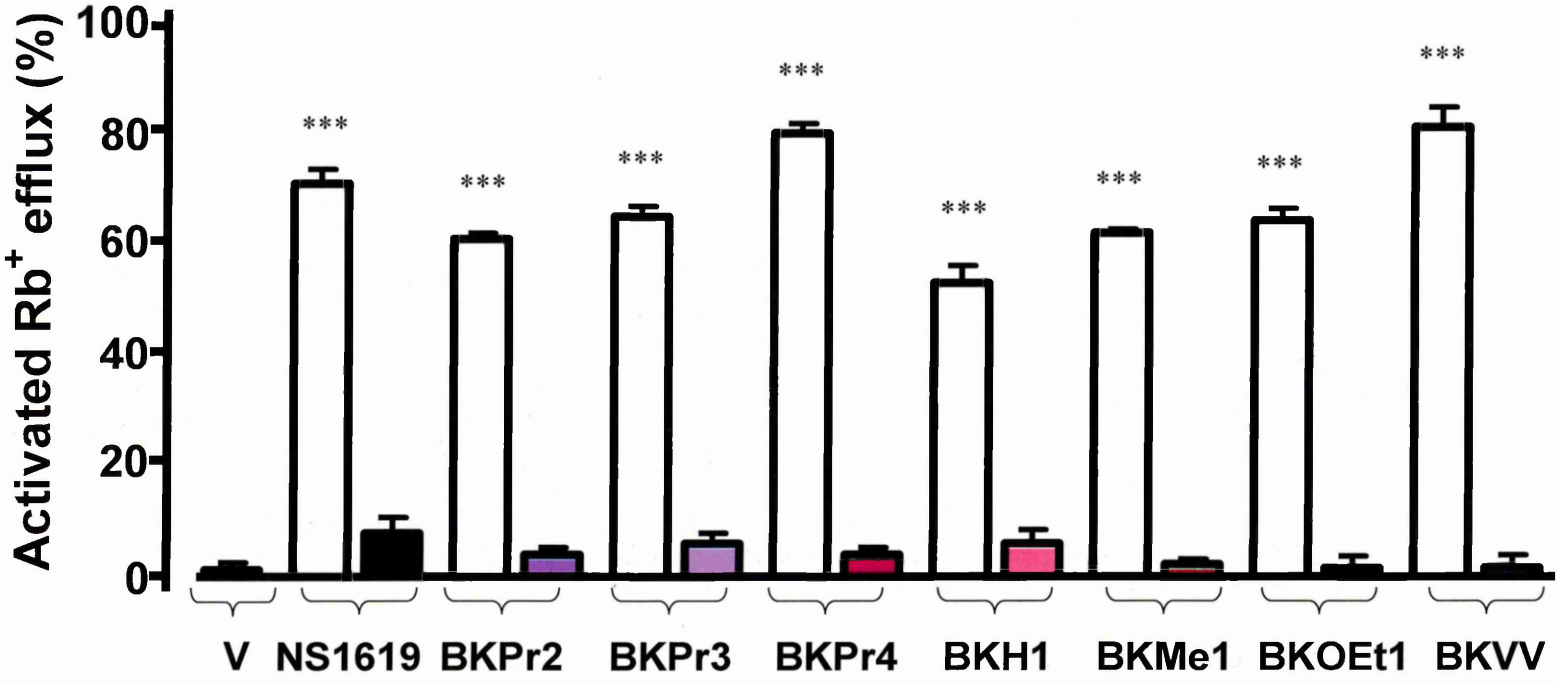


Figure 3.8: Effect of IbTX on the Rb^+ efflux response to benzanilides or NS1619 in BK_{Ca} α cells

BK_{Ca} α cells were exposed, for 10-minutes, to benzanilides (100μM) or NS1619 (100μM) in the presence (coloured bars) or absence (clear bars) of IbTX (0.1μM). V = Vehicle (DMSO 0.3% and IbTX 0.1μM). Data was normalised as the percentage increase in Rb^+ efflux above control (KCl buffer alone) for each concentration and is represented as 0% activated efflux (not shown). Data presented as means±SEM (n=24-32). Student's un-paired t-test ***$p<0.001$ indicate significant difference in Rb^+ efflux in the presence and absence of IbTX.

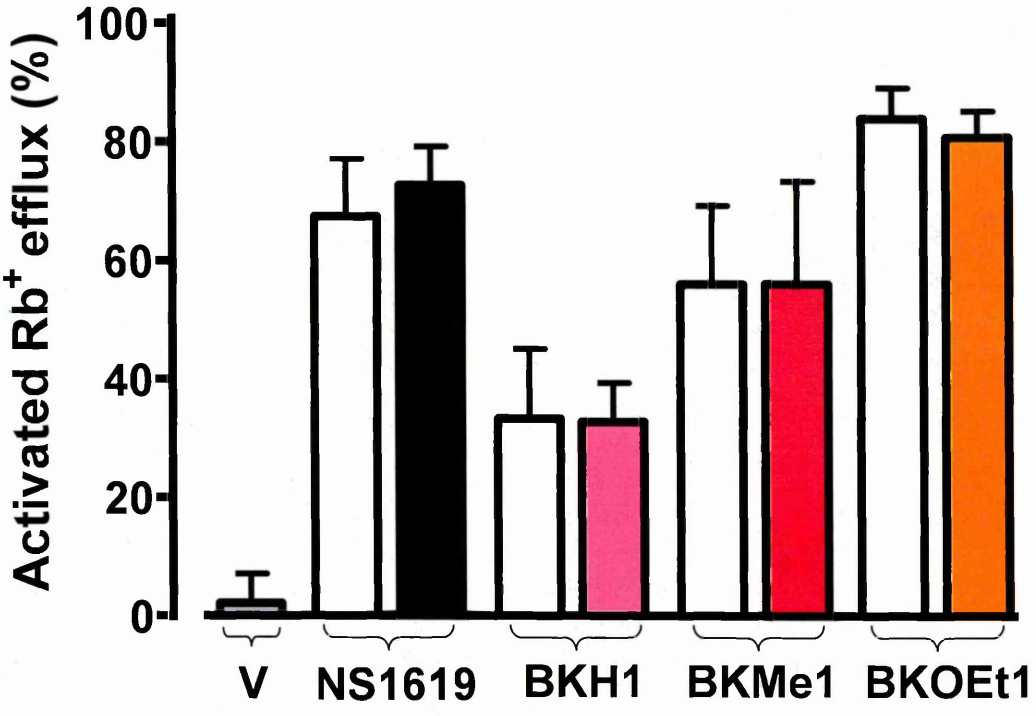


Figure 3.9: Effect of IbTX on the Rb^+ efflux response to benzanilides or NS1619 in BK_{Ca} α + β1 cells

BK_{Ca} α + β1 cells were exposed, for 10-minutes, to benzanilides (100μM) or NS1619 (100μM) in the presence (coloured bars) or absence (clear bars) of IbTX (0.1μM). V = Vehicle (DMSO 0.3% and IbTX 0.1μM) Data was normalised as the percentage increase in Rb^+ efflux above control (KCl buffer alone) for each concentration and is represented as 0% activated efflux (not shown. Data presented as means±SEM (n=24-32).

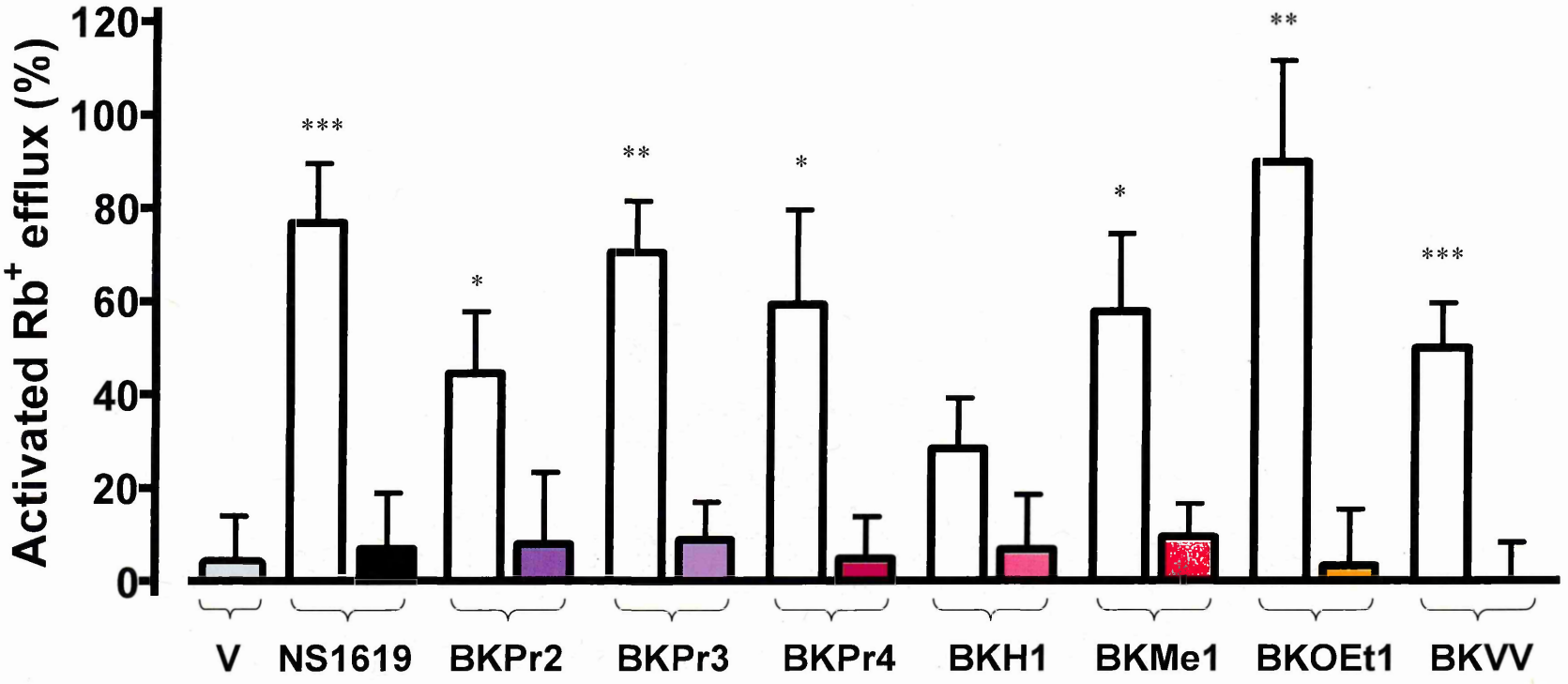


Figure 3.10: Effect of Pax on the Rb^+ efflux response to benzanilides or NS1619 in BK_{Ca} α + β1 cells

BK_{Ca} α + β1 cells were exposed, for 10-minutes, to benzanilides (100μM) or NS1619 (100μM) in the presence (coloured bars) or absence (clear bars) of Pax (10μM). V = Vehicle (DMSO 0.3% and Pax 10μM). Data was normalised as the percentage increase in Rb^+ efflux above control (KCl buffer alone) for each concentration and is represented as 0% activated efflux (not shown). Data presented as means±SEM (n=24-32). Student's un-paired t-test *p<0.05, **p<0.01, ***p<0.001 indicate significant differences in Rb^+ efflux in the presence and absence of pax.

3.5 Discussion

A series of novel benzanilides were examined for BK_{Ca} channel opener activity using an optimised Rb^+ efflux assay. All compounds evoked significant concentration related increases in Rb^+ efflux achieving activation profiles in the range of that determined for NS1619. The assay was able to discriminate the compounds ability to activate BK_{Ca} channels on the basis of potency and efficacy. In addition it was able to determine any differences in these parameters between cells expressing BK_{Ca} α alone or BK_{Ca} channel α + β subunits.

3.5.1 Determination of pharmacological properties of benzanilides

NS1619 showed equal potency and efficacy in the two cell lines. This is in agreement with other studies of this compound using electrophysiology, in that NS1619 activity is independent of β1 subunit expression (Olesen *et al* 1994b; Dworetsky *et al* 1996). BKOEt1 demonstrated high levels of potency (in the sub μM range) and was equi-potent in both cell lines. Compounds in the BKPr series were all significantly less potent in cells expressing the β1 subunit with apparent parallel shifts in the concentration response curve. In addition, the Hill slope determined for these compounds were similar to each other in the two cell lines examined. The other benzanilides (BKH1, BKMe1, BKOEt1 and BKVV) showed different shapes in the concentration response curves producing significant differences in Hill slope compared to unity.

A Hill slope of around unity for NS1619 (0.97-1.99) was determined, this is in agreement with previous published data that has shown a similar Hill slope (1.14-1.83) for the effects of NS1619 on the relaxation of uterine muscle strips and on activation of BK_{Ca} channel currents from uterine myocytes (Khan *et al* 1998).

Although there was a smaller range in efficacy values than potency significant differences in E_{max} were observed between the two cell lines for benzanilides with BKPr4, BKH1 and BKVV having significantly higher E_{max} values in BK_{Ca} α cells.

3.5.2 BK_{Ca} channel openers and subunit dependence

For DHS-1 (Giangiacomo *et al* 1998), $DiBAC_4$ derivatives (Morimoto *et al* 2007), tamoxifen (Dick 2001) and 17β estradiol (Valverde *et al* 1999) it has been shown that activation of BK_{Ca} channels specifically requires the presence of a β1-subunit whereas KB130015 has demonstrated increased activation in the presence of BK_{Ca} channel β1 subunit (Gessner *et al* 2007). The action of other BK_{Ca} activators such as NS1619 (Olesen *et al* 1994b), pimaric acid (Imaizumi *et al* 2002), NS1608 (Siemer *et al* 2000) and MaxiKdiol (Singh *et al* 1994) do not require the presence of a β1-subunit and its presence does not affect the strength of the activating effect.

A subunit dependence, with specificity towards the α-subunit, has been demonstrated for the action of compounds BKPr2, BKPr3, BKPr4 and BKH1. These compounds had decreased potency in stimulating Rb^+ efflux when tested on cells co-expressing the BK_{Ca} channel β-subunit. Synthetic molecules showing decreased potency in the presence of the β1 subunit have not been reported previously. However, ethanol has shown to cause potentiation of BK_{Ca} channels in skeletal muscle cell t-tubules and HEK293 cells expressing *mSlo* (Chu *et al* 1998). Whereas cells expressing the β1 subunit and to a lesser extent the β4 subunit reduced the effect of ethanol on activating BK_{Ca} channels (Feinberg-Zadek and Treistman 2007). Furthermore, it has been determined that ethanol can inhibit BK_{Ca} channel currents in channels composed of α and β-subunit (Dick 2007). Therefore, evidence suggests that the stimulatory effect of ethanol on BK_{Ca} channels is dependent upon expression of BK_{Ca} channel α-subunit only.

3.5.3 Potential explanations for differences in potency and efficacy

Addressing the issues of why certain compounds displayed differing abilities to activate BK_{Ca} channels in the presence of the β1 subunit could be explained by a number of theories.

3.5.3.1 Physical hindrance

The ability of the compounds to access the binding site on the channel may be hindered by the presence of the β-subunit. This physical hindrance may be in part down to the affinity of the molecules for a binding site near the point of β1 subunit coupling to the α-subunit. BK_{Ca} channel α-subunits couple to β1 subunits via the S_0 transmembrane domain (Wallner *et al* 1996; Meera *et al* 1997). Recent evidence has shown, using chimeric β1 and β2 subunits, that functional coupling primarily occurs through intracellular association of N' termini of the α-subunit and the C' terminus of the β-subunits, and to a lesser extent coupling occurs between residues in the transmembrane regions having some effect with the extracellular loop playing a minor role (Orio *et al* 2006). Therefore, the chemistry of these compounds could confer a binding site on the N' terminus of the α-subunit that potentially occurs on the intracellular side or within the transmembrane region of the channel.

However, the binding site could be occluded by the extracellular loop of the β1 subunit. The loop plays an important role in determining K^+ ion penetration and affects toxin binding (e.g β1 subunit is a requirement for ChTX binding), (Hanner *et al* 1998). In addition, the extracellular loop is glycosylated (Knaus *et al* 1994a). Therefore, the presence of secondary protein structures could occlude access to the channel pore for the compounds or provide other sites of interaction.

Compounds BKPr2, BKPr3 and BKPr4 are structurally similar and all showed a decrease in potency in the presence of the β1 subunit indicating that physical hindrance of a binding site by the β1 subunit. Further investigation of this could be achieved by expressing mutant BK_{Ca} channel α-subunits without a S_o domain or by selected mutagenesis of particular S_o domain residues.

Hill slopes determined from the Rb^+ efflux activation profiles were not different in the presence of the β1 subunit for BKPr2, BKPr3 and BKPr4 indicating that there is not a change in the co-operativity in molecules binding and activating the channel. Therefore a change in potency but not in Hill slope could indicate a change in the affinity for the binding site that is hindered by the presence of a β1 subunit.

The compound BKH1, over the concentration range tested, could not activate efflux above the 40% level in BK_{Ca} α + β1 cells where as BKMe1 showed slightly less potency in α + β1 cells and BKOEt1 was equi-potentent. Hill slope values for these compounds were different indicating that the β1 subunit may have affected the co-operativitiy in the association of molecules with the channel but had varying different effects on potency. This may suggest that this series of compounds could act at different sites to the BKPr series.

Another point to consider is that the compounds could mimic the effect of the interaction of the β1 subunit with the α-subunit. Seimer *et al* (2000) propose that NS1608 interacts with the So segment at a site for β-subunit association on the extracellular surface of the channel. This was because experimental data and the lipophilicity of NS1608 rule out the possibility of an association with intracellular C' termini of the channel. In addition, Hu and Kim (1996) proposed that the lipophilic trifluoromethyl carbocyclic part of the molecule lies within the site of action.

3.5.3.2 Physiological effect of the β-subunit on BK_{Ca} channels

The β1 subunit has been shown to enhance the Ca^{2+} sensitivity of the channel, in that 5-10 fold less Ca^{2+} is required with the β1 subunit for 50% activation of the channel at a particular membrane potential compared to α-subunits alone (McManus *et al* 1995; Meera *et al* 1996; Nimigean and Magleby 1999). The β1 subunit causes a decrease in the voltage dependence of the channel (Orio and Latorre 2005) and activation kinetics (Dworetzky *et al* 1996; Meera *et al* 1996; Tseng-Crank *et al* 1996). Therefore, the ability of compounds to activate the channel may be affected by the intrinsic voltage and calcium sensitivity or the

altered macroscopic kinetics in BK_{Ca} α + β1 cells. This could be investigated further using electrophysiology.

3.5.3.3 Two binding sites?

An alternative hypothesis to physical hindrance is the presence of two binding sites on the channel one that activates the channel at higher compound concentrations and the other site is activated at lower concentrations. The lower concentration binding site could be occluded by the presence of the β1 subunit and hence more compound would be required to produce the same effect. This in theory could be supported from the data if a biphasic relationship was observed. However, the compounds did not show a biphasic pattern.

Other openers such as phloretin, when examined on cloned m*Slo* and h*Slo,* in Xenopus oocytes demonstrated a biphasic relationship with an EC_{50} of 30μM and >200μM (Gribkoff *et al* 1996). Therefore, the concentration range used (0.1-100μM) may not have been sufficient to observe bi-phasic activity, in that a second low affinity binding site would probably require sub-mM concentrations of benzanilides.

Therefore, it would be of interest in terms of future work to determine whether the β1 subunit affects the potency of these compounds through physical hindrance or by the phenotypic effect of the β1 subunit on the functional state of the channel. Using specific blockers such as tetradrine (Dworetsky *et al* 1994) a BK_{Ca} channel β-subunit specific blocker and other BK_{Ca} channel blockers such as IbTX and ChTX could be used in binding assays (^{125}I-ChTX) to elucidate potential binding sites, however this may be limited to activators and blockers that act on the same site of the channel (Knaus *et al* 1994b; Kaczorowski *et al* 1999; Imaizumi *et al* 2002). In addition blockers could be used in determining the affinity of the compounds by using the principles of Cheng-Prussof for cells expressing different BK_{Ca} channel β-subunits with different α-subunit splice variants (Calderone 1998).

Furthermore, the assay could be used to determine the additive effects of different combinations of different openers. This may provide an insight into the

mechanism of action of these compounds. Gressner *et al* (2007) studied the additive effects, using electrophysiology, of NS1619 and KB130015 (a potential new BK_{Ca} channel opener) and determined no discernable difference when added together versus their effects when tested independently indicating that they could act in a similar mechanism.

In addition, the effect of different β-subunits (β1- β4) with their different structure and functional effect may provide further understanding of the compounds mechanism of action and elucidate further specificity of these compounds.

3.5.4 Therapeutic potential of benzanilide compounds

BKH1, BKPr3 and BKPr4 showed a decrease in potency and to some degree efficacy when tested on BK_{Ca} α + β1 cells. Such agents showing differential activity profiles in the absence and presence of the β1 subunit could be used as therapeutic agents in a number of clinical conditions. In particular these agents could be beneficial therapeutically for diseases associated with changes in α-subunit or in tissues with the expression of BK_{Ca} channel α-subunit only. Further, BK_{Ca}CO compounds that are dependent upon β-subunit for binding are not necessarily advantageous as therapeutics because changes (decreases) in expression of the β-subunit with age and disease have been reported so BK_{Ca} channel α-subunit specific activators would be more advantageous (Zakharov *et al* 2005).

BK_{Ca} channels are ubiquitously distributed in nearly all tissues (except in cardiomyocytes), however tissues display differential expression levels of α or β subunits. The predominant BK_{Ca} channel in brain regions and spinal chord are composed of BK_{Ca} channel α-subunits only (Knaus *et al* 1996; Chang *et al* 1997; Jiang *et al* 1999) except in the cerebellum and corpus callosum (Tseng-Crank *et al* 1996). Furthermore, BK_{Ca} channels have recently been discovered to be expressed in inner mitochondrial membrane of rat brain (Douglas *et al* 2006). Therefore, BK_{Ca} channel openers could be potentially used to treat neuronal ion channel disorders (Lawson 2000a).

3.5.4.1 Skeletal muscle

Tissues displaying α-subunit expression alone include skeletal muscle (Nimigean and Magleby 1999) which has demonstrated low or no β-subunit mRNA expression in human, canine, or rat skeletal muscle (Jiang *et al* 1999; Chang *et al* 1997; Tseng-Crank *et al* 1994). In addition, the β–subunit selective activating properties of 17β estradiol were unable to elicit BK_{Ca} channel current in rat skeletal muscle (Valverde *et al* 1999).

In contrast to smooth muscle BK_{Ca} channels, which have been shown to be critical regulators of vascular tone, the physiological role of skeletal muscle BK_{Ca} channels is much less defined (Marijic *et al* 2001). The majority of research into skeletal muscle BK_{Ca} channels has come from the work of Tricarico and colleagues. They have shown that in skeletal muscle BK_{Ca} channels are opened by changes in membrane potential and intracellular calcium as well as by protein kinase A (PKA) phosphorylation. Skeletal BK_{Ca} channels increase the duration between after-hyperpolarisations between action potential burst and buffer the accumulation of intracellular Ca^{2+} ions (Tricarico *et al* 1997; Tricarico *et al* 2000). In addition, BK_{Ca} channel activity in skeletal muscle is increased in aged rats (Tricarico *et al* 1997).

Enhanced BK_{Ca} channel activity has been implicated in the pathogenesis of hyperkalemia in particular following ischaemia-reperfusion in a rat model of skeletal ischaemia. Furthmore it is an unwanted side effect that can cause sudden death in patients affected by reperfusion disorders. Therefore modulation of K_{Ca} currents in skeletal muscle could prove therapeutically beneficial (Tricarico *et al* 2002).

The carbonic anhydrase inhibitor acetazolamide (ACTZ) has been used clinically to treat hypokalemic periodic paralysis (hypoPP) disorder with patients suffering episodes of flaccid paralysis with muscle weakness lasting from hours to days. Treatment restores muscle strength and reduced frequency of attacks (Links *et al* 1988; Tricarico *et al* 2000). The disorder is characterized by low plasma K^+ levels and muscle depolarisation in response to insulin (Tricarico *et al* 2000; Tricarico *et al* 2004).

Tricarico *et al* (2000) identified that ACTZ mode of action was not through blocking carbonic anhydrase enzymes but via the activation of K_{Ca} channels. The application of ACTZ to a hypokalemic rat model prevented paralysis that was reversed by K_{Ca} channel blockade (using ChTX) but not by K_{ATP} channel blockade (using glibenclamide). Furthermore, that excised patches of dissociated rat fibers from K^+ deficient mice showed that ACTZ activated BK_{Ca} currents (Tricarico *et al* 2000). Further to this structure-activity relationships for a series of carbonic anhydrase (including ACTZ) inhibitors have been reported (Tricarico *et al* 2004). However, ACTZ treatment has shown in some patients to be ineffective and can exacerbate the condition (Tricarico *et al* 2005).

Recently, the differential effects of ACTZ have been shown to result from the expression of two types of BK_{Ca} channel in rat skeletal muscles. BK_{Ca} channels from slow twitching soleus muscles are Ca^{2+} and ACTZ insensitive while the BK_{Ca} channel phenotype from fast twitching flexor digitorum brevis muscles are ACTZ sensitive and display normal Ca^{2+} sensitivities. A change in phenotype can occur following ACTZ treatment and prolonged muscle disuse (Tricarico *et al* 2005). Furthermore, BK_{Ca} and K_{ATP} channels have been implicated in muscle fatigue caused by potassium imbalances in skeletal muscle (Kristensen *et al* 2006).

BK_{Ca}CO's with selectivity to skeletal muscle (expressing BK_{Ca} channel α-subunits only) could provide a therapeutic advantage to current treatments for hypoPP and other disorders associated with muscle weakness and fatigue.

3.5.4.2 Endothelial cells

In porcine cultured endothelial cells BK_{Ca} channels expressing only the α-subunit has been reported (Papassotiriou *et al* 2000). In addition, 17β estradiol had no effect on BK_{Ca} channel currents in cultured human vascular endothelial cell line derived from umbilical cord (HUV-EC-C) where as other BK_{Ca} openers including niflumic acid and Evans blue elicited paxilline sensitive BK_{Ca} currents (Chiang and Wu 2001). It has been reported that endothelial BK_{Ca} channels are important elements in coupling of shear stress and the release of endothelial

vasoactive compounds such as NO and prostaglandins that elicit dilation of arterioles of skeletal muscle (Sun *et al* 2001). Furthermore, it has been demonstrated that the BK_{Ca} channel openers NS1619 and resveratol induce NO release from endothelium of rat aorta but fail to induce it in denuded aorta (Calderone *et al* 2007). Furthermore, increases in endothelial NO synthesis have been reported in cultured Human endothelial cells upon exposure to NS1619 and that this increase involves p42/p44 MAP kinase and calcium induced activation of eNOS system (Kuhlmann *et al* 2004).

In addition, it has been demonstrated that BK_{Ca}CO do not effect the proliferation of endothelial cells and that they could be advantageous in the treatment of hypertension and other cardiovascular diseases (Calderone *et al* 2007) without producing hypertension induced vascular remodeling. Therefore, BK_{Ca} channel subtype specific openers identified here could potentially be useful as therapeutic tools in the treatment of hypertension and other cardiovascular problems.

3.5.5 Blocking experiments

To determine the specificity of benzanilides in activating Rb^+ efflux from BK_{Ca} channels in the two cell lines the effects of benzanilides were tested in presence of the specific blockers channel blockers. IbTX (Garcia *et al* 1991) and Pax (Knaus *et al* 1994b) were able to block benzanilide induced increases in Rb^+ efflux in BK_{Ca} α and BK_{Ca} α + β1 respectively. IbTX was unable to block benzanilide induced increases in Rb^+ efflux in BK_{Ca} α + β1. The insensitivity of BK_{Ca} channels expressing the β1 subunit to block by IbTX has been documented previously in HEK293 cells (Lippiat *et al* 2003; Ahring *et al* 1997) and oocytes (Dworetsky *et al* 1996) expressing either α alone or α + β1 subunits, with an approximate 30-fold shift in IC_{50} for IbTX from ~30nM to ~300nM.

Therefore, blocking with the IbTX (100nM) concentration used may not have been sufficient to block BK_{Ca} channels in the BK_{Ca} α + β1 cell line. However, pax which was able to block BK_{Ca} α + β1 cells has shown to block α and α + β1 subunits with similar affinity (Ahring *et al* 1997). In addition, the IC_{50} for pax (1-200nM), (Sanchez and McManus 1996; Ahring *et al* 1997; Li and Cheung 1999)

was well below that used in the blocking experiments (10μM). For future work it may have been useful to have looked at different concentrations of blocker in the presence of fixed amounts of benzanilide. This could be used to build up a picture of affinity of the compounds and the use of different blockers with different binding sites could lead to an understanding of sites of interaction on the channel.

3.5.6 Considerations

Potency ($EC_{40\%}$) has been used to assess those benzanilides with the greatest BK_{Ca} channel opener ability. In the pharmaceutical industry potency in HTS is the predominant criterion used in the selection of potential lead candidate drugs (Abad-Zapatero and Metz 2005). However, current thought is that potent compounds do not always result in good drugs (Hopkins *et al* 2004; Abad-Zapatero and Metz 2005). In that other parameters should be taken into consideration, in particular molecular weight. In addition, physiochemical properties such as polar surface area, affinity (K_d) and IC_{50} have been used in the development of indexes to asses new compounds potential to become drugs (Hopkins *et al* 2004). For example, ligand efficiency which uses the number of non-hydrogen atoms as a normalising factor against differences in affinity measured for different compounds. Percentage efficiency index (PEI) and binding efficiencies index (BEI) based on molecular weight and surface-binding efficiency index (SEI) based on the polar surface area and or polarity of a compound (Abad-Zapatero and Metz 2005). Molecular modelling of these compounds could aid in assessing the physio-chemcial properties of the benzanilides. Any potential differences in the properties of these compounds could be related to variations in their structures and their differential potency profiles. This could provide information that could guide the synthesis of more potency and specific BK_{Ca}CO from this series.

Therefore, defining compounds on their ability to activate BK_{Ca} on the basis of potency is still a good measure but should consider the physiochemical effects of these compounds (Chapter 6) to determine their potential for lead selection.

3.6 Conclusions

This assay has identified 7 novel compounds as potential BK_{Ca} channel openers that display varying degrees of potency and efficacy and that some compounds show specificity to the BK_{Ca} channel α-subunit.

Chapter 4

Assessment of benzanilides as $BK_{Ca}CO$'s using whole cell electrophysiology

4.1 Introduction

Patch clamp electrophysiology was developed in the mid-70's by E. Neher and B. Sakmann and allowed the characterisation of single channels within a membrane; this has become the definitive method for ion channel studies (Neher and Sakmann 1976, Hamill *et al* 1981).

The use of the patch clamp technique coupled with developments in molecular genetics has been fundamental in the characterisation of the cellular functions of potassium channels (Calderone 2002; Korovkina *et al* 2002). Furthermore, the whole cell electrophysiology recording configuration (WCR) has provided a powerful technique for the assessment of K^+ channel modulators (Olesen *et al* 1994b; Gribkoff *et al* 1996; Holland *et al* 1996; Hu and Kim 1996; Hu *et al* 1997; Gribkoff *et al* 2001b; Imaziumi *et al* 2002; Wu *et al* 2002; Nardi *et al* 2003; Sakamoto *et al* 2005; Huang *et al* 2007). Currently, it remains the preferred technique for the verification of compounds identified using Rb^+ efflux assay (Tang *et al* 2001; Parihar *et al* 2003; Scott *et al* 2003; Willumsen *et al* 2003; Rezazadeh *et al* 2004) as K^+ channel modulators.

The Rb^+ efflux assay reported in chapter 2 provided a robust and informative assay to assess novel $BK_{Ca}CO$ compounds. The Rb^+ efflux studies reported in chapter 3 revealed a series of benzanilides that demonstrated differential potency and efficacy profiles. However, electrophysiology allows for detailed examination of the activation properties of the benzanilides on BK_{Ca} channels and is the preferred method for the analysis of ion channel function.

4.2 Chapter Aims

To further characterise the activation of BK_{Ca} channels by benzanilides using whole cell electrophysiology.

- Asses the effect of benzanilides on activity of BK_{Ca} channels by determining their effects on voltage-dependent activation, V_{50}'s, activation and deactivation times.

- Validation of Rb^+ efflux assay in determining the potency of benzanilides vs. that obtained with whole cell electrophysiology.

- Determine the specificity of activation of benzanilides to BK_{Ca} channels in this cell line.

4.3 Methods

4.3.1 Materials

Cell culture equipment

Scientific Laboratory Supplies, Nottingham U.K: Nunc Dishes (35mm)

General purpose reagents

VWR International Ltd, Lutterworth, U.K: Potassium hydroxide (KOH), methanol

Sigma Aldrich, Gillingham, U.K: (β-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA)

Electrophysiology

VWR International Ltd, Lutterworth, U.K: Acrodisc syringe filter (0.2μl).

Thermo Electron Corporation, Auchtermuchty, U.K: Ionplus Ca^{2+} module and electrode.

World Precision Instruments (WPI), Stevenage, U.K: CALBUF-1: Calcium standard buffers, 24AWG 34-Guage 70mm long microfilm syringe

Harvard Apparatus, Edenbridge, U.K: Borosilicate glass capillaries (1.2mm O.D X 0.69mm I.D).

4.3.2 Cell culture

BK_{Ca} α cells were maintained in continuous culture as described in chapter 2 section 3.3. Cells were prepared for electrophysiology by seeding 35mm nunc dishes with 10,000 cells/ml in DMEM (2mls). Cells were used 1-4 days post plating. Isolated cells were selected for WCR.

4.3.3 Electrophysiology

4.3.3.1 Microelectrode Fabrication

Patch pipettes were fabricated from borosilicate glass capillaries (1.2mm O.D. X 0.69mm I.D.) using a Sutter Instrument Company P-97 Flaming/Brown micropipette puller (Intracell, Shepreth, U.K.) yielding two symmetrical pipettes (with an optimised shape) from each pull. These were then fire polished using a Narishige Microforge MF-830 (Intracell, Shepreth, U.K) to obtain electrodes with a resistance of 2-5MΩ.

4.3.3.2 Drugs and solutions

Extracellular bath solutions contained; (mM) KCl 120, KOH 20, $MgCl_2$ 1.0, $CaCl_2$ 0.01, HEPES 10 and intracellular pipette solution contained; (mM) KCl 90, KOH 50, $MgCl_2$ 1.0, $CaCl_2$ 0.01, HEPES 10. The pH of both solutions was adjusted to 7.4 (KOH 1M) and calcium Ca^{2+} concentration was adjusted to 0.3μM with EGTA (10mM) using a Ca^{2+} selective electrode calibrated using Ca^{2+} buffer standards (0.1, 1.0 and 10μM). Solutions were warmed to room temperature (18-24°C) prior to experimentation and intracellular pipette solution was filtered using an acrodisc syringe filter (0.2μM) before filling pipettes.

Drugs were prepared from frozen stocks as described previously (chapter 2) and diluted to the required concentration in extracellular solution. Solutions were applied to the bath using a gravity fed perfusion system (at a rate of 10.0 ml/min) which allowed for complete exchange of bath solution within 2-minutes. Perfusion system tubing was cleaned by washing through with methanol (100%) and then with double-distilled water (resistivity >18 $M\Omega\,cm^{-1}$), this procedure was carried out following experimentation after the addition of BK_{Ca} channel modulators.

Control recordings were made in the presence of extracellular solution and during the perfusion of compounds, the perfusion system was turned off before recordings were acquired.

4.3.3.3 Data Recording

Whole cell currents were recorded using conventional patch clamping techniques (Hamill *et al* 1981). Currents were recorded in voltage clamp mode using an EPC-10 patch-clamp amplifier via an IPTC16 digital interface (HEKA, Lambrecht, Germany). Current signals, after compensation for whole-cell capacitance, series resistance and liquid junction potential, were low-pass filtered at 2.9 KHz using a 4-pole Bessel filter and sampled at 10 KHz and 1 KHz before being stored on the hard disk drive of the computer. BK_{Ca} channel current, under these conditions, did not run down during the following 30–40 min of the experimentation period.

4.3.3.4 Whole cell patch formation and Data acquisition

Single isolated cells were chosen to patch; following seal formation whole cell access was acquired by applying gentle suction to the pipette to disrupt the cell membrane, seals >2GΩ were accepted. Breakthrough was performed by applying gentle suction following seal formation and detecting a change of current and the appearance of small capacitance transients when voltage pulses were applied.

In whole cell the time for solution exchange between the pipette and the intracellular contents of the cell was ~10s based upon the parameters defined by Thomas and Smart (2005) for HEK293 cells. Therefore within 1-2 mins of membrane breakthrough complete solution exchange would have occurred.

Currents were elicited using a voltage step protocol (Figure 4.1); from a holding potential of 0mV for 50ms the voltage was ramped to -100mV for 100ms to deactivate BK_{Ca} channels, following this the voltage was stepped to the first voltage to be analysed for 500ms. Subsequently the voltage was then stepped to -100mV and held at this potential for 100ms to close all of the channels (instantaneous tail current), this was followed by a period of rest when the potential was returned to 0mV for 50ms. This cycle was repeated 21 times from

-100 to + 100mV with each cycle increasing the voltage applied to the membrane by 10mV.

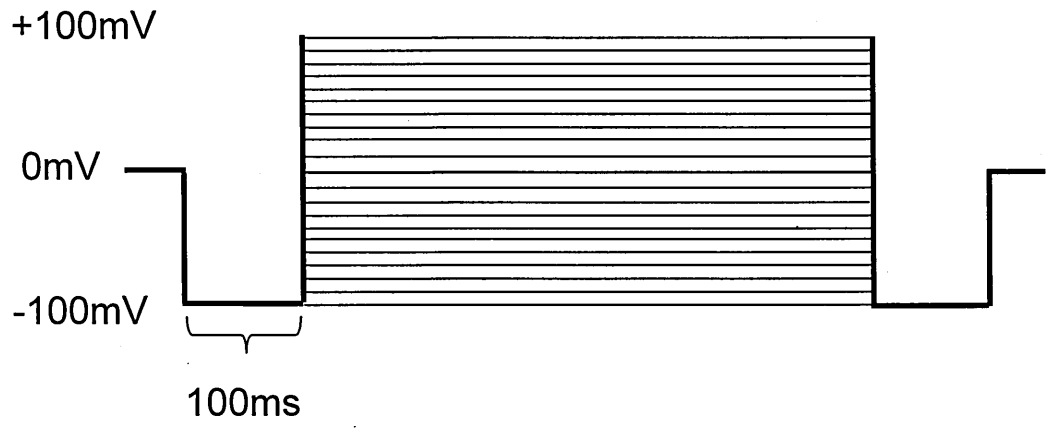


Figure 4.1: Voltage-step protocol for recording of BK_{Ca} channel currents

4.3.3.5 Data analysis

Data was analysed *offline* for peak current at each stepwise increase in membrane voltage using HEKA Pulsefit software and values exported to Microsoft Excel.

Conductance-voltage curves

Peak current values were converted to conductance using the formula $G= I/V_m$ where G is conductance, V is the membrane potential and I the current elicited. Conductance was normalised to the highest conductance within the patch (Gmax). Normalised data was pooled and used to generate normalised conductance (G/Gmax) – voltage (V) curves (G-V) to which Boltzmann sigmoidal function was fitted;

$$G/Gmax = (1+\exp[(V_{50} - V_m)/S])^{-1}$$

From which V_{50}, S and V_m representing voltage required for half-maximal activation, slope and membrane potential were determined respectively (Gribkoff *et al* 1996; Ahring *et al* 1997; Zakharov *et al* 2005). Current traces were exported from HEKA PulseFit to use as visual representations of the data.

Tail currents and deactivation time

For current-deactivation studies; tail currents were evoked following a +100mV activating pulse by a step to a deactivating -100mV potential and then repolarising to 0mV for 100ms. Tail currents were best fit using a one-component exponential model (Dworetsky *et al* 1996; Zhang *et al* 2001; Horrigan and Aldrich 2002) of the form;

$$I(t) = a_o + a_1 \exp(-t/\tau_1)$$

From which deactivation time constant (τ, ms) was determined. Time to peak currents were measured directly with both values obtained using PulseFit software.

Inhibition profile

The inhibitory profile of the blockers TEA, IbTX and pax was determined by calculating the relative inhibition of current (I/I_0) compared to control current recorded at +60mV membrane potential. The data was fitted by the following modified Hill equation (Sanchez and McManus 1996);

$$I/I_o = Bottom + (Top - Bottom)/1 + 10\text{^}((\log IC_{50} - X) * Hillslope))^{-1}$$

From which the IC_{50} (the concentration of blocker giving 50% inhibition) of a blocker was determined.

All data was fitted using GraphPad Prism 4 software and are presented as mean (±SEM) for n determinations. Data was analysed using either Student's un-paired or paired t-test or for multiple comparisons one-way ANOVA followed by Dunnett's post test analysis or Bonferroni's multiple comparison test (Wallenstein *et al* 1980). The accepted level of significance was $p \leq 0.05$.

4.4 Results

4.4.1 The effect of K^+ channel blockers on I_K from BK_{Ca} α cells

To determine that the control currents observed were due to the activation of K^+ channels (I_k) the non-specific K^+ channel blocker TEA (applied to extracellular bath solution) was used. The assessment of blocker action was determined as the % decrease in current at +60mV membrane potential. TEA was able to completely block I_K currents in the cell with an IC_{50} value determined as 5.24±1.00mM (n=4), (Figure 4.2).

To assess whether the K^+ current was carried by BK_{Ca} channels the specific blocker IbTX was applied to the extracellular solution. In symmetrical K^+ (140mM) IbTX at supra-maximal concentrations (0.5μM) was unable to completely block I_K, with an IC_{50} value determined as 0.22±0.15μM (n=4). However, in asymmetrical K^+ (5.4:140mM) the IC_{50} value was lower 0.08±0.07μM (n=4), with greater block observed at each concentration tested (Figure 4.3). Although at high concentrations (0.5μM) IbTX was unable to completely block I_k. In contrast, the BK_{Ca} channel blocker pax (0.001-10μM) blocked I_k currents in a concentration dependent manner from which an IC_{50} of 0.09±0.2μM (n=6) was determined. Complete inhibition of channel currents was observed at 10μM pax (Figure 4.4).

The blocker action for TEA, IbTX and pax was reversible upon washout with extracellular buffer and complete washout accomplished within 5-10 minutes of continual perfusion (50-100mls), (Data not shown).

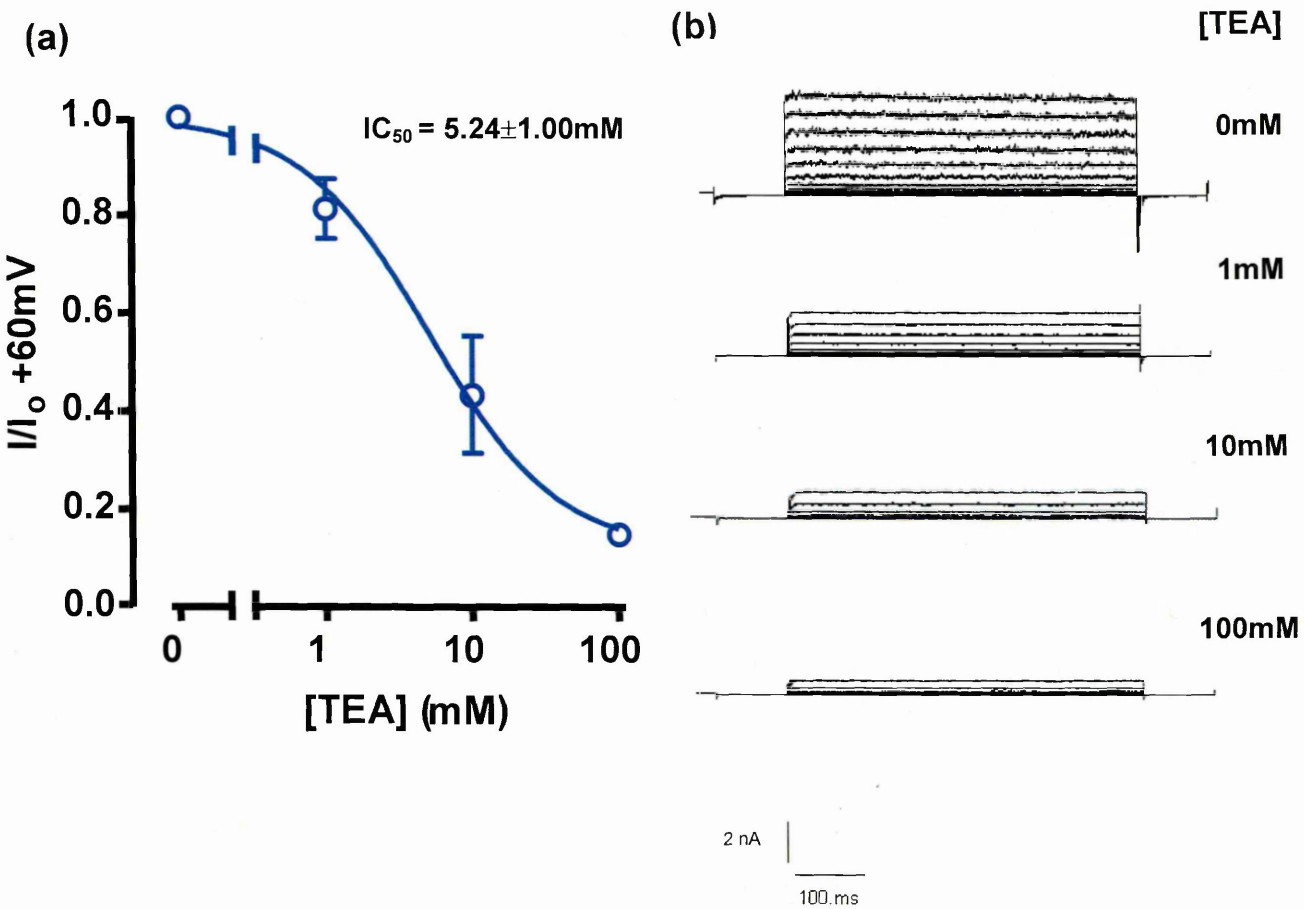


Figure 4.2: Effect of TEA on control whole cell currents from BK_{Ca} α cells

Whole cell currents were elicited under control conditions (0.0mM TEA, 0.3μM Ca^{2+} and symmetrical 140mM K^+) and following application of TEA (1-100mM). **(a)** Inhibition CRC determined where reduction of current relative to control (at +60mV membrane potential) for each concentration tested. **(b)** Representative current traces for the effect of different concentrations of TEA as indicated. Data presented as means±SEM, (n=3-4).

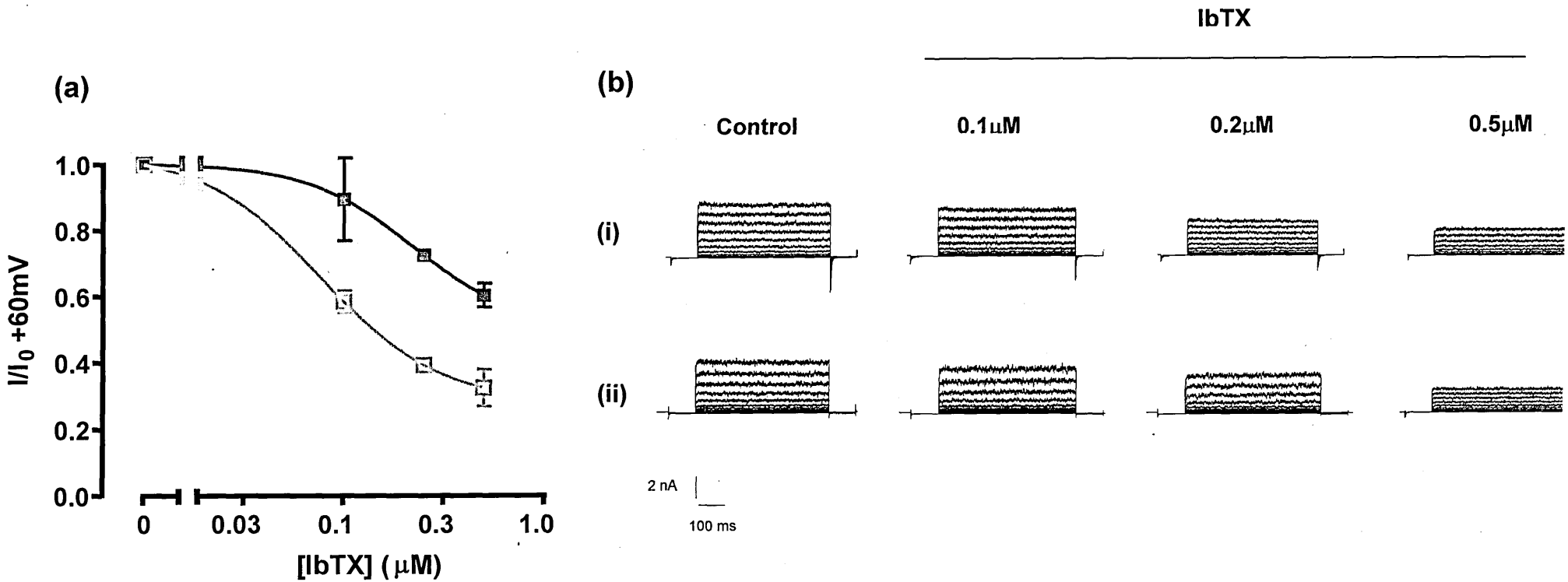


Figure 4.3: Effect of IbTX on control whole cell currents from BK_{Ca} α cells

Whole cell currents were elicited under control conditions and following application of IbTX (0.1-0.5μM). **(a)** Inhibition CRC determined as the inhibition of current relative to control (at +60mV membrane potential) for each concentration tested in either symmetrical (140:140mM) (■) or asymmetrical (5mM extracellular) potassium (□), from which the IC_{50} (Inhibitory concentration 50%) was determined. Data presented as means±SEM, (n=6-9). **(b)** Representative current traces for data determined in symmetrical **(i)** and asymmetrical **ii)** in the absence (control) and presence of differing IbTX concentrations.

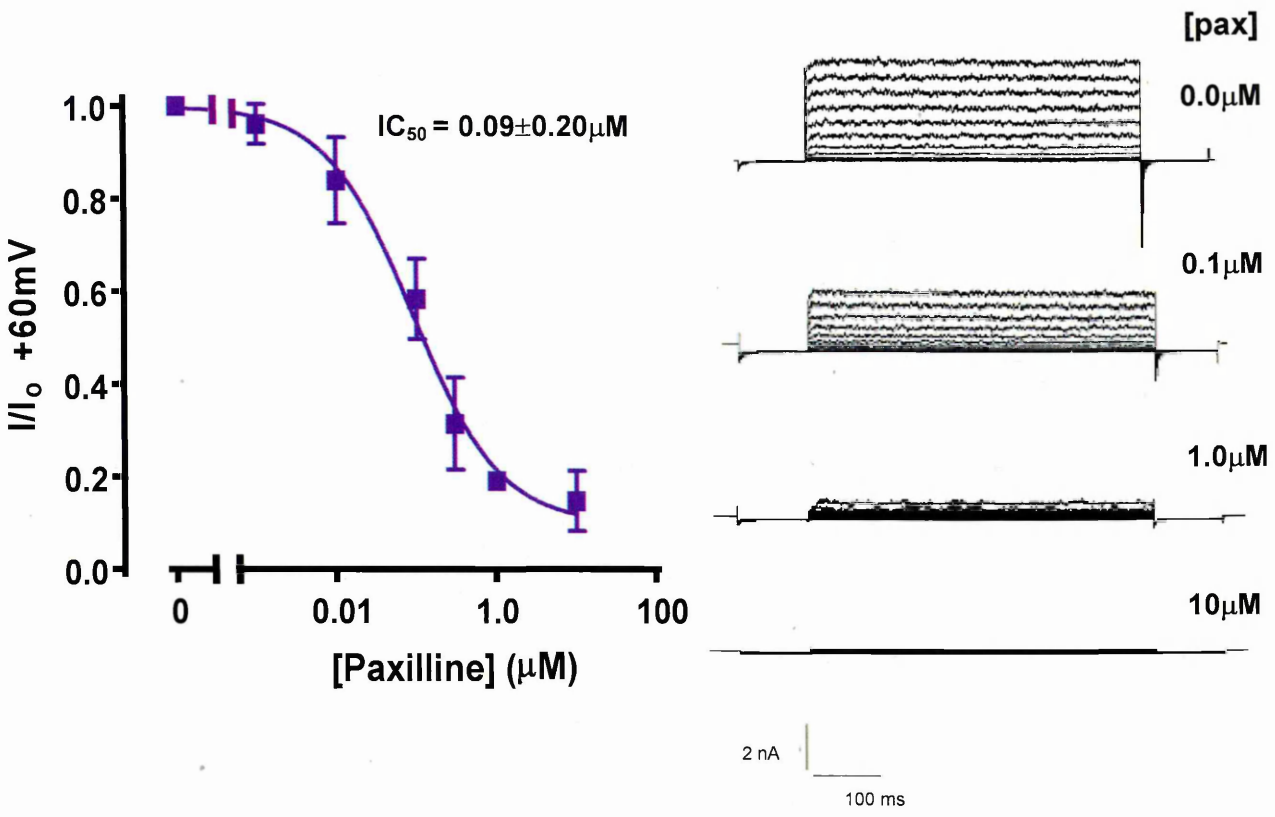


Figure 4.4: Effect of Paxilline on control whole cell currents from BK_{Ca} α cells

Whole cell currents were elicited under control conditions (0.0μM Pax, 0.3μM Ca^{2+} symmetrical ((140mM)) potassium) and following application of pax (0.001-10μM). **(a)** Inhibition CRC determined as the reduction of current relative to control (at +60mV membrane potential) for each concentration tested. Data presented as means±SEM (n=4-6). **(b)** Representative current traces for different concentrations of pax as indicated.

4.4.2 Benzanilide activation of whole cell BK_{Ca} channel currents

Benzanilides and NS1619 (30μM) were tested for their ability to elicit whole cell BK_{Ca} channel current activity from BK_{Ca} α cells. Each cell was exposed to one compound at a single concentration and compounds were tested in randomised order on cells from different passages.

Application of NS1619 (30μM) resulted in an increase in whole-cell BK_{Ca} channel currents (above control) that was more pronounced with further depolarisation (Figure 4.5b) producing significant displacement of G-V curve towards more hyperpolarised voltages (Figure 4.5a), indicative of BK_{Ca} channel activation. The effect was evaluated by comparing the voltage required to produce 50% activation (V_{50}). Under control conditions (0.3μM Ca^{2+}) a V_{50} of 85.3±1.5mV (n=9) was determined and was not modified by application of extracellular bath solution containing DMSO (0.3%) (V_{50} = 85.1±9.4mV, n=3). The addition of NS1619 (30μM) produced a significantly (Student's paired t-test, $p<0.001$) decreased V_{50} (13.4±3.3mV, n=9) value (Figure 4.5c).

All benzanilide compounds (30μM) independently tested produced significant increases in whole-cell BK_{Ca} channel currents above control that was more pronounced with further depolarisation (Figure 4.5b-4.12b). Each benzanilide, like NS1619, produced significant leftward displacement of the G-V curve from control in the hyperpolarising direction, indicative of BK_{Ca} channel activation (Figure 4.5a-4.11b). For the benzanilides, a range (29.9-63.0mV, n=6-9) in V_{50} values was produced. These were all significantly decreased compared to their respective V_{50} values determined under control conditions (Figure 4.5c-4.12c).

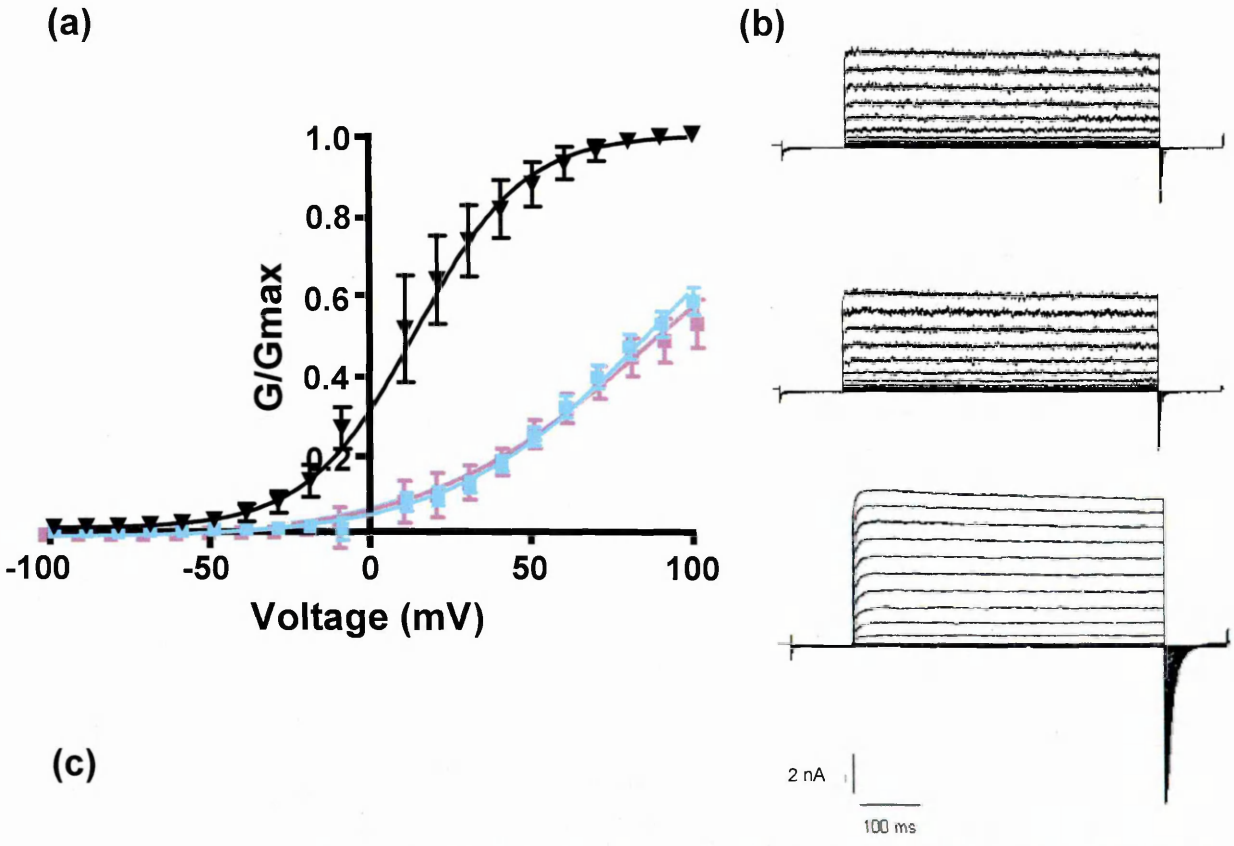
The effect of the benzanilides and NS1619 was reversible upon wash-out with extracellular solution. However, an extensive washing time was required of 15-20 minutes (~150mls of extracellular solution) continual perfusion to return the level of activation to control levels (Data not shown).

4.4.3 Time to peak current and deactivation kinetics

The time to peak current was measured as the time required to achieve maximum current following induction of voltage step following a holding potential of -100mV. NS1619 significantly ($p<0.01$, Student's paired t-test) increased the time (25.0±2.4ms, n=6) for current to reach a peak value relative to control (12.3±1.7ms, n=6), (Figure 4.5c). Under control conditions a range of activation times (11.1±2.5 to 15.1±3.4ms, n=6-9) was determined that was not significantly (Student's paired t-test, $p>0.05$) affected by application of each benzanilide (10.0±1.1 to 14.4±3.6ms, n=6-9), (Figure 4.6c-4.12c). Therefore, no individual benzanilide affected the time to reach peak current relative to the control measurement made prior to the application of compound.

The deactivation kinetics for benzanilides and NS1619 was assessed by determining the deactivation time constant from instantaneous tail current generated from the voltage step protocol by application of -100mV following a +100mV activating test pulse. NS1619 significantly ($p<0.01$, Student's un-paired t-test) increased the time (12.5±2.9ms, n=6) for the channel to deactivate relative to control measurements made prior to application of NS1619 (2.6±0.6ms, n=6).

Under control conditions a range of values (2.4±0.3 to 6.2±2.3ms, n=6-9) determined that were not significantly (Student's paired t-test, $p>0.05$) affected by application of each benzanilide (4.2±2.0 to 7.5±2.8ms, n=6-9). Therefore, no individual benzanilide tested affected the time for the channel to deactivate in response to an inactivating potential relative to the control measurement made prior to the application of compound.

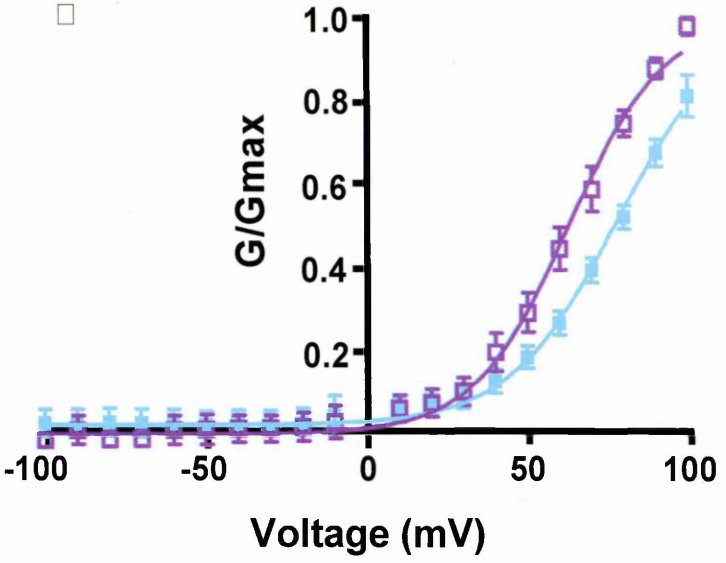


	V_{50} (mV)	Time to Peak Current (ms)	Deactivation Time (τ ms)
Control	85.3±1.5	12.3±1.7	2.6±0.6
DMSO (0.3%)	85.1±9.4	n.d	n.d
NS1619	13.4±3.3***	25.0±2.4***	12.5±2.9***

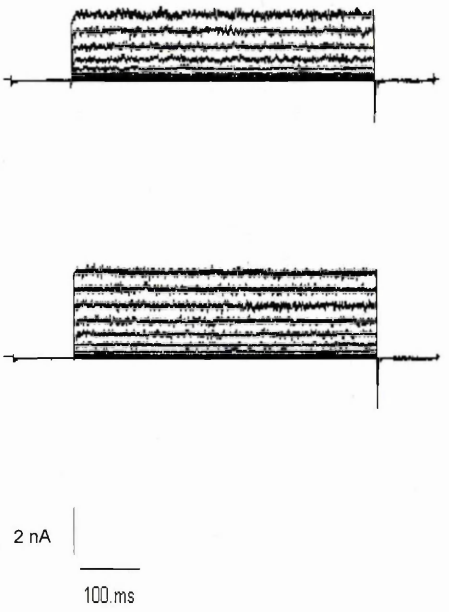
Figure 4.5: The effect of DMSO and NS1619 on BK_{Ca} channels

In whole cell configuration BK_{Ca} channel currents were elicited from BK_{Ca} α cells by applying test pulses in 10mV increments from -100mV to +100mV from a holding potential of −100mV. **(a)** Averaged normalised G–V relationships were determined under control conditions (■) and following exposure to DMSO (0.3% ■) then NS1619 (30μM ▼) from which V_{50} and slope factor were determined **(C)**. **(b)** Representative current traces obtained in the absence (control, Top) and presence of DMSO (middle) and then followed by NS1619 (30μM) (Bottom) from which time to peak current was measured and deactivation time constant determined from tail currents (at -100mV) **(c)**. Data presented as means±SEM (n=6-9). Student's paired t-test, ***p<0.001 indicate significant differences from control. n.d = not determined.

(a)



(b)



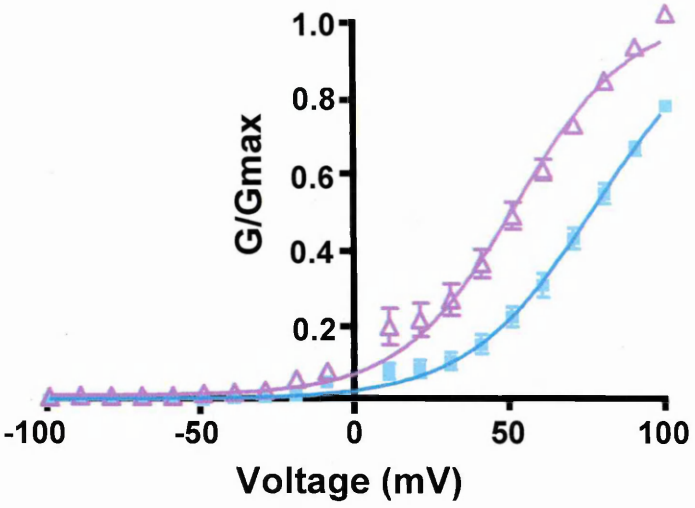
(c)

	V_{50} (mV)	Time to Peak Current (ms)	Deactivation Time (τ ms)
Control	78.1±2.9	11.5±1.4	2.4±0.3
BKPr2	63.1±2.1**	10.0±1.1	2.3±0.1

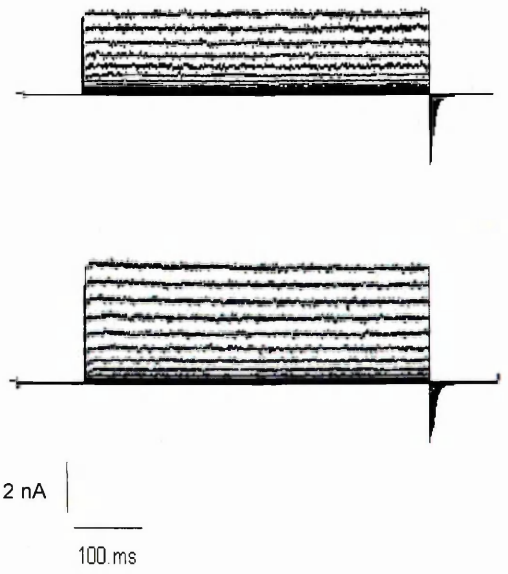
Figure 4.6: Stimulatory effect of BKPr2 on BK_{Ca} channels

In whole cell configuration BK_{Ca} channel currents were elicited from BK_{Ca} α cells by applying test pulses in 10mV increments from -100mV to +100mV from a holding potential of −100mV. **(a)** Averaged normalised G–V relationships were determined under control conditions (■) and during exposure of BKPr2 (30μM, □) from which V_{50} and slope factor were determined **(c)**. **(b)** Representative current traces obtained in the absence (control; top) and presence (bottom) of BKPr2 (30μM) from which time to peak current was measured and deactivation time constant determined from tail currents (at -100mV) **(c)**. Data presented as means±SEM, (n=6-9). Student's paired t-test, **p<0.01 indicate significant differences from control.

(a)



(b)



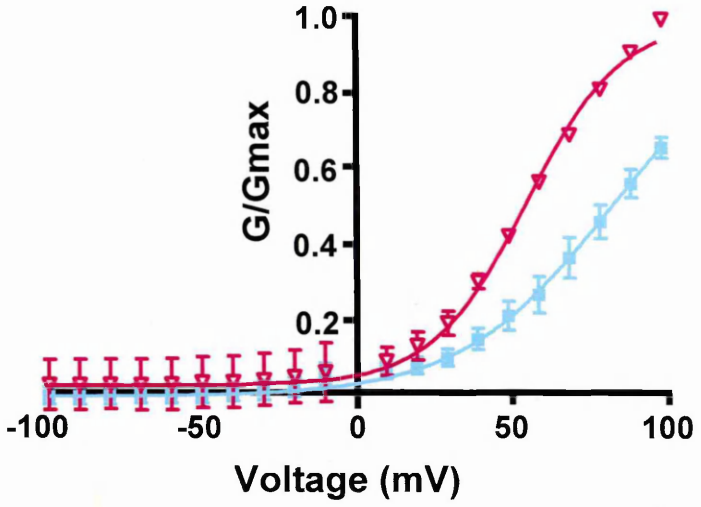
(c)

	V_{50} (mV)	Time to Peak Current (ms)	Deactivation Time (τ ms)
Control	77.1±1.4	14.3±1.5	3.4±0.4
BKPr3	51.4±2.7***	14.4±3.6	3.7±0.7

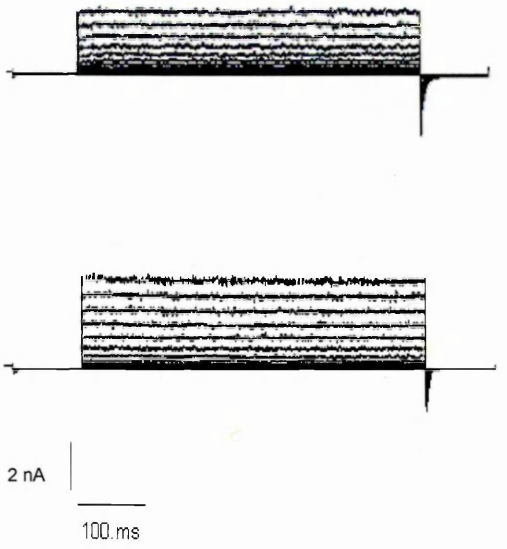
Figure 4.7: Stimulatory effect of BKPr3 on BK_{Ca} channels

In whole cell configuration BK_{Ca} channel currents were elicited from BK_{Ca} α cells by applying test pulses in 10mV increments from -100mV to +100mV from a holding potential of −100mV. **(a)** Averaged normalised G–V relationships were determined under control conditions (■) and during exposure of BKPr3 (30μM, △) from which V_{50} and slope factor were determined **(c)**. **(b)** Representative current traces obtained in the absence (control; top) and presence (bottom) of BKPr3 (30μM) from which time to peak current was measured and deactivation time constant determined from tail currents (at -100mV), **(c)**. Data presented as means±SEM, (n=6-9). Student's paired t-test, ***p<0.001 indicate significant differences from control.

(a)



(b)



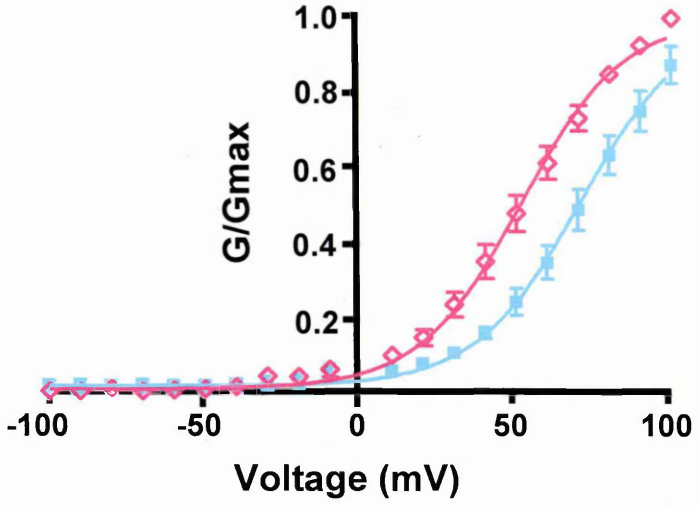
(c)

	V_{50} (mV)	Time to Peak Current (ms)	Deactivation Time (τ ms)
Control	83.6±2.8	13.6±2.5	3.3±0.5
BKPr4	56.0±2.3***	12.3±2.1	3.1±0.4

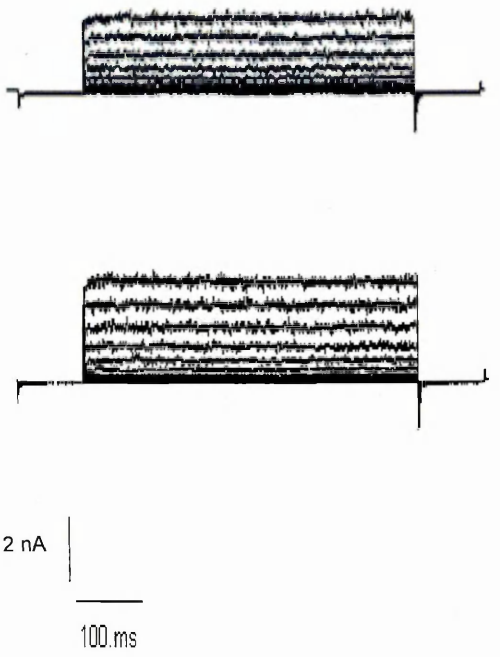
Figure 4.8: Stimulatory effect of BKPr4 on BK_{Ca} channels

In whole cell configuration BK_{Ca} channel currents were elicited from BK_{Ca} α cells by applying test pulses in 10mV increments from -100mV to +100mV from a holding potential of −100mV. **(a)** Averaged normalised G–V relationships were determined under control conditions (■) and during exposure of BKPr4 (30μM, (▽) from which V_{50} and slope factor were determined **(c)**. **(b)** Representative current traces obtained in the absence (control; top) and presence (bottom) of BKPr4 (30μM) from which time to peak current was measured and deactivation time constant determined from tail currents (at -100mV), **(c)**. Data presented as means±SEM, (n=6-9). Student's paired t-test, ***p<0.001 indicate significant differences from control.

(a)



(b)



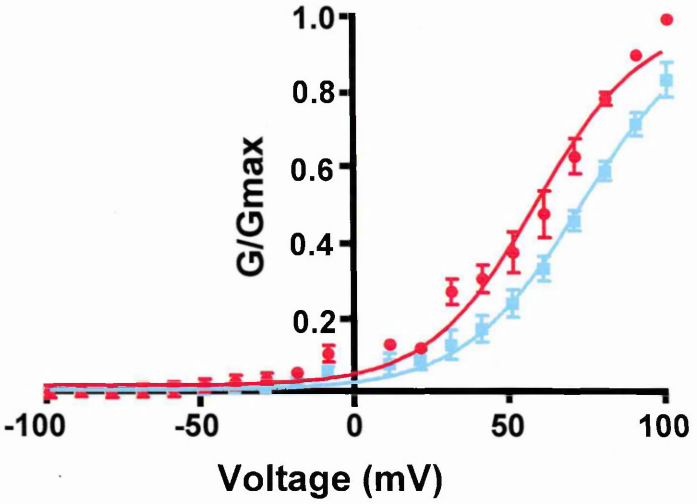
(c)

	V_{50} (mV)	Time to Peak current (ms)	Deactivation Time (τ ms)
Control	70.9±1.8	15.1±3.4	3.0±0.4
BKH1	51.6±1.8***	13.3±1.7	3.5±0.4

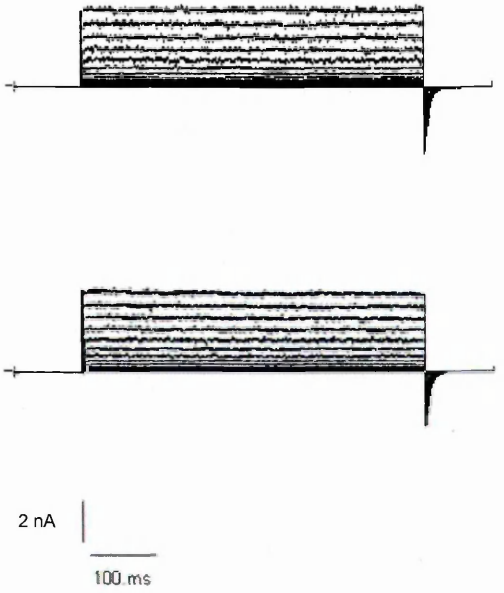
Figure 4.9: Stimulatory effect of BKH1 on BK_{Ca} channels

In whole cell configuration BK_{Ca} channel currents were elicited from BK_{Ca} α cells by applying test pulses in 10mV increments from -100mV to +100mV from a holding potential of −100mV. **(a)** Averaged normalised G–V relationships were determined under control conditions (■) and during exposure to BKH1 (30μM, ◇) from which V_{50} and slope factor were determined **(c)**. **(b)** Representative current traces obtained in the absence (control; top) and presence (bottom) of BKH1 (30μM) from which time to peak current was measured and deactivation time constant determined from tail currents (at -100mV), **(c)**. Data presented as means±SEM, (n=6-9). Student's paired t-test, ***p<0.001 indicate significant differences from control.

(a)



(b)



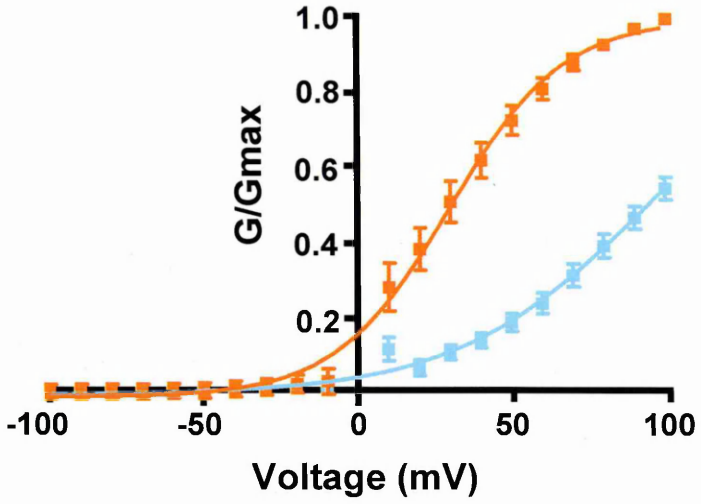
(c)

	V_{50} (mV)	Time to Peak Current (ms)	Deactivation Time (τ ms)
Control	72.5±1.7	14.6±3.6	3.6±1.6
BKMe1	57.7±2.9**	11.1±1.4	4.2±2.0

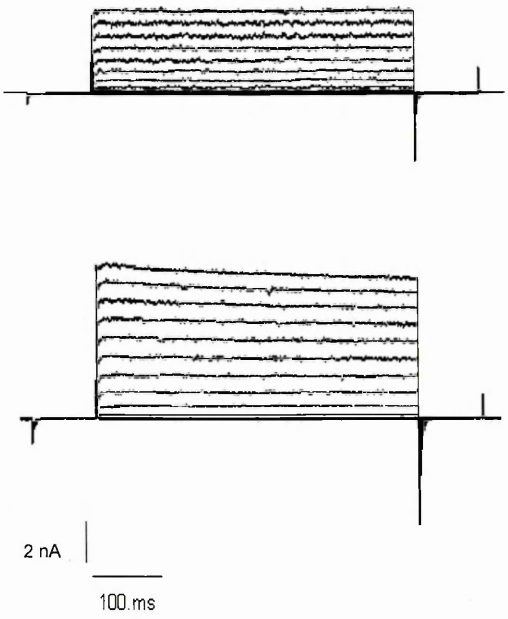
Figure 4.10: Stimulatory effect of BKMe1 on BK_{Ca} channels

In whole cell configuration BK_{Ca} channel currents were elicited from BK_{Ca} α cells by applying test pulses in 10mV increments from -100mV to +100mV from a holding potential of −100mV. **(a)** Averaged normalised G–V relationships were determined under control conditions (■) and during exposure of BKMe1 (30μM, (●) from which V_{50} and slope factor were determined (c). **(b)** Representative current traces obtained in the absence (control; top) and presence (bottom) of BKMe1 (30μM) from which time to peak current was measured and deactivation time constant determined from tail currents (at -100mV), **(c)**. Data presented as means±SEM, (n=6-9). Student's paired t-test, **p<0.01 indicate significant differences from control.

(a)



(b)



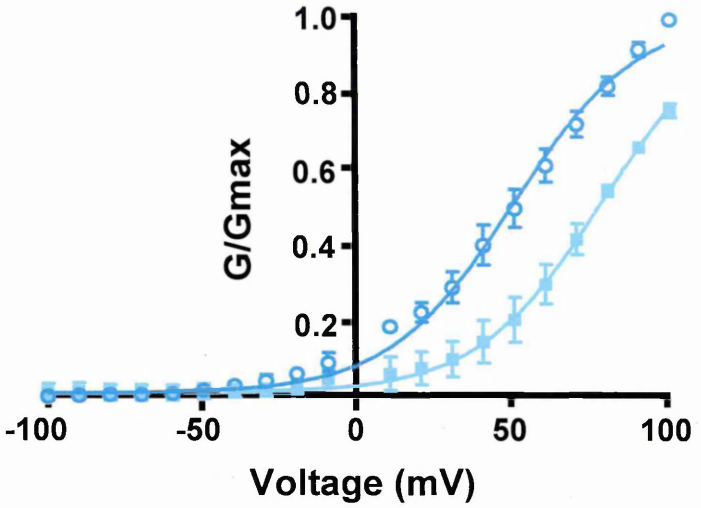
(c)

	V_{50} (mV)	Time to Peak Current (ms)	Deactivation Time (τ ms)
Control	93.9±1.2	11.1±2.5	3.5±0.7
BKOEt1	29.9±1.4***	10.9±1.0	3.1±0.5

Figure 4.11: Stimulatory effect of BKOEt1 on BK_{Ca} channels

In whole cell configuration BK_{Ca} channel currents were elicited from BK_{Ca} α cells by applying test pulses in 10mV increments from -100mV to +100mV from a holding potential of −100mV. **(a)** Averaged normalised G–V relationships were determined under control conditions (■) and during exposure of BKOEt1 (30μM, (■) from which V_{50} and slope factor were determined **(c)**. **(b)** Representative current traces obtained in the absence (control; top) and presence (bottom) of BKOEt1 (30μM) from which time to peak current was measured and deactivation time constant determined from tail currents (at -100mV), **(c)**. Data presented as means±SEM, (n=6-9). Student's paired t-test, ***p<0.001 indicate significant differences from control.

(a)



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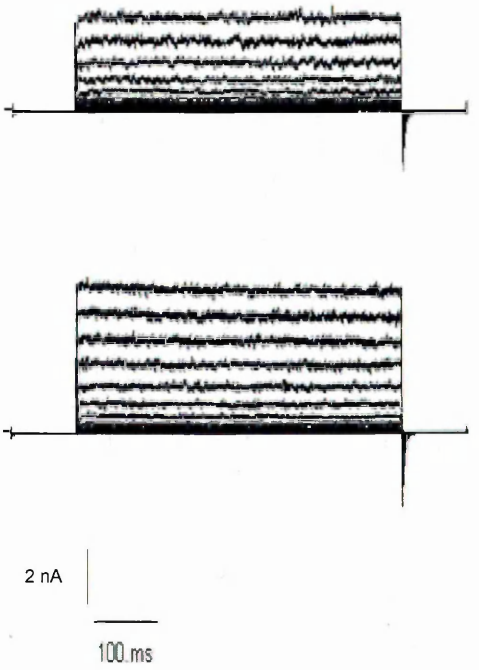


(c)

	V_{50} (mV)	Time to Peak Current (ms)	Deactivation Time (τ ms)
Control	77.7±1.7	14.9±5.6	6.2±2.3
BKVV	49.7±1.6***	12.9±2.9	7.5±2.8

Figure 4.12: Stimulatory effect of BKVV on BK_{Ca} channels

In whole cell configuration BK_{Ca} channel currents were elicited from BK_{Ca} α cells by applying test pulses in 10mV increments from -100mV to +100mV from a holding potential of −100mV. **(a)** Averaged normalised G–V relationships were determined under control conditions (■) and during exposure of BKVV (30μM, (○) from which V_{50} and slope factor were determined **(c)**. **(b)** Representative current traces obtained in the absence (control; top) and presence (bottom) of BKVV (30μM) from which time to peak current was measured and deactivation time constant determined from tail currents (at -100mV), **(c)**. Data presented as means±SEM, (n=6-9). Student's paired t-test, ***p<0.001 indicate significant differences from control.

4.4.4 Order of effectiveness of benzanilides

The rank order of efficacy for the benzanilides and NS1619 was assessed by the degree of displacement in the G-V curve represented by the change in V_{50} values from control for each compound examined (Figure 4.13).

The order of activation based on the degree in shift of V_{50} was:

NS1619 > BKOEt1 > BKPr3 / BKPr4 / BKVV > BKH1 > BKMe1 / BKPr2

The benzanilides (30μM), in non-saturating Ca^{2+} concentrations and in symmetrical K^+ produced a range of shifts (10-60mV) of V_{50} from control; that for NS1619 was 68mV. BKPr3 showed similar efficacy to BKPr4 but both of these compounds were significantly more effective than the structurally related BKPr2. However, of all the benzanilide compounds tested under these conditions BKOEt1 was the most effective and demonstrated a 2-fold greater shift in V_{50} relative to the next most effective benzanilide.

4.4.5 Selective antagonists

To examine whether current increases evoked by BKOEt1 were due to the activation of BK_{Ca} channels, experiments in the presence of selective channel blockers (IbTX or pax) were performed. Co-application of IbTX (0.1μM) failed to significantly ($p>0.05$, Student's paired t-test) block BKOEt1 stimulated increases in current (V_{50} control = 100.9±3.5mV; BKOEt1 = 34.5±5.6mV; + IbTX= 43.5±7.4mV) from BK_{Ca} α cells. However, co-incubation with pax (10μM) completely abolished all BK_{Ca} channel currents (Figure 4.14). Paxilline (10μM) also completely abolished the increases in current evoked by each of the other benzanilides tested (Data not shown).

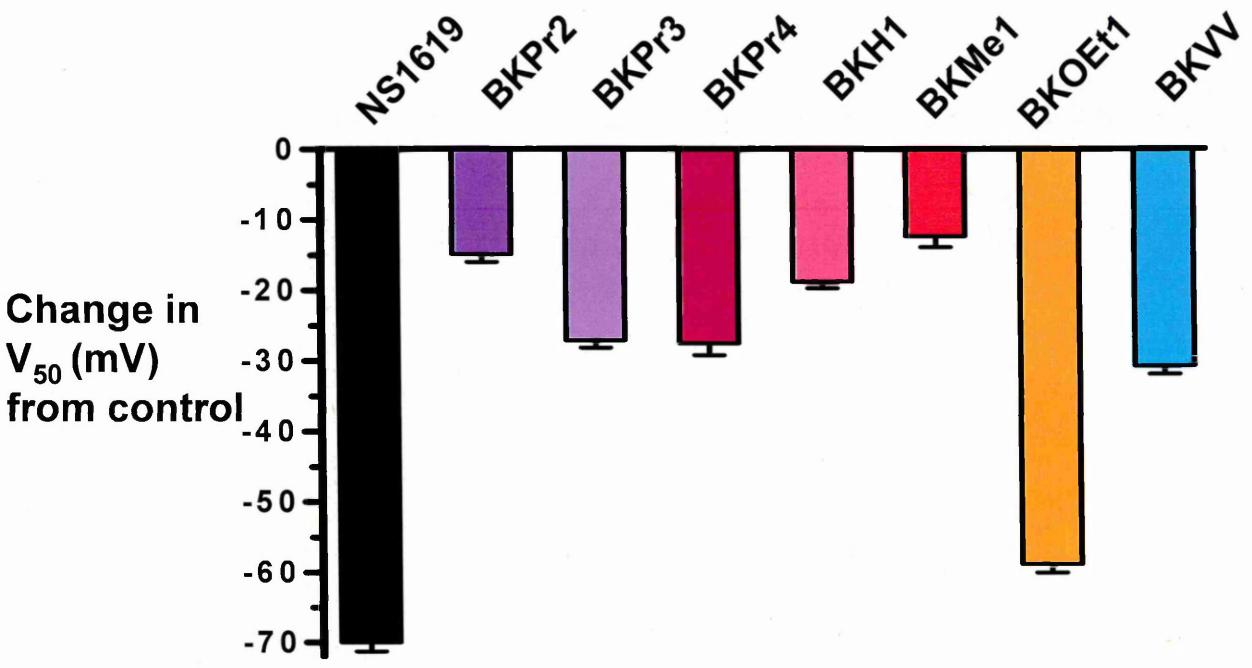
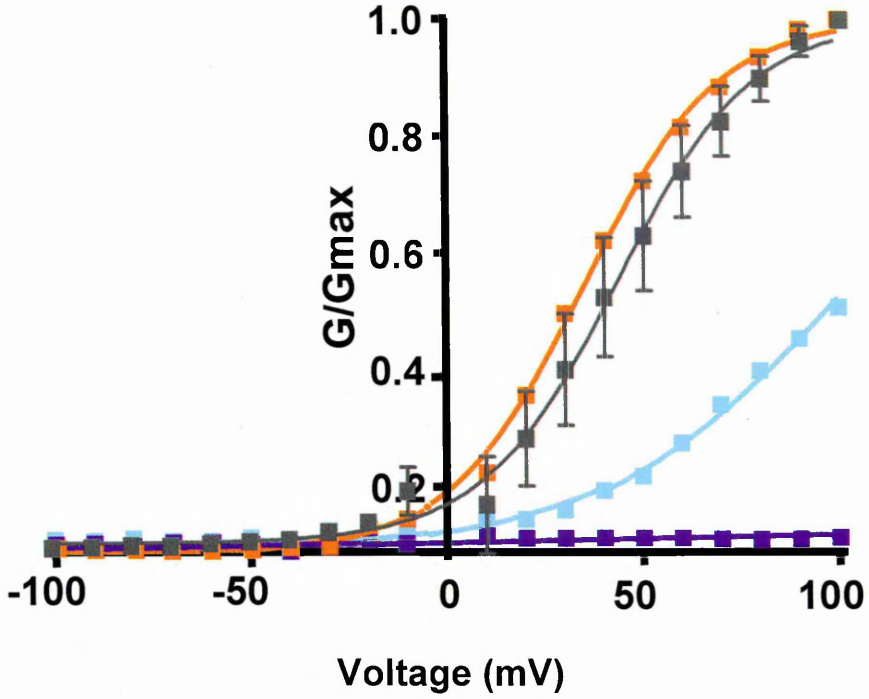


Figure 4.13: Effect of test compounds on V_{50} values relative to control

The changes in V_{50} values from control conditions as a consequence of compound (30μM) activation of BK_{Ca} channels. Negative values indicate a shift in G-V curve to more hyperpolarising voltages. Data was presented as Means±SEM (n=6-9).

(a)



(b)

	Control	BKOEt1 (30μM)	BKOEt1 + IbTX (0.1μM)	BKOEt1 + Pax (10μM)
V_{50} (mV)	100.9±3.5	34.5±5.6	43.5±7.4	n.d

Figure 4.14: The effect of IbTX or pax on blocking BKOEt1 mediated activation of BK_{Ca} channels

Whole cell BK_{Ca} channel currents were elicited from BK_{Ca} a cells by applying voltage steps in 10mV increments from -100 to +100mV from a holding potential of -100mV. **(a)** Normalised G-V relationships were determined under control conditions (■) and following the perfusion of BKOEt1 (30μM, ■) and then either co-application of BKOEt1 with either IbTX (0.1μM, ■) or Pax (10μM, ■). **(b)** The V_{50} values determined under different incubations as indicated. Data presented as means±SEM, (n=3). n.d not-determined.

4.5 Discussion

A series of novel benzanildies were examined for BK_{Ca} channel opener activity using whole cell patch clamp electrophysiology.

4.5.1 Benzanilides as BK_{Ca}CO's

All benzanilides evoked significant, paxilline sensitive, increases above control in BK_{Ca} channel current and increased with further depolarisation. This produced leftward shifts in the hyperpolarising direction of the G-V curve indicative of BK_{Ca} channel activation and similar to NS1619 the reference BK_{Ca}CO tested.

The benzanilides (30μM) were able to shift V_{50} from control in a range of 10 to 60mV. This shift was in the range of or greater than those achieved by other BK_{Ca} channel openers such as tamoxifen ~30 mV (Dick *et al* 2001), pimaric acid ~21mV shift (Imaizumi *et al* 2002), naringenin ~20mV shift (Saponara *et al* 2006) and KB130015 shift of ~40mV (Gessner *et al* 2007). Furthermore, a series of novel openers based on derivatives of hexa-hydro-dibenzazepinones have displayed shifts of between 5 and 25mV in V_{50} values (Tashmina *et al* 2006).

NS1619 produced a 68mV shift in V_{50} from control, similar to that observed previously (~70mV) in bovine myocytes (Olesen *et al* 1994b) and for *hSlo* channels (~42mV) expressed in HEK293 cells (Gessner *et al* 2007). However, the benzanilide series of compounds were not as potent as DHS-1 which still remains one of the most potent BK_{Ca} channel opener with a ~120mV shift (Giangiacomo *et al* 1998), although this was examined in isolated bovine aortic smooth muscle cells which express the BK_{Ca} channel β1-subunit. Therefore, the benzanildies demonstrate effectiveness in a similar range to those observed for a diverse range of synthetic compounds.

4.5.2 Activation and deactivation properties

Benzanilide compounds did not effect the time for BK_{Ca} channels to reach peak current, conversely, NS1619 increased the time to reach peak current.

The benzanilides did not affect the deactivation time of the channel determined from instantaneous tail current following an inactivating pulse (at -100mV). From analysis of tail current traces it can be observed that for NS1619 there is an expansion of width of the tail current. However, this difference is not observed with benzanilides.

This is an interesting finding as a number of other BK_{Ca} channel openers have shown to increase the deactivation time constant. For example mallotoxin/rottlerin (0.5μM) increased the deactivation time constant to ~2.0ms from a control value of ~0.5ms (in 0.02μM Ca^{2+}) determined by applying a -80mV inactivating pulse following either a +50mV or +200mV activating voltage pulse from whole cell instantaneous tail currents recorded in HEK293 cells expressing mSlo (Zakharov *et al* 2005).

In addition, NS1608 in has been shown to increase deactivation time from ~2ms under control conditions (0.5μM Ca^{2+}) to ~5.0ms (5.0μM) as determined from instantaneous tail currents generated from a -100mV inactivating pulse (Strobaek *et al* 1996). In addition, KB130015 (100μM) has shown to increase deactivation time constant from ~0.5ms to 1.5ms in HEK293 cells expressing hSlo in response to a -150mV deactivating potential (Gessner *et al* 2007).

From analysis of current-traces it can be determined that benzanildies did not significantly increase the maximal level of instantaneous current relative to control suggesting that the compounds had no affect on conductance or the number of channels that were open and that no more channels could be opened.

Therefore, benzanilides did not effect the activation time or deactivation consequently further electrophysiological investigation would be of interest to further elucidate the BK_{Ca}CO properties of benzanilides.

4.5.3 Comparison of order of effectiveness of benzanilides obtained in whole cell vs Rb^+ efflux

The following rank order of potency was determined for the compounds and NS1619 from the degree of shift in V_{50} from control:

NS1619 > BKOEt1 > BKPr3 / BKPr4 / BKVV > BKH1 > BKMe1 / BKPr2

That determined by Rb^+ efflux screening was ($EC_{40\%}$):

NS1619 / BKOEt1 / BKPr3 / BKPr4 / BKVV > BKH1 / BKMe1 / BKPr2

A good agreement was observed between the order of potency determined for benzanilides in BK_{Ca} α-cells using $EC_{40\%}$ values obtained from Rb^+ efflux studies and that based on the V_{50} values determined using whole cell electrophysiology. Compounds BKH1, BKMe1 and BKPr2 with both methods were the least potent compounds. However, electrophysiology seemed to be able to discriminate between compounds with higher potency with more resolving power than in Rb^+ efflux.

Comparing the rank order of benzanilide activation of BK_{Ca} channels based on calculated V_{50} and $EC_{40\%}$ for the two methods tested may not be reliable; this is because $EC_{40\%}$ represents potency calculated by testing a range of concentrations where as V_{50} represents the effect on voltage at a single concentration of compound tested. However, there is good agreement between the two methods.

Discrepancies between potency profiles based on calculated values of IC_{50} or EC_{50} from electrophysiology and those determined from Rb^+ efflux studies (both non-radioactive and radioactive efflux) have been previously reported (Tang *et al* 2001; Wang *et al* 2003; Rezazadeh *et al* 2004). For example, Tang *et al* (2001) have shown that in the assessment of hERG channel blockers a 5-20 fold higher potency was observed with Rb^+ efflux than when measured with electrophysiology, although a similar rank order in potency was achieved. This

was further supported by the work of Rezazadeh *et al* (2004) who observed similar discrepancies in potency values for a series of hERG channel blockers and that the non-radioactive Rb^+ efflux assay was less able to discriminate between compounds with highest potency as well as electrophysiology, although a similar rank order of agreement was determined.

Furthermore, Scott *et al* (2003) demonstrated a similar rank order in potency between electrophysiology and Rb^+ efflux for inhibitors of $K_v7.2$ (KCNQ2) channels but differences in potency values. In addition, Wang *et al* (2003) showed that EC_{50} values determined by Rb^+ efflux and electrophysiology for KCNQ/M channels showed positive correlation; although potency values were approx 2-10 times higher in Rb^+ efflux than in electrophysiology.

In an attempt to understand why these differences occur Rezazadeh *et al* (2004) examined the potency of hERG channel blockers determined in patch clamp using either Rb^+ or K^+ as the conducting ion. The substitution of K^+ for Rb^+ resulted in a ~10-fold reduction in blocker potency. They hypothesise that this discrepancy could be either due to Rb^+ conduction through the pore occurring via a different mechanism to that of K^+ and this interferes with the site of blocker binding or that conditioning of the drug binding site by Rb^+ could occur; but the potential mechanisms of this are not fully understood (Rezazadeh *et al* 2004).

Whether this explanation can be applied to compounds that act as K^+ channel openers has not been addressed. BK_{Ca} channel openers discovered to date have not shown to affect conduction of K^+ through the channel pore. K^+ channel blockers main mechanism of action is to occlude ion passage through the channel pore, therefore whether BK_{Ca}CO's could themselves affect Rb^+ conduction through the pore via competition between the two remains unknown and therefore probably can not account for discrepancies in rank order of effectiveness of BK_{Ca}CO.

Another difference between the two assays that may contribute to potency differences is that in electrophysiology the voltage across the membrane can be controlled and that in Rb^+ efflux the membrane potential across the cell is dependent upon the K^+ ion concentration and basal activity of the cell

(contribution of other background channels). Therefore, discrepancies observed may be due to incomplete control of the membrane voltage in the Rb^+ efflux assay.

Potency differences observed could be due to the difference in conducting ion, sensitivity of the Rb^+ efflux assay itself or via incomplete control of membrane potential. Further examination would be required to elucidate these differences, maybe through determining the resting membrane potential of the cell or by assessing the effect of BK_{Ca}CO's in the presence of different depolarising strength (20-50mM K^+) KCl buffers. Further the effect of benzanilides on BK_{Ca} channel activity as measured using whole cell electrophysiology could be assessed again but by substitution of K^+ conducting ion for Rb^+ and comparing the rank order of efficacy achieved with the two conducting ions.

4.5.4 Determination of I_k currents in BK_{Ca} α cells

In BK_{Ca} α cells, currents elicited under control conditions were completely blocked by application of TEA to the external side of the channel therefore the current evoked is mediated through K^+ channels. Application of IbTX failed to completely block all I_K channel current at concentrations 10-50 times greater (0.1-0.5μM) than the reported IC_{50} of 1-10nM (Galvez *et al* 1990; Kaczorowski *et al* 1996; Gutman *et al* 2003) from BK_{Ca} α cells. However, when the K^+ concentration on the outside of the cell was reduced (5.4mM) IbTX blocked BK_{Ca} channel currents more effectively. However, complete block was not achieved at concentrations several times greater than the reported IC_{50} for IbTX. The effects of IbTX were reversible following extensive washout. A potential explanation for this discrepancy can be sought from the initial work by Giangiacomo *et al* (1992) and Candia *et al* (1992) on the mechanism of IbTX action.

IbTX blocks BK_{Ca} channels in a reversible bimolecular reaction and can only block from the external side of the channel through its association with the external mouth of BK_{Ca} channels. Channel block is characterised, at the single channel level, by long lived non-conducting states separating bursts of activity. From analysis of the kinetics of IbTX block it has been observed that the positively charged IbTX molecule associates with negatively charged residues

of the mouth of the channel and that this electrostatic attraction increases the apparent toxin concentration near the channel pore. Furthermore, changes in external K^+ ion concentration from 12.5 to 310mM resulted in a greater than 10-fold reduction in the association constant of IbTX. The increase in K^+ ion concentration affected local charges on the external pore of the channel that consequently affects the binding of an IbTX molecule (Giangiacomo *et al* 1992; Candia *et al* 1992). Therefore, the decreased potency could be due to application of IbTX in a high extracellular K^+ solution and therefore the increased potency observed in asymmetrical (low external K^+) could be due to lowering of extracellular potassium concentration.

Nevertheless, in asymmetrical K^+ even a high concentration of IbTX (0.5μM) was unable to fully block BK_{Ca} channel activated currents; this could indicate that BK_{Ca} channel is not the sole channel conducting I_K currents from these cells. This is important as mRNA and protein has been detected in native HEK293 cells for a number of other potassium channels such as members of the K_v channel family (K_v1.1-1.6, 3.1, 3.3, 3.5 and 4.1) and SK channels (Thomas and Smart 2005). Furthermore, unknown background potassium and chloride ionic currents have been detected in wild type HEK293 cells (Zhu *et al* 1998).

The IC_{50} for IbTX has generally been calculated from experiments on primary isolated cells from tissue that have a lower BK_{Ca} channel expression, in that the cloned cell lines used here BK_{Ca} channel expression is regulated by a strong promoter producing constitutive expression. Consequently the concentration of IbTX used could have not have been enough to physically block all the channels activated under control conditions and following addition of benzanilides. However, this depends upon the stoichometry of IbTX binding to the channel and the total number of channels in the cell that need to be activated to evoke a full response.

To address whether the inability of IbTX to block BK_{Ca} channels was due to its physiochemical properties or that under control conditions other K^+ channels were being activated pax was used. Pax inhibited I_k currents in a concentration dependent manner with complete block of I_k current at 10μM and prevented BKOEt1 from activating the channel.

4.5.5 BKOEt1 as a BK_{Ca} channel opener

From the series of benzanilides examined BKOEt1 was identified as the most potent BK_{Ca} channel opener. To determine that the effects of BKOEt1 were specific to BK_{Ca} channels the effect of the blockers IbTX and pax were studied. Co-application of IbTX failed to antagonise BKOEt1 mediated increases in BK_{Ca} current where as co-application of pax abolished BK_{Ca} channel activation. A previous study has shown that supramaximal concentrations of IbTX have not been able to completely antagonise the response of NS1619, phloretin and niflumic acid on BK_{Ca} channels expressed in Oocytes (Gribkoff *et al* 1996). This observation is in line with the inability of IbTX to block control BK_{Ca} channel currents from BK_{Ca} α cells. Pax was able to prevent BK_{Ca} channel activation at 10μM, this compares with previous observations in that where IbTX has failed to block NS1619 responses in cloned cell lines pax was more successful (Gribkoff *et al* 1996). Gribkoff *et al* (1996) suggested that pax should be the preferred blocker of choice for complete BK_{Ca} channel antagonism (Gribkoff *et al* 1996).

4.6 Conclusions

All compounds tested activated BK_{Ca} channels in BK_{Ca} α cells producing different levels of channel activation. All benzanilides tested did not affect the kinetics of activation or deactivation. Furthermore, the whole cell activating profile of benzanilides showed good agreement with that obtained using the Rb^+ efflux assay. Identification of the mechanism of action of these compounds as BK_{Ca} channel opener may lead to clues into the structure activity relationship between the benzanilide series structure and there differential activating profiles. Using both the electrophysiology and Rb^+ efflux techniques BKOEt1 was identified as a novel and potent BK_{Ca} channel opener.

Chapter 5

Characterisation of BKOEt1 as a novel BK_{Ca} channel opener

5.1 Introduction

The most effective novel benzanilide identified as a BK_{Ca} channel opener using Rb^+ efflux assay and whole cell electrophysiological screen was BKOEt1.

Further characterisation of the $BK_{Ca}CO$ properties of BKOEt1 were performed using whole cell and single channel electrophysiological recording. In particular potency, open pore probability and effects on single channel conductance were determined. Furthermore, the Ca^{2+} dependence of activation and the effect of BKOEt1 on intracellular Ca^{2+} was assessed with the latter determined using a Fura-2/AM fluorescent intracellular calcium assay.

The non-radioactive Rb^+ efflux assay was used to examine the Rb^+ efflux activation profile of BKOEt1 in rat bladder myocytes and then to examine whether the Rb^+ efflux observed was specific to BK_{Ca} channels by determining the ability of BKOEt1 to activate Rb^+ efflux in the presence of different K^+ channel blockers

The properties of BKOEt1 were compared to other known BK_{Ca} channel openers. Analysis of this compound could offer new information about the action of this series of benzanilides on activating BK_{Ca} channels. Further this would contribute novel electrophysiological data pertaining to compounds based on the generalised pharmacophore pattern proposed for $BK_{Ca}CO$'s.

5.2 Chapter Aims

Characterise the BK_{Ca} channel opener properties of BKOEt1 by:

- Further characterising the whole cell activating properties of BKOEt1 in BK_{Ca} α cells.

- Examine the effect of BKOEt1 on single channel electrophysiological properties of BK_{Ca} channels.

- Assess the calcium dependence of activation and the effect of BKOEt1 on intracellular Ca^{2+}

- Investigate the effect of BKOEt1 on Rb^{+} efflux from K^{+} channels in rat bladder myocytes.

5.3 Methods

5.3.1 Materials

Cell culture and isolation

Sigma Aldrich, Gillingham, U.K: Collangenase, pluronic acid, probencid protease type XXIV, trypan blue and trypsin inhibitor

Invitrogen (GIBCO), Paisley, U.K: Penicillin G, Streptomyocin

Fisher Scientific UK, Loughborough, U.K: Nunc LabTek 8-well chamber slides

Ion channel modulators and pharmacological agents

Sigma Aldrich, Gillingham, U.K: Apamin, Fura-2AM

Axxora Ltd, Nottingham, U.K: Margatoxin (marg)

Tocris Biosciences, Bristol, U.K: Forskolin, TRAM-34, ryanodine

Immunocytochemistry reagents

Sigma Aldrich, Gillingham, U.K: Mouse anti-smooth muscle α–actin mAb, bovine serum albumin (BSA)

Invitrogen (Molecular Probes), Paisley, U.K: Alexa Fluor 568 anti-mouse IgG and Alexa Fluor 568 donkey anti-mouse IgG

Santa Cruz, California, USA: Goat anti-vimentin C-20 polyclonal antibody and Mouse anti-cytokeratin 18 monoclonal antibody

Vecta laboratories, Peterborough, U.K: 4'-6-Diamidino-2-phenylindole (DAPI)

5.3.2 HEK293 cells

A different recombinant clone of BK_{Ca} α cells was used to that in chapter 2 and 3. This had the human BK_{Ca} channel α-subunit but with an additional 132bp segment between the S_0 and S_1 intracellular loop. This splice variant behaves biophysically as the zero variant and the rest of the channel has the same sequence and major structural features of the zero variant (Korovkina *et al* 2001). The variant was used because it displayed less BK_{Ca} channel current density in patches and thus enabled more reliable recordings of single channel BK_{Ca} currents. Some studies have reported that BK_{Ca} channels containing the 132bp region can lead to a decrease in cell surface expression of the channel (Korovkina *et al* 2006). These cells termed BK_{Ca} α_{+132} cells were continuously cultured and plated as described previously for electrophysiology (Chapter 3, section 3.2).

5.3.3 Bladder myocytes

5.3.3.1 Myocyte dissociation

Myocytes were dissociated from whole rat bladders using partial enzymatic digestion followed by mechanical dissociation. Male Wistar rats (200-300g) were killed by cervical dislocation following stunning. The abdominal cavity was opened by a midline incision exposing the internal organs, the entire bladder was removed and transferred to a dissecting dish containing ice cold calcium free Hanks buffered saline (HBSS) (mM: KCl 5.3, KH_2PO_4 0.44, $NaHCO_3$ 4.17, NaCl 137.9, Na_2HPO_4 0.338, Glucose 5.56). The prostate and serosal connective tissue was removed and a longitudinal incision from the bladder dome to the base was made, the bladder was unfolded and the urothelium layer removed by scraping with a scalpel. The tissue was cut into approx $5mm^2$ pieces and transferred to a Petri dish containing HBSS (10mls) and placed in a at 4°C, for 20-30mins.

Tissue was transferred to a Nunc dish containing digestion cocktail mix. This was made by diluting a pre-prepared frozen stock in Ca^{2+} free HBSS (1ml) to yield a solution containing a final concentration of (mg ml^{-1}) collagenase type IA 3.0, protease type XXIV 0.2, bovine serum albumin (BSA) 2.0, trypsin inhibitor 2.0, the solution was sterilised using an acrodisc (0.2μM) filter. Tissue was

incubated with the digestion cocktail mix for 15 min at 37°C in a tissue culture incubator, then the tissue pieces were transferred to a Nunc dish containing 1ml of filtered Ca^{2+} free HBSS and a sterilised magnetic flea (10mm), cells were gently agitated for 10 min using a magnetic plate stirrer. Following this dissociated cells were collected and transferred to a sterile eppendorf tube in preparation for cell culture and HBSS (1ml) was added to the remaining cells and collected following agitation for another 10-minutes.

5.3.3.2 Myocyte cell culture

The cell culture technique and equipment used is that as described in chapter 2, section 3.3. Dissociated cell suspensions of myocytes were transferred to a T75 cell culture flask containing DMEM (20mls) supplemented with FCS (10%), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}). Cells were grown and passaged for two generations (P_2), (~2 weeks growth in total); cells were passaged as before, however, the trypsinisation was modified compared to that described for HEK293 cells to include a longer incubation (5-min) and in a greater volume of Trypsin-EDTA (5mls) due to the adherent strength of myocytes compared to HEK293 cells.

5.3.3.3 Cell preparation for Rb^+ efflux assay

Cells were prepared as before (Chapter 2, section 3.3) but seeded at an approximate density of 5000 cells/well (200μl of media) of a 96-well plate from which two plates were prepared from one confluent flask of bladder myocytes.

5.3.4 Immunocytochemistry

Immunocytochemistry (ICC) was used to characterise the cell types that were cultured following dissociation from the bladder, antibody (Ab) for markers used include smooth muscle α-actin (for myocytes), vimentin (mesenchymal cells such as fibroblasts and connective tissue) and cytokeratin (epithelial cell marker).

Antigen	1° Ab	2° Ab
Smooth muscle α-actin	Mouse anti-smooth muscle α–actin mAb (Sigma-Aldrich, Cat No A5228)	Alexa Fluor 568 anti-mouse IgG (H + L, 2mg ml^{-1}) (Molecular Probes, Invitrogen Cat No A11061)
Vimentin (C-20)	Goat anti-Vimentin C-20 polyclonal Ab, (Santa Cruz Biotechnology Cat No sc-7557)	Alexa Fluor 488 donkey anti-goat IgG (H + L, 2mg ml^{-1}) (Molecular Probes, Invitrogen Cat No A11055)
Cytokeratin (C-18)	Mouse anti-cytokeratin 18 mAb (Santa Cruz Biotechnology Cat No sc-32329)	Alexa Fluor 568 donkey anti-mouse IgG (H + L, 2mg ml^{-1}) (Molecular Probes, Invitrogen Cat No A11055)

Table 5.1: Primary and secondary antibodies used in ICC

For ICC myocytes were seeded into 8 well chamber slides at a density of 5000 cells/well in DMEM (200μl) and grown for 48-hours. Following this media was discarded and the cells washed once with ice cold PBS (200μl). Cells were fixed in ice cold methanol (100%, 400μl) that was added to each chamber and the slides were transferred to -20°C for 15 mins, the methanol was then decanted and cells washed once with PBS and cells left to air dry at room temperature. Cells were then blocked (0.1% BSA in PBS) for 30 min cells prior to incubation with 1^0 Ab (1:50 and 1:200 dilution) for 18 hours at 4^0C in a humidified chamber. Cells were then washed (3 times for 5 min in PBS) prior to incubation with 2^0 Ab; this was prepared by dilution (1:200) in a solution of 1% BSA in PBS of which 200μl was added to each well. This was left to incubate at 37°C for 1 hour in the dark followed by washes (3 times, 5 mins) with PBS. Incubation with equal volume of PBS in place of 1^0 Ab was used as negative controls.

Cover slips were mounted with Vecta-shield mounting media containing 4'-6-diamidino-2-phenylindole (DAPI) that forms fluorescent complexes with dsDNA enabling visualisation of the cell nucleus. Images were obtained using an Olympus BX60 fluorescent microscope using a Cool Snap Pro colour digital camera (Media Cybernetics, Silver Spring, USA) with LabWorks image acquisition and analysis software.

5.3.5 Rb^+ efflux assay

Rat bladder myocytes grown to confluence in 96-well plates were prepared for Rb^+ efflux experiments as described previously. The methodology was used as that described in Chapter 2, section 3.4.

5.3.6 Whole cell electrophysiology

The whole cell configuration and experimental protocol used was as described in Chapter 4, section 3.3.

5.3.7 Single channel recording

Single channel currents were recorded from excised inside-out patches of BK_{Ca} α_{+132} cells using standard patch-clamp recording procedures (Hamill *et al* 1981). Pipettes were fabricated (Chapter 4, section 3.3.1) for inside-out excised patches pipettes and were fire polished to a final resistance of 8-12MΩ. Pipette and extracellular solutions were prepared as previously (Chapter 4, section 3.3.2) described (symmetrical 140mM potassium) with the pipette solution containing 0.3μM Ca^{2+} and bath solutions containing a range of Ca^{2+} (0.1-10μM). Excised patches with seals within a range of 2-60GΩ were used, not all patches obtained demonstrated channel opening and current activity. In these instances rapidly cycling the membrane voltage between positive and negative potentials on occasion stimulated channel opening. However, air exposure of the pipette was required in those instances when cycling between membrane potentials failed to stimulate activity.

5.3.7.1 Data recording and acquisition

Currents were recorded in voltage clamp mode using an EPC-10 patch-clamp amplifier via an IPTC16 digital interface (HEKA, Lambrecht, Germany). Current signals, after compensation for capacitance, series resistance and liquid junction potential, were low-pass filtered at 2.9 KHz using a 4-pole Bessel filter and sampled at 10 KHz and 1 KHz before being stored on the hard disk drive of the computer. Single channel data was acquired from patches with <8 channels by measuring the current response during continuous recordings (for at least 60 seconds) to varying membrane potentials (-30 to + 100mV) as specified in the figure legends before and after the application of differing drug treatments.

5.3.7.2 Electrophysiology data analysis

Open pore probability

Data was analysed offline using TACFit software (Bruxton, Seattle, USA). Current threshold for event detection was set to 0.5 times the amplitude. From which all-points current amplitude histograms were compiled. Current levels were identified by peaks in the histograms to which Normal (Gaussian) distribution curves were fitted. Open pore probability (P_o) is determined as the area under the amplitude histogram for peaks representing 'open' channels divided by the total area. The relationship between membrane potential and P_o was fitted using the Boltzmann equation (Lee et al 1995a):

$$NP_o = P\max/(1 + \exp[(V_{50} - V_m)/S])$$

Where NP_o, V_{50}, V_m and S represent normalised open pore probability, voltage required for half maximal activation, membrane potential and slope factor respectively. P_{max} is the maximum P_o determined for a particular concentration (Ca^{2+} or BKOEt1) tested across all voltages tested.

Voltage sensitivity

The effect of BKOEt1 on voltage sensitivity of the channel was assessed by determining the slope of ln [P_0/1-P_0] vs membrane voltage. From which the *e*-fold shift in P_o per mV change in voltage was determined (Lee *et al* 1995a; Dichiara and Reinhart 1995; Khan *et al* 1998) in the absence (control; 0.3μM Ca^{2+}) and presence of BKOEt1 (3-100μM).

The effect of BKOEt1 on V_{50} was assessed by determining the slope of V_{50} vs ln [BKOEt1 μM]. The change in V_{50} per *e*-fold increase in concentration was determined (Lee *et al* 1995a) for BKOEt1 when applied to either the extracellular aspect of the channel in whole cell recording or to the intracellular aspect of excised inside-out patches.

Single channel conductance

Single channel conductance was calculated from the slope of the function G = I/V_m. where G is conductance, V_m is membrane potential and I is the current elicited from the opening of single ion channel at that particular membrane potential.

Potency and co-operativity

The EC_{50} (concentration equal to half maximal effect) was determined using the following modified Hill function (Gribkoff *et al* 1996; Gessner *et al* 2007):

$$\Delta V_{50} = \text{Bottom} + (\text{Top} - \text{Bottom})/1 + 10^{[(\log EC_{50} - X)*\text{HillSlope})]}$$

Where X is the concentration of BKOEt1 and ΔV_{50} is the shift in V_{50} from control.

5.3.8 Intracellular Fura-2 calcium assay

Fura-2, based on the chemistry of the Ca^{2+} chelator EGTA, has been used in numerous applications as a fluorescent indicator of Ca^{2+} (Malgaroli *et al* 1987). The acetoxymethyl ester of Fura-2 (Fura-2/AM) is a cell permeable molecule that once cleaved by intracellular esterases will fluoresce following excitation, when chelated with free intracellular Ca^{2+} (Malgaroli *et al* 1987). Briefly BK_{Ca} α_{+132} cells were loaded with Fura-2/AM in the presence of pluoronic acid to help disperse and solubilise the dye; cells are washed and left for a period of time to allow esterase cleavage of the dye. Probencid is included in the buffers to block dye leakage out of the cell through non-specific anion pumps. Cells were then exposed to drug treatment and subsequent fluorescence is measured over time.

Cell culture media was removed from wells and cells were then washed (1 x PBS, 200µl) before being loaded (200µl/well) with Fura2/AM (3µM) prepared in Krebs buffer (mM: NaCl 118.4, KCl 4.7, $NaHCO_3$ 24.9, D-Glucose 11.7, $MgSO_4$ 1.15, KH_2PO_4 1.15, $CaCl_2$ 1.9, pH 7.2) containing pluoronic acid (0.01%) in a 37°C incubator in the dark for 40 minutes. For depolarisation conditions a high

K^+ Krebs buffer was prepared containing 50mM KCl isosmotically adjusted by decreasing the NaCl concentration (68.4mM). Cells were then washed twice with Krebs buffer (200μl) containing probencid (2.5mM) and a 150μl volume of Krebs containing probencid was added to the wells. Fluorescence readings for each well were then recorded using a Wallac Victor^2 1420 multi-label counter (PerkinElmer Ltd, Turku, Finland) fitted with the appropriate filters. Following addition of 150μl of Krebs buffer to each well the fluorescence was measured and the plate was placed in a 37°C incubator in the dark for 30 min to allow cleavage of the ester. Treatments were prepared during this time at a concentration 4x greater than the final concentration required in 50μl aliquots. These were added (using a multi-channel pipette) to the wells (final volume 200μl) of the micro-titre plate to yield the correct concentration. A fluorescence reading was taken prior and at subsequent times (1, 5, 10 and 20 min) after the addition of the drug treatments.

5.3.9 Data analysis

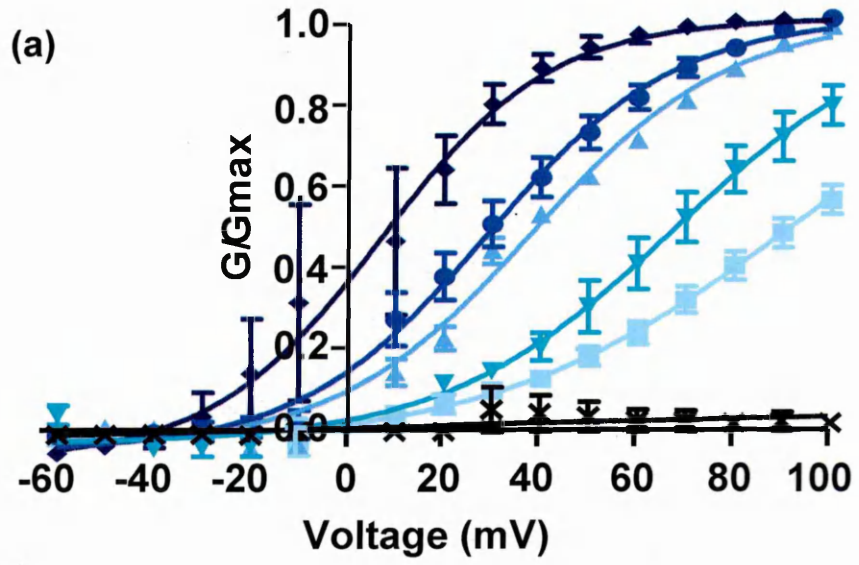
All data was fitted using GraphPad Prism 4 software and are presented as mean (±SEM) for n determinations. Current traces were exported from HEKA PulseFit or TacFit to use as visual representations of the data.

Data was analysed using either Student's un-paired or paired t-test or for multiple comparisons one-way ANOVA followed by Dunnett's post test analysis or Bonferroni's multiple comparison test (Wallenstein *et al* 1980). The accepted level of significance was $p \leq 0.05$.

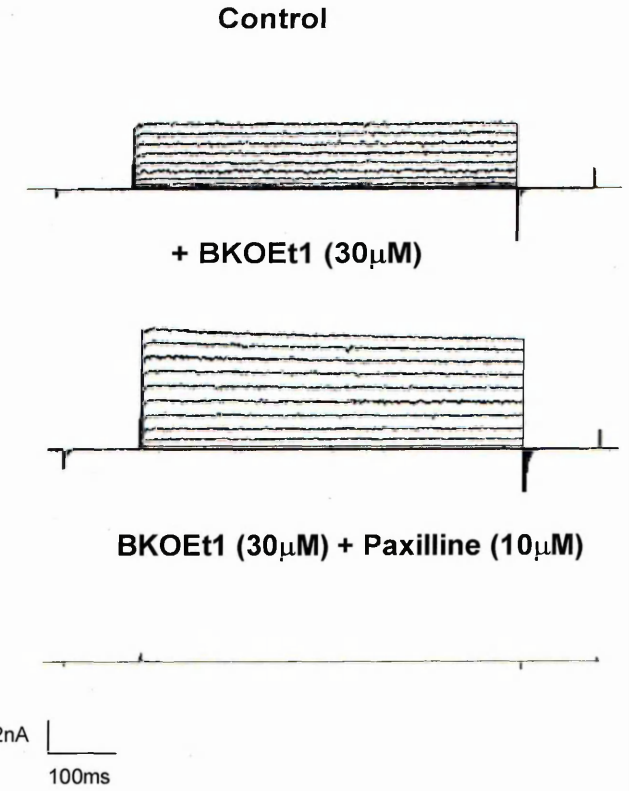
5.4 Results

5.4.1 Effect of BKOEt1 on whole cell BK_{Ca} channel currents

The effect of BKOEt1 on whole cell BK_{Ca} channel currents tested over an extended concentration range was examined. Patches were obtained in symmetrical (140mM) K^+ with 0.3μM Ca^{2+} in the pipette and bath solutions. Cell exposure to BKOEt1 (3-100μM) led to increases in BK_{Ca} channel current within 1-minute of application (Figure 5.1b). BK_{Ca} channel activation was assessed by comparing the voltage required to produce 50% activation (V_{50}) as determined from normalised averaged conductance-voltage curves generated for each concentration that was tested (Figure 5.1a). Increasing concentrations of extracellular BKOEt1 shifted V_{50} from a control value of 90.1±1.0mV to 77.5±0.7mV, 43.9±6.3 mV, 27.7±1.4 mV and 6.8±3.7 mV for 3, 10, 30 and 100μM respectively to more hyperpolarised potentials (Figure 5.1c). This resulted in a 16.99±4.50mV change in V_{50} per *e*-fold increase in extracellular BKOEt1 concentration (Figure 5.5). From the degree of shift in V_{50} from control an EC_{50} of 9.79±0.78μM and a Hill slope of 1.26±0.38μM was determined (Figure 5.3). The effect of BKOEt1 (30μM) was sensitive to block upon co-application with paxilline (10μM) which blocked all BK_{Ca} channel currents (Figure 5.1c).



(b)



(c)

BKOEt1 (μM)	0 ■	3 ▼	10 ▲	30 ●	100 ◆
V_{50} (mV)	90.1±2.0	77.5±0.7***	43.9±6.3***	27.7±1.4***	6.8±3.7***

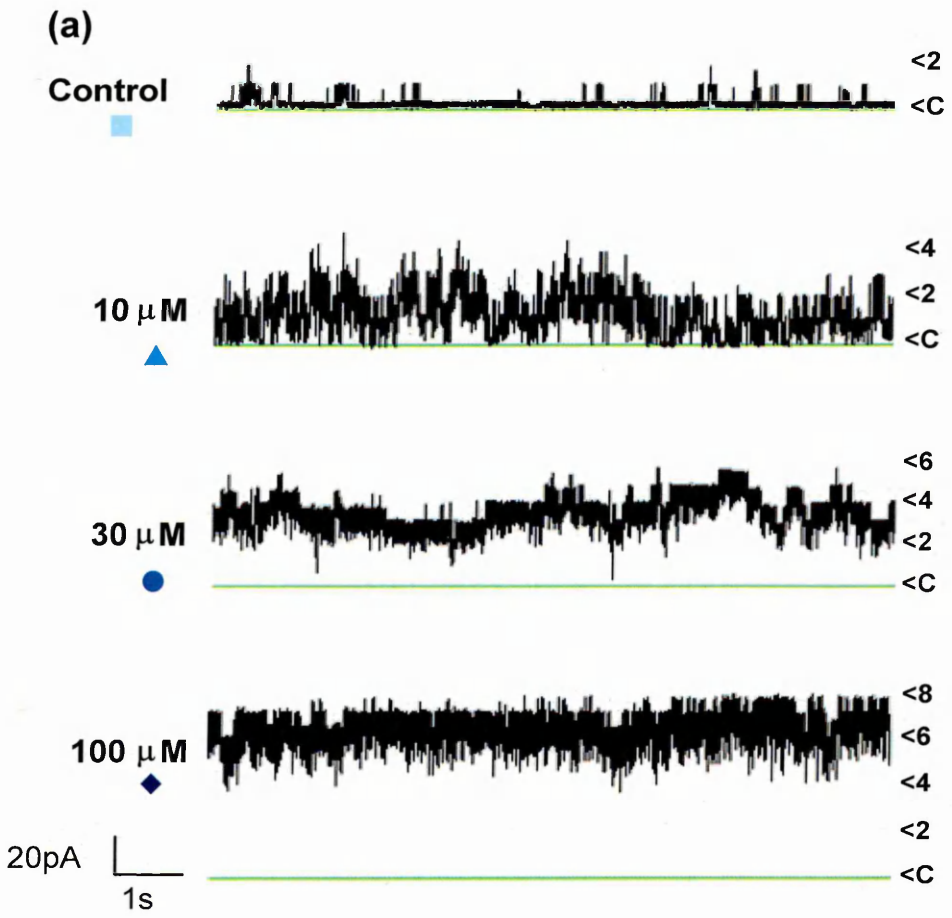
Figure 5.1: The effect of BKOEt1 on whole cell BK_{Ca} channel currents

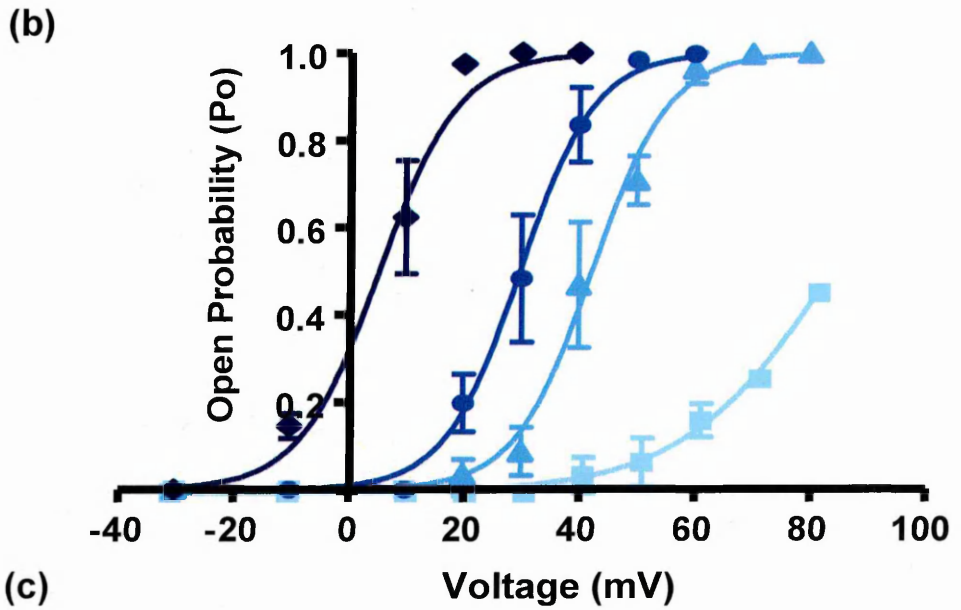
Currents were elicited from BK_{Ca} α cells by applying testpulses in 10mV increments from -100mV to + 100mV from a holding potential of -100mV. **(a)** Normalised G-V relationshps were determined under control conditions and during exposure to BKOEt1 (3-100μM) from which V_{50} values were determined and tabulated **(c)** or from $BKOEt_1$ co-applied with paxiline (10μM). Representative current traces obtained in the absence (top) and presence (middle) of BKOEt1 alone or with paxilline (bottom) **(b)**. Data presented as means±SEM , n=4-9. One-way ANOVA with Dunnetts, ***p<0.001, indicate levels of significance in V_{50} values relative to control.

5.4.2 Effect of BKOEt1 on single BK_{Ca} channel currents

The effect of BKOEt1 on single BK_{Ca} channel currents was examined in inside-out patch configuration. Patches were obtained with pipette and bath solutions containing low Ca^{2+} (0.3μM) and symmetrical (140mM) K^+. The effect of voltage (-30 to +80mV for at least 60s for each voltage) on BK_{Ca} channel currents under control bath conditions and following the perfusion of increasing concentrations of BKOEt1 (3, 10, 30 and 100μM) was determined from which P_o was measured (Figure 5.2a). Cumulative application of BKOEt1 increased BK_{Ca} channel opening in a concentration dependent manner with an increase in P_o from control (0.058±0.02) to 0.469±0.17, 0.836±0.09, 1.00±0.09 for 10, 30 and 100μM at +40mV holding potential (Figure 5.2c) the effect was reversible upon washout (Data not shown).

The relationship between P_o and voltage was fitted with Boltzmann relationship from which V_{50} values were determined (Figure 5.2b). BKOEt1 increased P_o at the potentials examined relative to control. This produced shifts in V_{50} to more negative potentials (Figure 5.2b). This resulted in a 16.1±2.6mV change in V_{50} per *e*-fold increase in intracellular BKOEt1 concentration (Figure 5.4). Furthermore, the relationship between shift in V_{50} (from control) vs log [BKOEt1] concentration was fitted by a modified Hill equation from which an EC_{50} of 9.14±0.65μM and a Hill slope of 1.05±0.36μM was determined (Figure 5.3). Linearisation of open pore probability data showed that the channel underwent an *e*-fold change in P_o per change in membrane potential of 13.48±3.2mV under control conditions and 5.46±1.37mV in 10μM, 5.06±1.36mV in 30μM and 5.8±5.00mV in 100μM BKOEt1 respectively (Figure 5.4a,b).



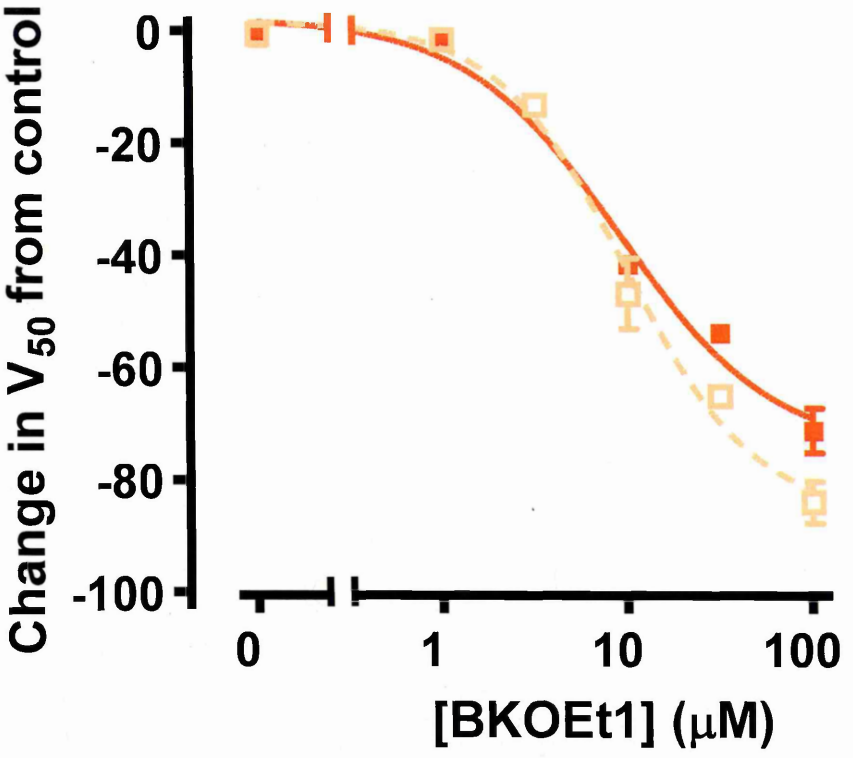


BKOEt1 (μM)	0	10	30	100
V_{50} (mV)	83.0±0.4	42.1±1.0***	29.8±1.2***	5.5±2.0
P_o (+40mV)	0.058 ± 0.02	0.469 ±0.17 *	0.836 ±0.09**	1.00 ±0.0

Figure 5.2: The effect of BKOEt1 on open pore probability

(a) Recordings of single channel BK_{Ca} current at +40mV under control conditions (■) or following perfusion of BKOEt1 (10 ▲, 30 ● and 100μM ◆) intracellulary. **(b)** The open pore probability (P_o) determined from inside-out patch recordings (60s) of single channel BK_{Ca} currents at various holding potentials (-30 to +80mV), (n=6-8). **(c)** Shows the V_{50} and P_o (at +40mV) values determined for control and for BKOEt1. One-way ANOVA with Dunnett's, *p<0.05, **p<0.01, ***p<0.001 indicate levels of significance in V_{50} and P_o values relative to control

(a)



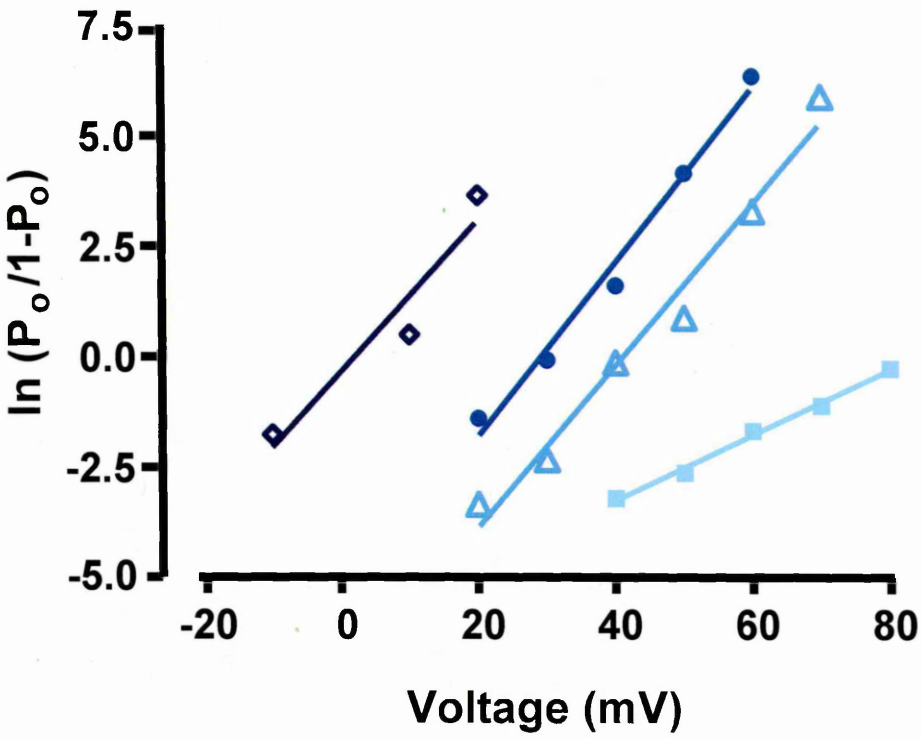
(b)

	EC_{50} (μM)	Hill Slope
WCR	9.79±0.78	1.26±0.38
I/O	9.14±0.65	1.05±0.36

Figure 5.3: Potency and co-operativity of BKOEt1 at activating BK_{Ca} channels

(a) The effect of BKOEt1 (0-100μM) on the shift in V_{50} from control was determined in WCR (□) and from excised inside-out patch (■) configuration from which the EC_{50} (half maximal concentration) and Hill slope was calculated **(b)**. Data presented as means±SEM, (n=4-6). BKOEt1 at 1μM represents data obtained from 1-2 observations.

(a)



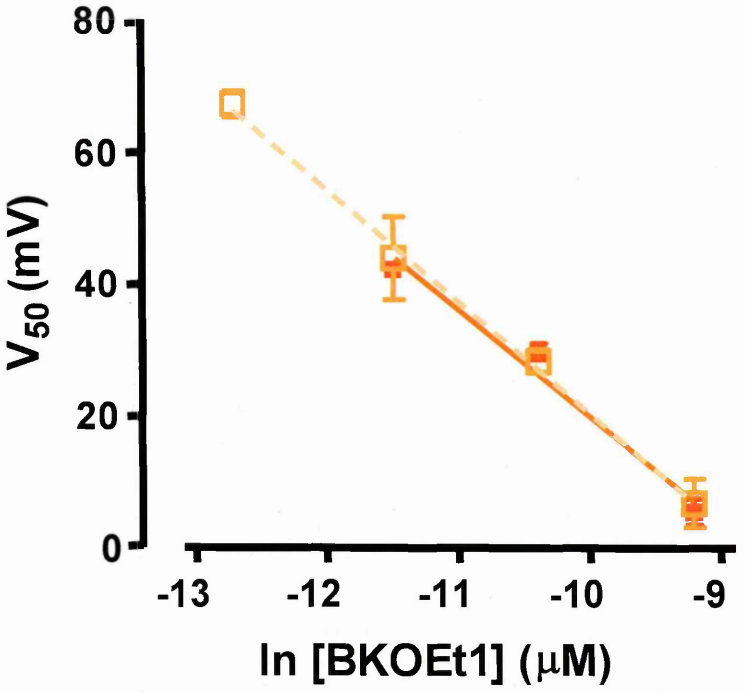
(b)

	BKOEt1 (μM)			
	Control	10	30	100
e-fold change in P_o / mV change in voltage	13.5±3.2	5.5±1.4***	5.1±1.4***	5.8±4.0*

Figure 5.4: The effect of BKOEt1 on voltage sensitivity of BK_{Ca} channel activation

(a) To examine the effect of voltage on P_o under control (■) and the presence of increasing BKOEt1 (10 △, 30 ●, and 100 ◇) concentrations applied to the intracellular aspect of excised inside-out patches **(b)** Corresponding slope values indicating the *e*-fold increase in P_o per mV change in membrane potential. Data presented as means±SEM (n=6-8). Data fitted by linear regression analysis. One-way ANOVA with Dunnett's, *p<0.05, ***p<0.001 indicate levels of significance in V_{50} and P_o values relative to control.

(a)



(b)

	Extracellular	Intracellular
Change in V_{50} (mV) per e-fold ↑ BKOEt1	16.99±4.50	16.12±2.62

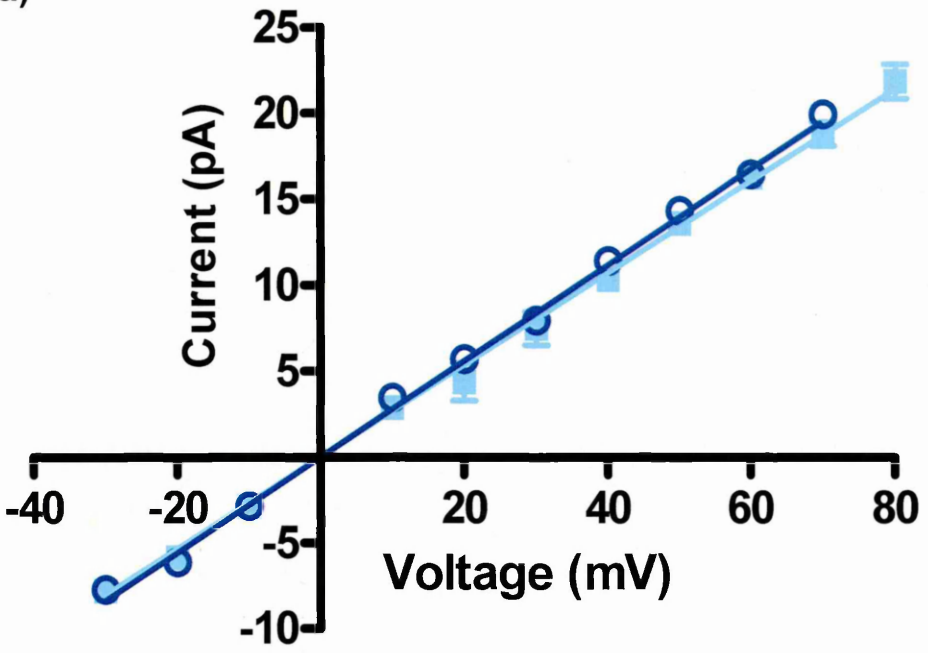
Figure 5.5: The effect of BKOEt1 on V_{50} when applied to the outside or inside aspect of the channel

(a) The effect of increasing concentrations of BKOEt1 on V_{50} was determined when applied in whole cell configuration to the extracellular aspect of the channel (dashed line) and when exposed to the intracellular aspect (solid line) of excised inside-out patches. V_{50} values are those determined previously (Figure 5.1c and 5.2c). **(b)** From the slope of the line V_{50} vs ln [BKOEt1] (μM) the change in V_{50} per *e*-fold increase in BKOEt1 was determined. Data presented as means±SEM (n=6-8). Data fitted by linear regression analysis. There was no significant difference (Student's paired t-test, $p>0.05$) between change in V_{50} values determined for the effect of BKOEt1.

5.4.3 Single channel current voltage relationships: determination of conductance

The effect of BKOEt1 on single channel conductance was determined by measuring the current amplitude from a single ion channel at different membrane potentials (-30 to +80mV) before and after the application of BKOEt1 (30μM), from which the slope of the relationship of current vs voltage yielded the conductance (Figure 5.6a). The conductance value obtained in the presence of BKOEt1 (278.9±2.2pS) was not significantly (Student's paired t-test, $p>0.05$) different to that determined under control conditions (277.1±3.2pS), (Figure 5.6b). Therefore, BKOEt1 did not alter the single channel conductance of BK_{Ca} channels.

(a)



(b)

	Control	BKOEt1 (30μM)
G (pS)	267.1±3.2	278.9±2.2

Figure 5.6: Determining the effect of BKOEt1 on BK_{Ca} channel conductance

(a) Current from a single open channel recorded at different voltages (-30 to +80mV) before (■) and after (O) application of BKOEt1 to the intracellular aspect in excised inside-out patches (30μM) form which single channel conductance (G) was determined **(b)** from linear regression analysis of the slope of the relationship I vs. V. Data presented as means±SEM, (n=6). There was no significant difference determined (Student's paired t-test, $p>0.05$) between conductance values measured before and after application of BKOEt1.

5.4.4 The effect of Ca^{2+} on BKOEt1 mediated activation of BK_{Ca} channels

To determine whether the effect of BKOEt1 was dependent upon the concentration of intracellular Ca^{2+} the open pore probability (at +30mV) was measured from excised inside-out patches in the presence of a range of Ca^{2+} concentrations (0.3-10μM) and then in the presence of BKOEt1 (30μM). In the presence of 0.3, 1.0 and 3.0μM Ca^{2+} the corresponding P_o value increased in a concentration dependent manner with the corresponding P_o values determined as 0.0±0.0, 0.039±0.029, and 0.321±0.128 (Figure 5.7). Following application of BKOEt1, P_o was significantly increased (Student's paired t-test, $p>0.05$) above that achieved for 0.3, 1.0 and 3.0 μM Ca^{2+} alone achieving similar P_o levels of 0.885±0.009, 0.823±0.067, 0.875±0.078 respectively. BKOEt1 (0.892±0.09) did not significantly ($p<0.05$, Student's paired t-test) increase P_o above that achieved in the presence of 10μM Ca^{2+} (0.723±0.120), (Figure 5.7).

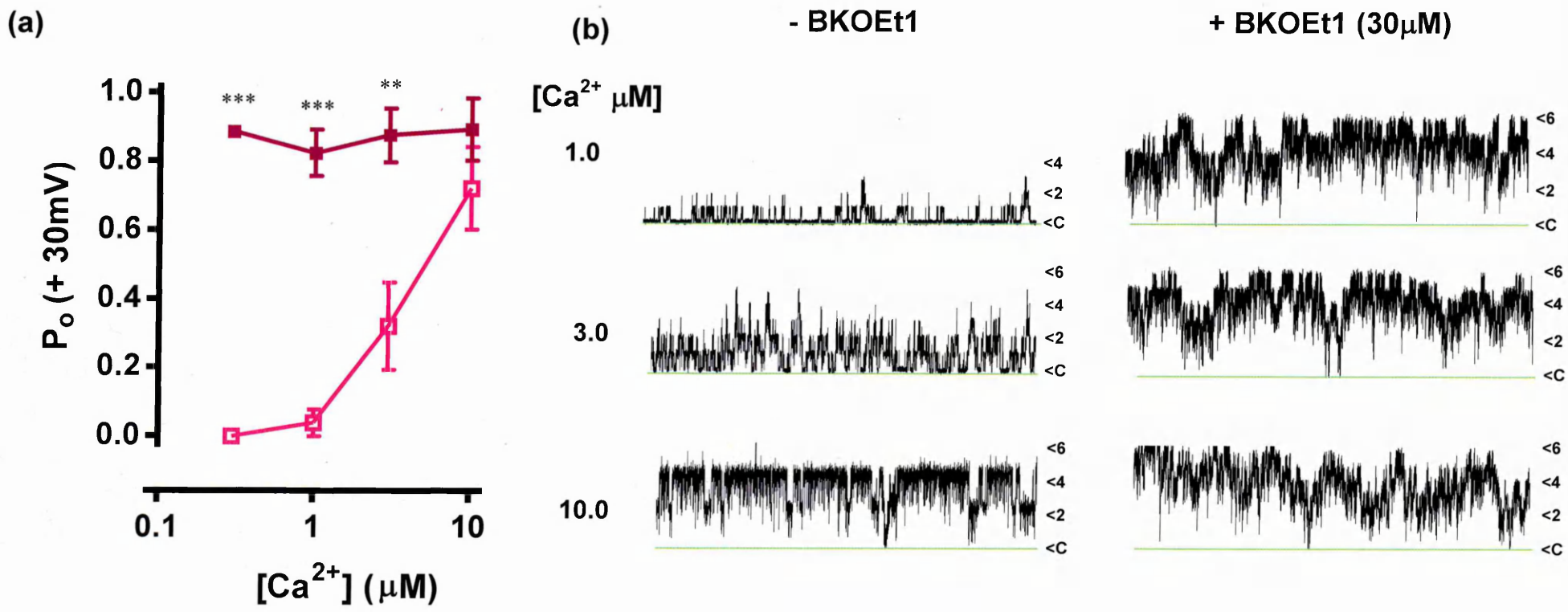


Figure 5.7: The effect of Ca^{2+} on BKOEt1 dependent activation of BK_{Ca} channels

(a) The effect of increasing intracellular Ca^{2+} (0.3-10μM) on P_o determined in the absence (□) and presence of BKOEt1 (30μM ■) from inside out patch recordings (>60s) of single channel currents Data presented as means±SEM (n=3). **(b)** Representative recordings of single channel BK_{Ca} current (at +30mV) in the presence of a single Ca^{2+} concentration then following the application of BKOEt1. Student's paired t-test, **p<0.01, ***p<0.001 indicate differences between P_o in Ca^{2+} alone and following application of BKOEt1.

5.4.5 Intracellular Ca^{2+} Fura-2 assay

Fura-2/AM was used to detect changes of intracellular calcium over time in the presence and absence of different treatments. The technique was used to elucidate whether BKOEt1 affected the level of intracellular Ca^{2+} to identify whether BKOEt1 mediated activation of BK_{Ca} channels was secondary to VDCC activation or that it activated BK_{Ca} channels via secondary cellular signalling mechanisms resulting in increases in Ca^{2+} from intracellular stores (Figure 5.8).

Calcium ionophore (10μM) was used as a positive control and resulted in a ~4-fold increase in fluorescence that was not significantly modified over time. In addition, FCS (10%) stimulated intracellular signalling pathways and peaked at 5-mins following exposure. Background fluorescence was determined from cells that had not been loaded with Fura-2/AM and appropriate 'negative' controls included the addition of Krebs buffer in the presence of vehicle (DMSO), (Figure 5.8).

Treatment of cells with depolarising Krebs buffer (high K^+, 50mM) resulted in a significant (One-way ANOVA with Bonferroni's, $p<0.05$) increase in fluorescence that peaked at 1 min following application and decreased over time, this indicated that the increase in intracellular Ca^{2+} could be attributed to VDCC channel activation and subsequent Ca^{2+} influx from the outside of the cell (extracellular concentration of Ca^{2+} = 1.9mM). Examination of the effect of forskolin (50μM), an activator of adenylate cyclase, leading to the formation of cAMP and subsequent increase in Ca^{2+} through VDCC, did not result in a significant increase in fluorescence above background. Ryanodine was used to stimulate intracellular Ca^{2+} release from stores via activating ryanodine receptors (RyR) in the endoplasmic reticulum, treatment with 0.1μM led to a significant (One-way ANOVA with Bonferroni's, $p<0.05$) increase in fluorescence that peaked at 5-mins following application (Figure 5.8). However, treatment of cells with BKOEt1 (100μM) or NS1619 (100μM) did not elicit any significant increase in fluorescence above background (Krebs buffer + vehicle) for all time points examined (Figure 5.8).

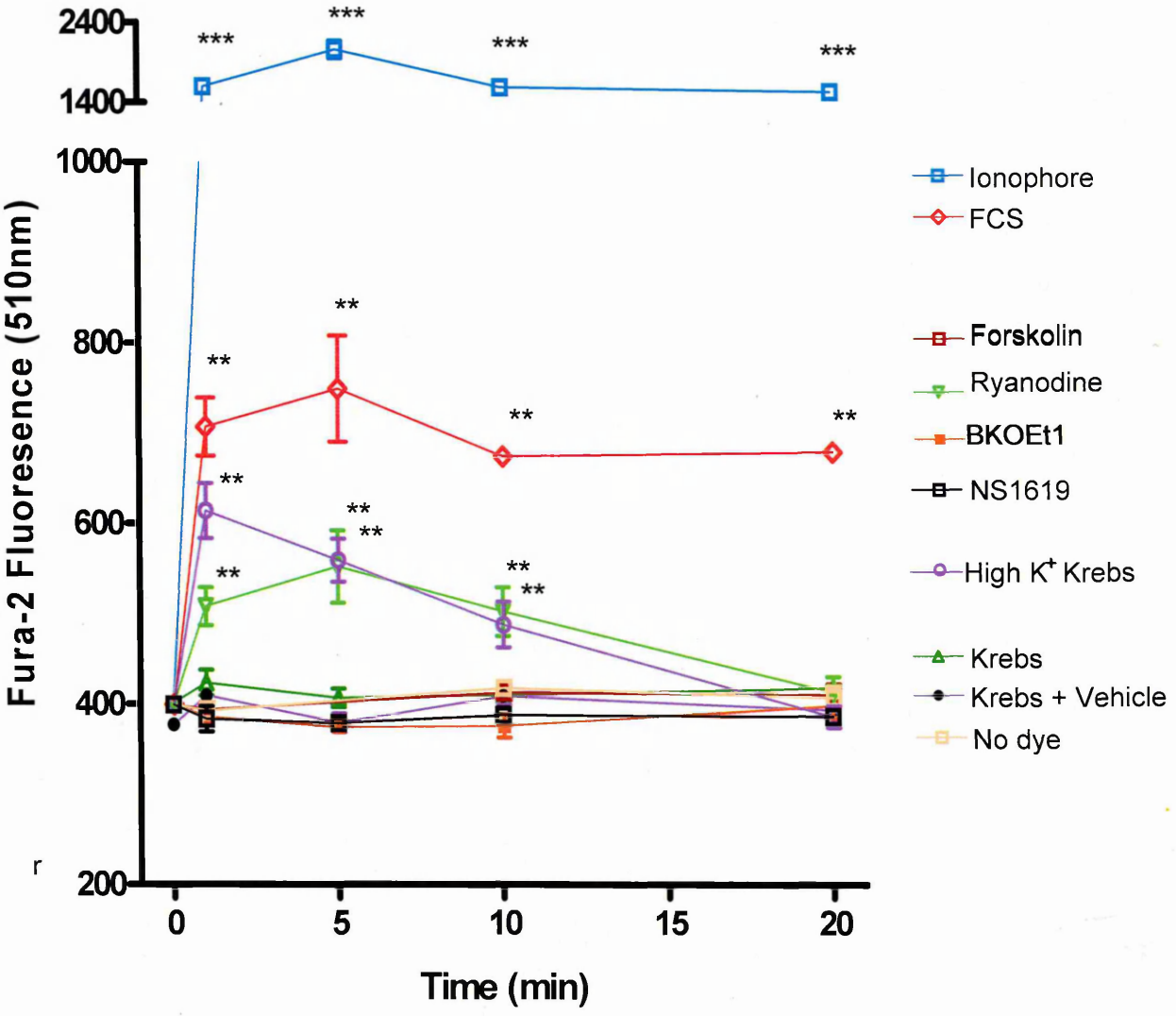


Figure 5.8: The effect of BKOEt1 on intracellular calcium in HEK293 cells

BK_{Ca} α_{+132} cells grown to confluence on 96-well plates were loaded with Fura-2/AM and the time dependent effect of differing treatments (in Krebs buffer) on increasing intracellular calcium was observed by measuring changes in fluorescence excited at 340nm and measured at 510nm. Controls included no dye treatment, those exposed to Krebs buffer alone or including vehicle. Calcium ionophore A23187 (10μM), Fetal calf serum (FCS, 10%) were used as positive controls. The effect of ryanodine (0.1μM), forskolin (50μM), high K^+ Krebs buffer (50mM), NS1619 (100μM) and BKOEt1 (100μM) was assessed. Results expressed as means±SEM, (n=16-24). One-way ANOVA with Bonferroni's, **p<0.01, ***p<0.001, was used to determine differences in fluorescence before and at each time point following the addition of treatments.

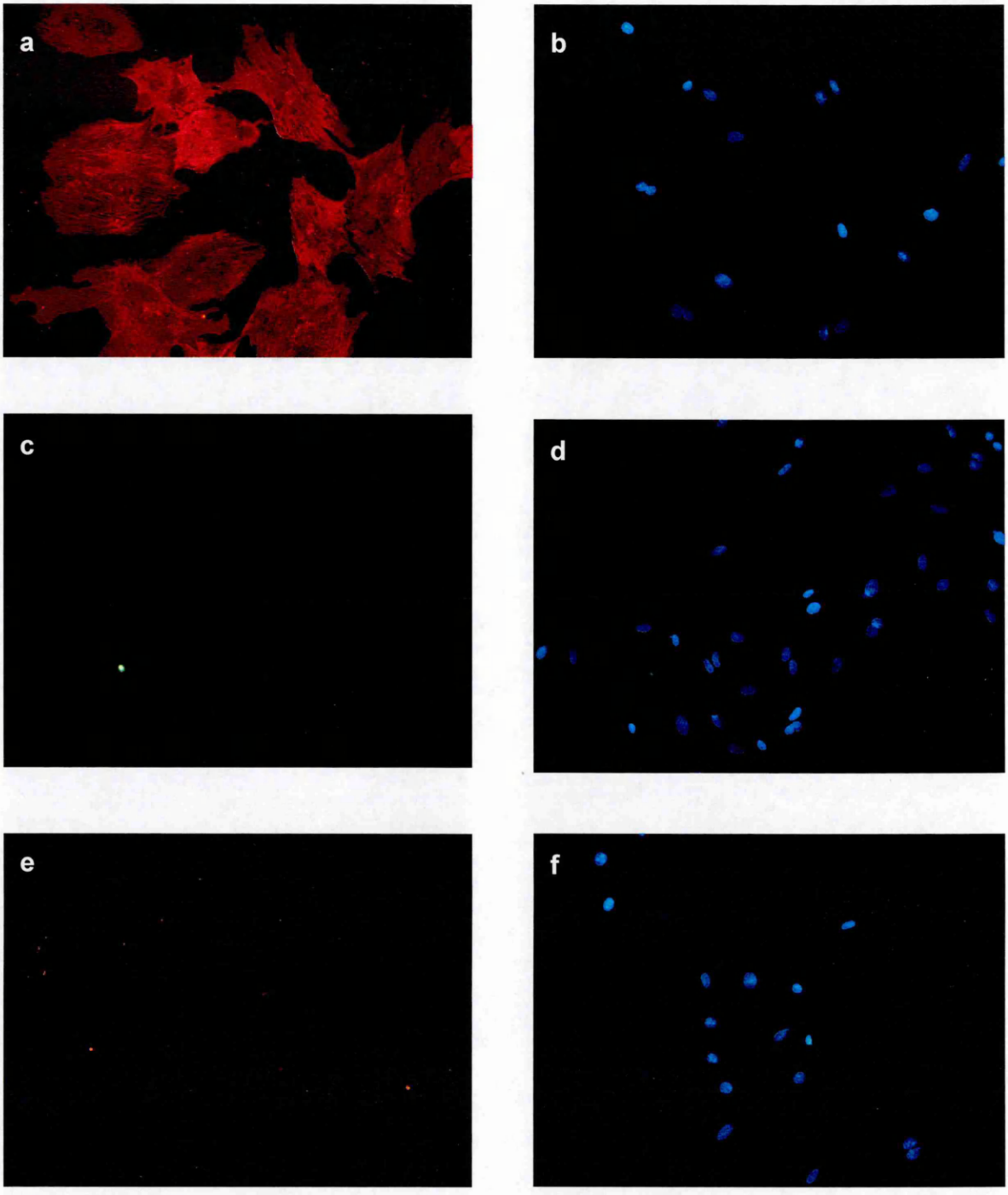
5.4.6 Rat bladder myocytes

5.4.6.1 Myocyte dissociation

One rat bladder (50-60mg) yielded approximately 5000 cells that were greater than 90% viable as determined using trypan blue staining. Cells isolated from one bladder were cultured and reached confluence within 6 days, cells were then seeded into 8-well chamber slides for ICC.

5.4.6.2 Characterising cell types in culture

ICC demonstrated that all dissociated cells in the field of view, compared to DAPI staining, were positive for anti-smooth muscle actin indicative of a myocyte cell type (Figure 5.9). Furthermore, no staining was observed for markers of epithelial cells (cytokeratin) and mesenchyme cell types such as fibroblasts and connective tissue cells (vimentin); as expected corresponding negative controls displayed no positive staining (Figure 5.9 a-j).


a
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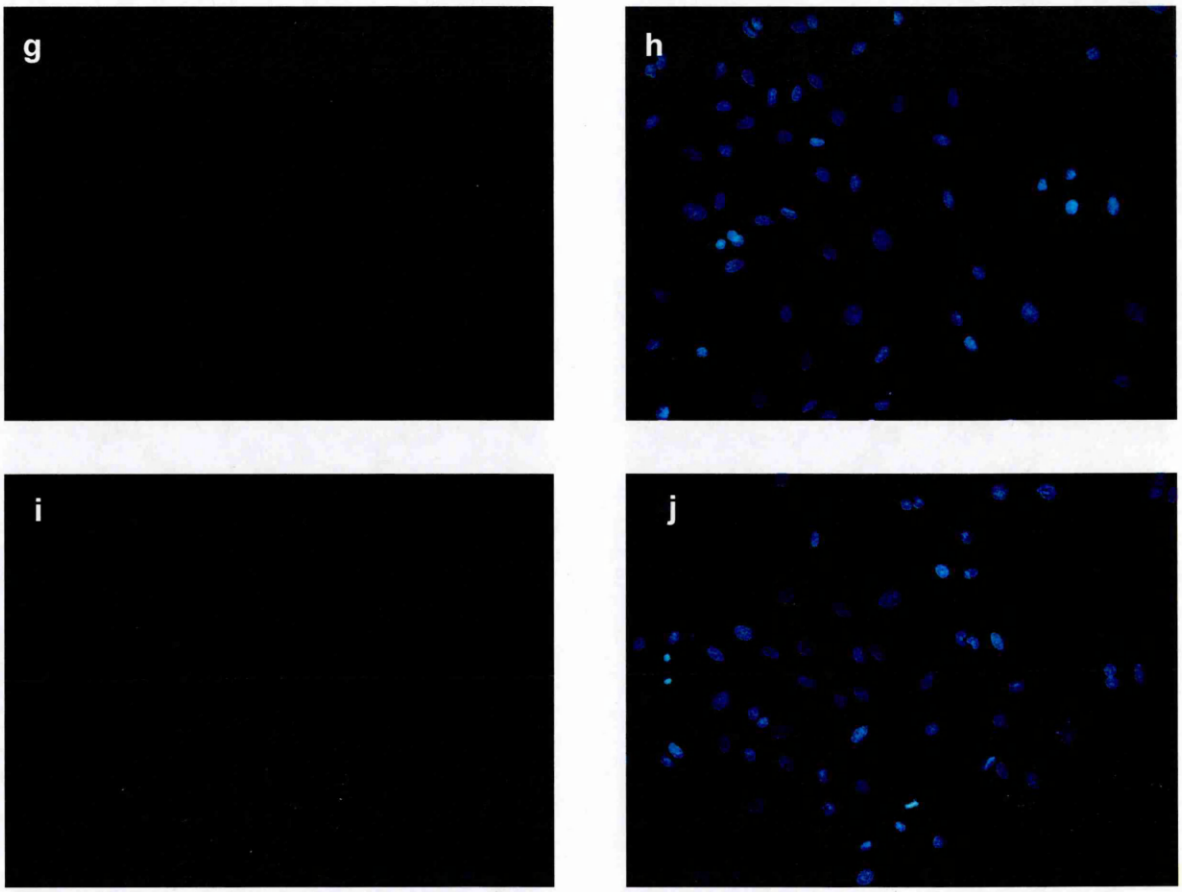


Figure 5.9: Rat bladder myocyte characterisation

ICC staining of rat bladder myocytes (P_2) visualised using fluorescent microscopy (x200) for either smooth muscle α-actin (a), vimentin (c), cytokeratin (e) and corresponding nuclear staining with DAPI (b, d, f). Images (g) and (i) are the respective negative controls for Alexa-Fluor 468 and 568 2^0 Ab (PBS v/v replacing the 1^0 Ab) and concurrent counter stain with DAPI (h and j).

5.4.7 Rb^+ efflux studies

5.4.7.1 Rb^+ uptake by rat bladder myocytes

Rat bladder myocytes were exposed to RbCl buffer (0.5-5.0 hours) and immediately lysed following removal of extracellular buffer by washing. The Rb^+ content of the lysate of the myocytes progressively increased with time of incubation in Rb^+ buffer (Figure 5.10). The Rb^+ content appeared to become saturated from 3.5 hours incubation and a four hour incubation period was sufficient to reach maximal loading (19.4±0.7μM, n=16; Figure 5.10). Four washes were sufficient to remove all Rb^+ from the extracellular compartment of the cells.

Rat bladder myocytes were assessed for their viability after 4 hour exposure to RbCl buffer using the trypan blue dye exclusion procedure, from which it was calculated that ≈85% of cells were still viable.

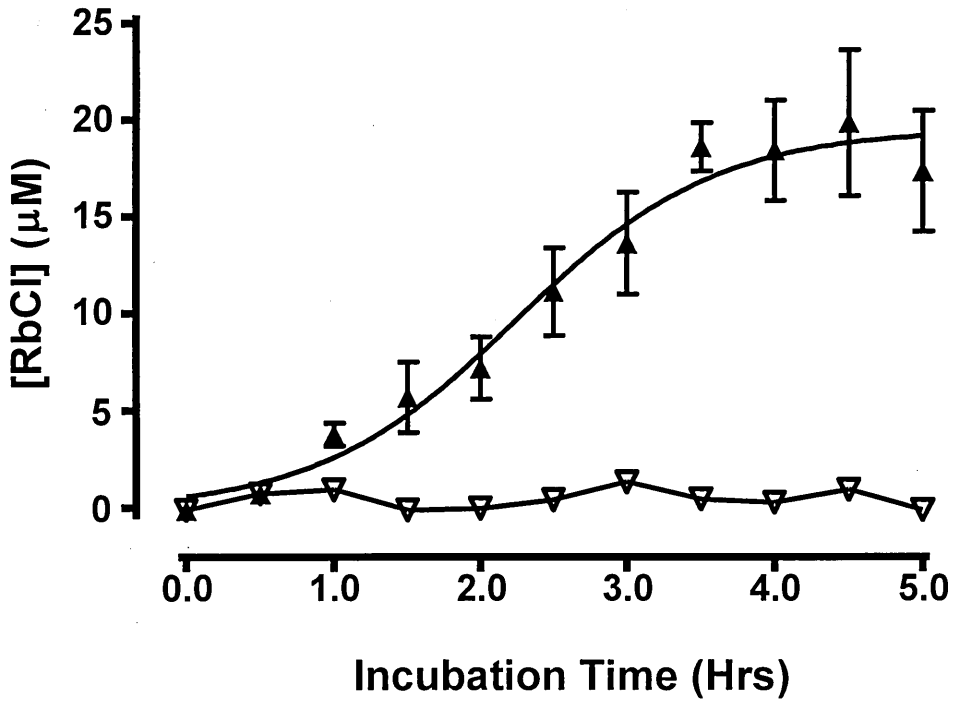


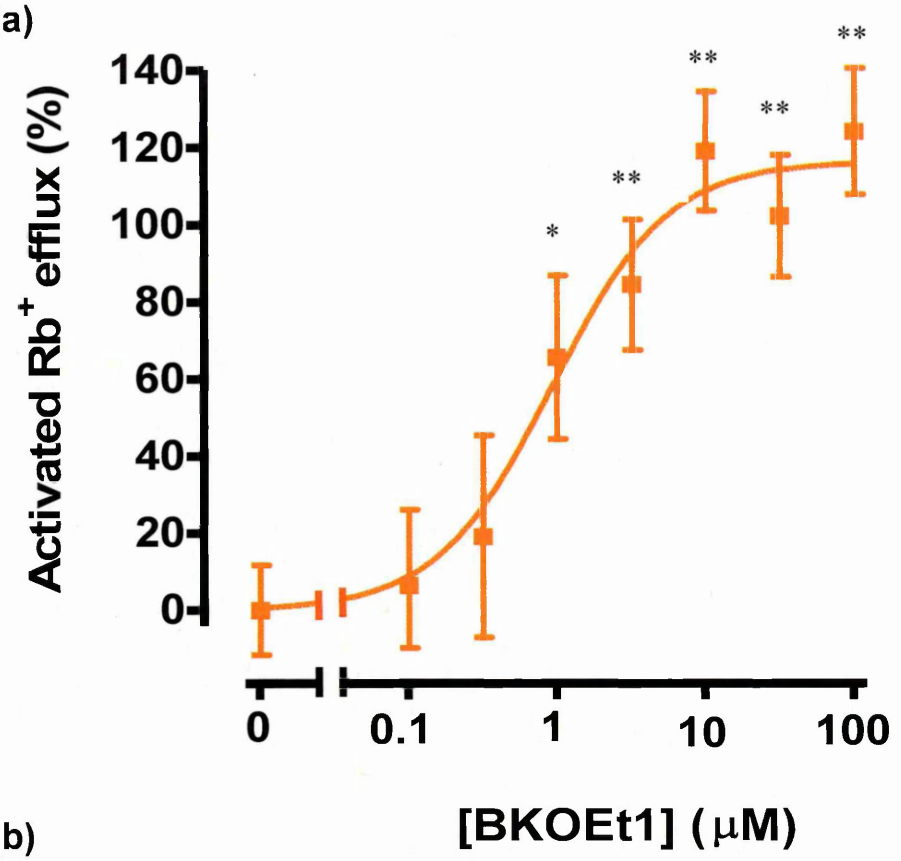
Figure 5.10: Time course of Rb^+ loading in rat bladder myocytes

Cells were incubated with RbCl buffer. At timed intervals up-to 5-hours the extracellular buffer was removed and cells were washed 4 times with PBS before lysis with Triton-X100. The Rb^+ content of lysate (▲) and fourth wash (▽) samples. Data expressed as means±SEM, (n=16).

5.4.7.2 BKOEt1 mediated increases in Rb^+ efflux from myocytes

The effect of BKOEt1 on stimulating Rb^+ efflux (10 min in KCl buffer) from rat bladder myocytes was assessed. BKOEt1 (0-100μM) evoked significant (One-way ANOVA with Dunnett's test, $p<0.05$) concentration-related increases in Rb^+ efflux above vehicle control (Figure 5.11a). BKOEt1 activated Rb^+ efflux to a maximum (E_{max}) level of 124±16.35%, with a corresponding potency ($EC_{40\%}$) of 0.45±0.20μM and a Hill slope of 1.12±0.64 (Figure 5.11b).

(a)



(b)

	$EC_{40\%}$ (μM)	E_{max} (%)	Hill Slope
BKOEt1	0.45±0.20	124±16.35	1.12±0.64

Figure 5.11: The concentration response effect of BKOEt1 on stimulating Rb^+ efflux from rat bladder myocytes

Rat bladder myocytes were exposed to BKOEt1 (0.1-100μM) for 10-minutes. The data was normalised as the percentage increase in Rb^+ efflux above control achieved at each concentration tested **(a)**. From which potency ($EC_{40\%}$; defined as the concentration required to increase Rb^+ efflux by 40%), efficacy (E_{max}; defined as the maximum increase in Rb^+ efflux) and Hill slope was determined **(b)**. Data presented as means±SEM (n=16-24). One-way ANOVA with Dunnett's test, *p<0.05, **p<0.01, indicate differences in Rb^+ efflux at each concentration tested relative to control (0μM BKOEt1, KCl buffer + vehicle).

5.4.7.3 Specificity of BKOEt1: Effect of K^+ channel blockers

The specificity of BKOEt1 to BK_{Ca} channels was tested in myocytes by examining its Rb^+ efflux activation effects in the presence of different K^+ channel blockers (Figure 5.12). All K^+ channel blockers tested (in the absence of BKOEt1) did not lower Rb^+ efflux below that achieved in the presence of KCl buffer alone. Addition of BKOEt1 (100μM) alone to KCl buffer activated Rb^+ efflux significantly (One-way ANOVA with Bonferroni's, $p<0.05$) above that observed in the presence of vehicle alone (KCl buffer + DMSO 0.3%) reaching a level (102.2±12.4%) that had been demonstrated previously (Figure 5.11b).

The BK_{Ca} channel blocker pax (10μM) was able to significantly block BKOEt1 mediated increases in Rb^+ efflux (21.3±11.9%), however complete block to control levels was not achieved.

However, BKOEt1 (100μM) mediated increases in Rb^+ efflux were significantly blocked when co-applied with the non-specific channel blockers 4-AP (1mM) and TEA (10mM) to a level (5.2±9.7%) that was not significantly different from background (KCl + vehicle; 3.5±5.7%), (Figure 5.12).

In the presence of BKOEt1 the SK channel blocker apamin (0.1μM) significantly lowered (47.4±14.5%) efflux compared to when BKOEt1 was tested alone; however it did not completely block Rb^+ efflux to control levels. A lowered (64.8±15.9%) but not significantly different response to BKOEt1 was observed in the presence of the IK blocker TRAM-34 (0.1μM), (Figure 5.12).

In the presence of the K_v channel blocker marg (0.1μM); the effect of BKOEt1 was significantly lowered (53.1±21.6%), although marg did not completely block BKOEt1 effects to control levels. In contrast, the K_{ATP} (10μM) channel blocker glib failed to modify (93.9±22.9%) the effect of BKOEt1 on increasing Rb^+ efflux (Figure 5.12).

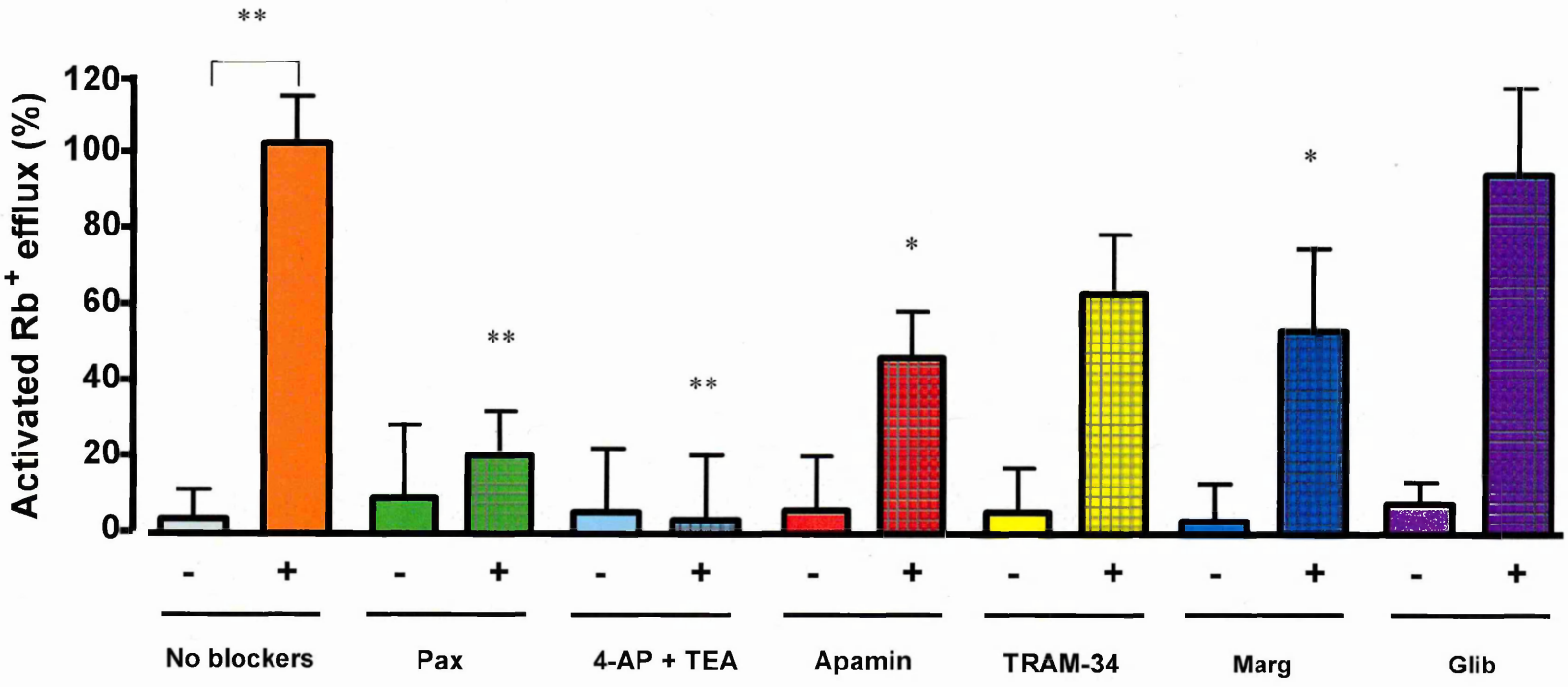


Figure 5.12: The effect of K^+ channel blockers on BKOEt1 induced Rb^+ efflux from bladder myocytes The effect of exposure of rat bladder myocytes (10-min) to KCl buffer in the presence of vehicle (DMSO 0.3%, grey bar) or BKOEt1 (100μM) alone (orange) was examined. Further cells were exposed to KCl buffer containing blockers (- clear) pax (BK_{Ca}, 10μM), 4-AP and TEA (non-specific K^+ channel blockers, 1 and 10mM respectively), apamin (SK, 0.1μM), TRAM-34 (IK, 0.1μM), marg (K_v, 0.1μM) or glib (K_{ATP}, 10μM) or with the addition of BKOEt1 (100μM, (+) hashed bars). Data presented as means±SEM (n=16-32). One-way ANOVA with Bonferroni's, *p<0.05, **p<0.01, indicate significance levels between BKOEt1 response alone and that observed in the presence of blockers.

5.5 Discussion

5.5.1 Pharmacological properties of BKOEt1

BK_{Ca} channel openers can be grouped into those with high or low affinity, high affinity compounds activate the channel with an EC_{50} within the sub-μM to nM range such as with DHS-1 (McManus *et al* 1993; Giangiacomo *et al* 1998) and di-Cl-DHAA (Sakamoto *et al* 2006). BKOEt1 can be regarded as a low-affinity BK_{Ca} channel opener with an EC_{50} (~10μM) within the micro-molar range. This can be classed like other lower affinity BK_{Ca} channel openers including NS1619 (10-30μM), (Olesen *et al* 1994b; Lee *et al* 1995b), KB130015 (20μM), (Gessner *et al* 2007), zonisamide (34μM) (Huang *et al* 2007), TBIC (8.9μM) (Ha *et al* 2006) and pimaric acid (1-10μM) (Imaizumi *et al* 2002).

The EC_{50} for BK_{Ca} channel activation by BKOEt1 was nearly identical when applied to either side of the channel. This property is similar to that demonstrated by NS1619, NS004 (McKay *et al* 1994; Hu *et al* 1996), BMS-204352/MaxiPost (Gribkoff *et al* 2001b) and pimaric acid (Imaizumi *et al* 2002). Other BK_{Ca}CO's activate the channels at a site(s) located on the intracellular side of the channel such as with DHS-I (McManus *et al* 1993; Giangiacomo *et al* 1998), maxiKdiol (Singh *et al* 1994) and L-735,334 (Lee *et al* 1995c). In addition, tamoxifen (Dick *et al* 2002), mallotoxin (Zakharov *et al* 2005; Wu *et al* 2007) and diosgenin (Wang *et al* 2006) are thought to activate BK_{Ca} channels from a site(s) located on the extracellular side of the channel.

A Hill coefficient of around unity was determined for BKOEt1 when applied to either the extracellular or intracellular aspect of the channel. This indicated binding of one BKOEt1 compound molecule per channel (per tetrameric channel composition) was required for activation. A similar Hill co-efficient of around unity has been reported for EET, an endothelium derived factor that has shown to activate BK_{Ca} channels (Zhang *et al* 2001). In addition, LY-171,883, a leukotriene D4 antagonist that has been shown BK_{Ca}CO properties in GH_3 cells (Li *et al* 2002) and certain benzofuroindole derivatives (Gormemis *et al* 2005) including TBIC (Ha *et al* 2006) have revealed Hill co-efficient around unity.

In contrast, compounds such as NS1619 (Lee *et al* 1995b; Khan *et al* 1998), KB130015 (Gessner *et al* 2007), magnolol (Wu *et al* 2002), cilostrazol (Wu *et al* 2004) and DHS-1 (Giangiacomo *et al* 1998) have displayed Hill co-efficients greater than unity (2-3) suggesting that BK_{Ca} channels contain more than one-binding site or potentially one binding site per α-subunit.

A number of explanations could be sought to determine potential sites of interaction. For example, BKOEt1 could activate BK_{Ca} channels at one site on the channel that is equally accessible to drug molecules from either side of the channel with equi-effectiveness. Alternatively there could be two sites on the channel one located extracellular and another intracellular with each site demonstrating similar Hill slope and potency values for BKOEt1. Further, the BKOEt1 binding site could be formed by more than one subunit. A similar shift in V_{50} per *e*-fold change in concentration of BKOEt1 was determined in both electrophysiology configurations, supporting one binding site that has a similar apparent equal affinity for BKOEt1 when applied to the extracellular and intracellular aspect.

In both electrophysiology configurations BKOEt1 activated BK_{Ca} channels in a relatively rapid time (within 1 min of application). However, the time taken to 'wash off' the effect of BKOEt1 was much greater in whole-cell than in excised inside-out patches. This coupled to the relatively quick wash-off when BKOEt1 was applied to the intracellular aspect would indicate either a transmembrane or an intracellular site of action (Data not shown). Another explanation could be the presence of a transmembrane binding site that would be equally accessible from either side of the channel. Furthermore, BKOEt1 has a high degree of lipophilicity with a calculated value of LogP of 4 (See chapter 6, section 3.2) within the theoretical range for a molecule to be able to cross the lipid bilyaer; this property could account for the equal onset time observed in both electrophysiology configurations.

The most well known and characterised BK_{Ca}CO is NS1619. The effect of which was delayed in activation on-set time by 1-4 minutes in whole-cell configuration (McKay *et al* 1994; Olesen *et al* 1994a,b; Edwards *et al* 1996). In addition, NS1619 had a slow response to wash off as well as slow association of the

drug for the channel in whole cell configuration (Olesen *et al* 1994b). Interestingly rapid activation of channels is apparent in excised membrane patches exposed to NS1619 (Olesen *et al* 1994b; Holland *et al* 1996). A similar observation to that determined for BKOEt1. Holland *et al* (1996) propose that this difference in time to activation may be due to the binding site of NS1619 to the channel being intracellular. Therefore, impairment across the membrane might be associated with the slow onset of action in whole cell mode. Holland *et al* (1996) suggest that this slow process of activation in whole cell verses inside-out is in part due to the availability and quickness of an effective NS1619 concentration reaching the intracellular side of the cell. Although contradictory to this NS1619 has been shown to display high degrees of lipophilicity through lipid bi-layers.

A similar observation was made for the indole carboxylate CGS-7181 developed by Novartis Pharmaceuticals that demonstrated a longer time to achieve a stimulatory response in whole cell (>5min) than in excised inside out-patch (2 min). In addition, it showed prolonged washout effects in whole cell (15 min) than in excised out patches (3-5 min). This led the authors to speculate an intracellular activating site (Hu *et al* 1997).

BKOEt1 (30 and 100μM) mediated activation of BK_{Ca} channels in both whole cell and excised inside-out patches of BK_{Ca} α cells was completely blocked by application of paxilline (10μM). Therefore it could be suggested that the site of action of BKOEt1 is independent to that of paxilline, this could be confirmed by use of ^{125}I-Paxilline binding assay or by further work investigating the kinetics of block by paxilline and determining whether BKOEt1 affects the association rate of the blocker to the channel.

Further work would be required to investigate whether the binding site of BKOEt1 is located intracellular, transmembrane or extracellular. This could be achieved by the use of either cell impermeable analogues of BKOEt1 or via creation of radioactive or fluorescent labelled BKOEt1 which could be used as tracer molecules to provide clues as to the site of action.

5.5.2 The effect of BKOEt1 on single BK_{Ca} channel properties

BKOEt1 when applied to the internal face of the channel increased P_o rapidly producing an *e*-fold increase in P_o per ~5mV change in voltage, which was observed with all concentrations of BKOEt1 tested. However, this value was significantly different to that obtained under control conditions with an ~15mV change in voltage per *e*-fold increase in P_o. Therefore, BKOEt1 affected the voltage sensitivity of the channel. A change in depolarising voltage of between 10-20mV per *e*-fold increase in P_o has been reported for BK_{Ca} channels (Dichiara and Reinhart 1995; Diaz *et al* 1998; Cui and Aldrich 2000; Niu and Magleby 2002; Magleby 2003). Therefore, the voltage dependence of BK_{Ca} channel activation reported here under control conditions (~15mV) falls within the range previously determined by others for the voltage dependence of BK_{Ca} channel activation.

In contrast to BKOEt1, NS1619 does not affect the voltage sensitivity of BK_{Ca} channels. Lee *et al* (1995b) reported that for an ~30mV change in membrane potential there was an *e*-fold increase in P_o and that this was un-affected in the presence of NS1619. Furthermore, mallotoxin does not affect the voltage dependency compared to that observed under control conditions with a reported *e*-fold increase in P_o per 10.6±0.5mV change in membrane voltage (Wu *et al* 2007). Further, TBIC has been shown to shift the conductance-voltage curve without affecting voltage sensitivity (Ha *et al* 2006).

The effect of BKOEt1 on P_o was more pronounced at more positive voltages relative to that obtained under control conditions. This is similar to that observed for most BK_{Ca}CO's including BMS-204352 (Gribkoff *et al* 2001b). Interestingly di-Cl-DHAA and pimaric acid are the only two reported BK_{Ca}CO's that demonstrate inversed potentiation of P_o, in that bigger increases in P_o are observed relative to control at more negative voltages that become diminished at more positive voltages (Imazumi *et al* 2002; Sakamoto *et al* 2006).

A relatively quick onset time to activate BK_{Ca} channels in whole-cell electrophysiology was observed for BKOEt1, and this coupled to the rapid increase in P_o demonstrated for a small change in voltage is in line with a 1:1 stoichometry determined for BKOEt1 based on Hill slope value.

Single channel conductance was not altered by incubation with BKOEt1. BK_{Ca} channel openers identified to date have not affected the conductance of BK_{Ca} channels. An exception is tamoxifen which has shown to cause a decrease in single channel conductance in BK_{Ca} channels expressing BK_{Ca} channel α and β1 subunits without affecting its ability to increase P_o (Dick *et al* 2001; Duncan 2005; Coiret *et al* 2007).

5.5.3 The effect of Ca^{2+} on BKOEt1 activation of BK_{Ca} channels

BKOEt1 was able to activate BK_{Ca} channels in the near absence of Ca^{2+}, furthermore, its effects were not additive with increasing concentrations of intracellular Ca^{2+} in that BKOEt1 was unable to further activate BK_{Ca} channels above the level achieved with 10μM intracellular calcium. A possible explanation could be that BKOEt1 binds to a site of Ca^{2+} interaction on the channel such as the Ca^{2+} bowl, RCK domains or other Ca^{2+} regulatory elements located on the channel (Magleby 2003). However, as suggested this is unlikely due to the equal affinity and potency observed when the compound is applied to either aspect of the channel. Therefore it is likely that the presence of 10μM Ca^{2+} would have opened all the channels and as a result BKOEt1 (at the concentration used) could not activate the channel further. It would have been interesting to have used a higher concentration of BKOEt1 (100μM). Although at +30mV membrane potential the P_o in 10μM Ca^{2+} was >0.8 therefore potentially not leaving a sufficient window to further increase channel activation.

BK_{Ca}CO's have shown differential activating effects in the presence of Ca^{2+}, for example, the openers KB130015 (Gessner *et al* 2007), tamoxifen (Dick *et al* 2001), NS1608 (Strobaek *et al* 1996; Siemer *et al* 2000), naringenin (Saponara *et al* 2007) and pimaric acid (Imaizumi *et al* 2002) have shown to activate BK_{Ca} channels in the absence of Ca^{2+} whereas NS1619 (Lee *et al* 1995b) and DHS-1 do not (Knaus *et al* 1993; Giangiacomo *et al* 1998). In addition, zonisamide was able to activate BK_{Ca} channels 1.6 fold above that achieved in the presence of 0.1, 1.0 and 10μM Ca^{2+} (Huang *et al* 2007) and NS1619 has been shown to

activate BK_{Ca} channels above the level observed in the presence of 10μM Ca^{2+}; a feature not observed with BKOEt1 (Olesen *et al* 1994b; Lee *et al* 1995b)

Furthermore, BMS-204352/MaxiPost demonstrated an additive effect, in that with increasing intracellular Ca^{2+} (across the physiological range) the greater the strength of BK_{Ca} channel activation, this hence led to its subsequent clinical development for the treatment of stroke (Gribkoff *et al* 2001b).

BKOEt1 did not affect the level of intracellular Ca^{2+} in HEK293 cells, indicating that its mechanism of action does not occur secondary to an increase in intracellular Ca^{2+} either by release from stores or via VDCC activation. Stimulating channel activation by application of ryanodine and its subsequent release of Ca^{2+} from intracellular stores did lead to a significant increase in fluorescence above background, this is in line with previous observations that application of ryanodine to HEK293 cells resulted in an increase in Ca^{2+} concentration (of around 0.3-0.5μM), (Querfurth *et al* 1998). Furthermore, depolarising the cell with high K^+ Krebs buffer resulted in an initial increase in fluorescence related to activation of VDCC and Ca^{2+} influx that subsequently declined over time; this in part could be due to repolarisation of the membrane by activation of BK_{Ca} channels following VDCC channel activation, although 50mM KCl is a strong depolarising stimulus. Calcium ionophore had the greatest effect upon fluorescence producing a 4-fold increase in fluorescence this is similar to the observations of Querfurth *et al* (1998) who demonstrated a 4-fold increase in Ca^{2+} fluorescence in HEK293 cells upon application of ionophore.

In this assay NS1619 did not affect the level of intracellular Ca^{2+}, this is in contrast to the work of Yamamura *et al* (2001), who showed that NS1619, in porcine coronary smooth muscle cells, released calcium from caffeine/ryanodine-sensitive calcium storage sites and this partially contributed to activation of BK_{Ca} channels leading to membrane hyperpolarisation. Furthermore, Khulmann *et al* (2004b) demonstrated that NS1619 induced Fura-2 fluorescence could be blocked by inhibition of Ins(1,4,5)P3 induced Ca^{2+} release. The potential of NS1619 and BKOEt1 to affect the level of intracellular

calcium may have not been observed in BK_{Ca} α cell line due to the absence of these cellular receptors in HEK293 cells.

A number of compounds, mainly from natural sources, have shown to activate BK_{Ca} channels indirectly. One such example is the plant steroid diosgenin (3b-hydroxyl-5-spirostene) which activated BK_{Ca} channels as a consequence of increases in intracellular calcium resulting from VDCC channel activation (Wang *et al* 2006). Furthermore, Huang *et al* (2005) determined that the effect of thymol (2-isopropyl-5-methylphenol) a compound isolated from the plant oil of *Thymus Vulgaris* (Thyme) only activated BK_{Ca} channel like diosgenin in cell-attached patches and not when applied to the intracellular aspect, they concluded that the effects of thymol were probably acting to activate ryanodine receptors in these cells.

5.5.4 Rubidium efflux studies: Characterising the effect of BKOEt1 on Rb^+ efflux from myocytes

The specificity of BKOEt1 to BK_{Ca} channels was assessed by adapting the non-radioactive Rb^+ efflux assay to screen rat bladder myocytes. From which the potential of BKOEt1 to activate K^+ channels was examined by testing its effects on Rb^+ efflux in the presence of different K^+ channel blockers.

Rat bladder myocytes were chosen as they express a number of different K^+ ion channel subtypes including BK_{Ca} (Nakahara *et al* 2004; Malysz *et al* 2004), IK (Argentieri *et al* 2006), SK (Nakahara *et al* 2004), K_{ATP} (Butera *et al* 2005; Argentieri *et al* 2006) and K_v channels (Ohya *et al* 2000). The bladder is composed of many different types of cell layers including smooth muscle, urothelium and fibroblast cells (Andersson and Arner 2004). A protocol for the dissociation of myocytes from rat bladder was optimised and to determine that the predominate cell types in culture were smooth muscle cells ICC was performed.

ICC demonstrated that cells stained positive with smooth muscle actin and not for the markers cytokeratin and vimentin. However, epithelial and fibroblast cells

were not used as positive controls. As a result, the presence of other cell types can't be ruled out completely. However, all cells in the field of view stained for smooth muscle α-actin as determined by a direct comparison with the pattern of DAPI staining. Therefore, it was likely that a homogenous population of rat bladder myocytes were obtained that were used for Rb^+ efflux experiments.

Rb^+ loading characteristics of myocytes were determined and the number of extracellular washes was optimised prior to efflux experiments. Rat bladder myocytes, as with BK_{Ca} α cells, showed time dependent Rb^+ loading reaching a maximum at around 4 hours, this was approximately twice as long (to reach peak loading) than in BK_{Ca} α cells. Furthermore, the amount of Rb^+ loaded was approximately four-fold less than that achieved in HEK293 cells. Similar to BK_{Ca} α cells four washes was sufficient to remove all extracellular Rb^+

In rat bladder myocytes BKOEt1 evoked significant concentration dependent increases in Rb^+ efflux above control from which $EC_{40\%}$ and E_{max} values were calculated, these values were in line with those determined in BK_{Ca} α cells, although there was greater variability in the data obtained from myocytes than from BK_{Ca} α cells.

The Rb^+ efflux response from myocytes to BKOEt1 was prevented by complete K^+ channel block with 4-AP and TEA. Therefore, BKOEt1 did not induce any non-specific Rb^+ efflux from cells in that all Rb^+ efflux (observed above control) can be attributed to K^+ channel activation. The assay revealed that BKOEt1 had a partial effect upon SK and K_v channels as it failed to activate Rb^+ efflux to maximum levels (within E_{max} range) in the presence of apamin and margatoxin respectively. BKOEt1 response was not affected by K_{ATP} channel blocker Glib or the IK channel blocker TRAM-34. BKOEt1 mediated increases in Rb^+ efflux were not completely blocked by pax. Therefore, BKOEt1 could have ancillary pharmacology with K_v and SK channels. This would require further investigation using electrophysiology.

Ancillary pharmacology has been ascribed for numerous different synthetic BK_{Ca}CO's. For example, as described previously (Chapter 1) NS1619 and NS004 have shown to activate numerous ion channels including CFTR-Cl

channel (Holland *et al* 1996; Patel *et al* 1998; Al Nakkash *et al* 2001). In addition, BMS-204352 has shown to have activated KCNQ/Kv7.x (Korsgaard *et al* 2005), di-Cl-DHAA shown to suppress VDCC currents (Sakamoto *et al* 2006) and BMS-223131 has shown potent cytochrome P450 activating properties (Vrudhula *et al* 2005).

There has been no previous instance in the literature of using Rb^+ efflux as a screening assay for K^+ channel modulators on primary dissociated cells from a tissue. Although more traditional radioactive rubidium (^{86}Rb) efflux assay has been used to assess the effect of K^+ channel modulation and function from whole or tissue strips from aorta (Greenwood and Weston 1993; Linde *et al* 1997), skeletal (Lindinger *et al* 2001) and bladder detrusor muscle (Edwards *et al* 1991; Trivedi *et al* 1994). In addition a non-radioactive, non- 96-well plate format Rb^+ efflux assay has been used to asses the function of potassium channels in lymphocytes isolated from human blood (Raphael *et al* 2005). Therefore this a novel reporting of applying a medium-throughput 96-well plate assay using primary/dissociated cells from tissue to assess the activating properties of and the specificity of KCO's.

5.6 Conclusions

BKOEt1 is a novel compound that has shown to potently activate BK_{Ca} channel currents in cloned cell lines expressing BK_{Ca} channel subunits and stimulate paxilline sensitive Rb^+ efflux from rat bladder myocytes. BKOEt1 affects the voltage sensitivity of BK_{Ca} channels increasing open pore probability. An exact site of interaction remains unknown, although it could potentially act at a site on the channel associated with the α-subunit in the transmembrane or intracellular part of the channel particularly at a site associated with Ca^{2+} regulation of the channel. Further work with mutated BK_{Ca} channel cell lines would be of interest and could provide information regarding this question. The properties of BKOEt1 as a BK_{Ca}CO's are summarised in table 5.2. Molecular modelling could provide clues as to the chemical or structural features required for BKOEt1 to be a BK_{Ca}CO (Chapter 6).

Summary of the Properties of BKOEt1 as a BK_{Ca} channel openers
A low-affinity activator of BK_{Ca} channels whose effects are reversible upon washout and can be blocked with paxilline (**Chapter 5.4.1 and 5.4.2**).
Increases the voltage sensitivity of BK_{Ca} channels and produces large and significant shifts of V_{50} to more hyperpolarising potentials (**Chapter 5.4.2**).
Increases P_o rapidly for small increases in membrane voltage (**Chapter 5.4.2**).
Activatation is equally effective when applied to either aspect of the membrane with similar apparent affinity and potency (**Chapter 5.4.1 and 5.4.2**).
Does not affect single channel conductance (**Chapter 5.4.3**).
Does not affect time to reach peak current or the deactivation time constant as determined from whole cell recordings (**Chapter 4.4.3**).
Activate BK_{Ca} channels in near absence of Ca^{2+} and its effects are not additive with increasing Ca^{2+} (**Chapter 5.4.4**).
Does not activate BK_{Ca} channels via a secondary effect by causing increases in intracellular Ca^{2+} or activation of VDCC (**Chapter 5.4.5**).
Hill slope of unity suggests one binding site per channel complex located on the α-subunit with a probable intracellular or transmembrane location (**Chapter 5.4.1 and 5.4.2**).
In Rb^+ efflux assay, the effect of BKOEt1 was not affected by co-expression of the β1-subunit (**Chapter 3.4.2**).
Stimulated paxilline sensitive Rb^+ efflux from rat bladder myocytes (**Chapter 5.4.5.2**).
Rb^+ efflux studies identified that BKOEt1 may activate K_v and SK channels (**Chapter 5.4.7.3**)

Table 5.2: Summary of the properties of BKOEt1 as a BK_{Ca}CO

Chapter 6

Pharmacophore modelling of benzanilides and general discussion

6.0 Molecular Modelling

6.1 Introduction

A pharmacophore is defined as an ensemble of steric and electrostatic features of different compounds which are necessary to ensure optimal supramolecular interactions with a specific biological target structure to trigger or block a biological response (Langer and Wolber 2004). Pharmacophore modelling provides a powerful tool in the design of pharmaceutical compounds with improved potency, selectivity and/or pharmacokinetic properties (Li and Harte 2002). The introduction of computer aided molecular design in the pharmaceutical industry has transformed the discovery and development of new classes of drugs. HTS bioassays produce a 0.1% 'hit' rate; pharmacophore modelling has enabled improvement to this figure by providing insight into the interactions between a ligand and target using 3D pharmacophores (Langer and Wolber 2004).

The determination of 3D pharmacophores is dependent upon on the availability of the 3D structure of ligand and target binding site. Further, 3D pharmacophores can be built when only the structure of target is known (without a ligand interacting) using programs such as LigandScout (Wolber *et al* 2006; Langer and Wolber 2004). However, if only ligand information is available the identification of pharmacophore relies upon the alignment of active molecules to identify common structural features and patterns. The Catalyst program by Accelrys Inc is the most common program used for this application (Langer and Wolber 2004).

Building of 3D pharmacophores for ligand-ion channel interaction is difficult. This is in part due to the complexities of ion channel structure and the dynamics of the gating process. This deems building realistic models that reflect the effect of binding of a ligand to a receptor unrealistic (Li and Harte 2002).

Neuronal sodium channel blockers have received the most investigation in terms of structure activity relationship (SAR) studies for ion channels; these

have been formulated by using data provided by Na^+ flux assays or by measuring the affinity of peptide blockers for the channel (Li and Harte 2002).

The lack of a protein structure of BK_{Ca} channels limits the ability to generate 3D pharmacophores of ligand-protein interaction. Pharmacophore models have been used for data mining and design of combinatorial libraries for ion channel interacting compounds for example with the identification of flavanoids as BK_{Ca}CO's (Li *et al* 1997). Further modelling of a series of carbonic anhydrase inhibitors has revealed the properties required for them to exhibit BK_{Ca}CO properties (Tricarico *et al* 2004).

Using a molecular mechanic approach benzanilides and NS1619 were examined for various molecular properties that could be used in determining the discriminate ability of certain compounds to activate BK_{Ca} channels. Further, to provide information on suggested pharmacophore features of this series of compounds and to use this information to guide the synthesis of further compounds.

Molecules were energy minimised and then aligned to identify structural differences or commonalitites between them. The pharmacodynamic profiles, electrostatic potentials and hydrogen bonding (H-bond) ability were determined. Compounds BKPr2-BKPr3-BKPr4 and BKMe1-BKH1-BKOEt1 are grouped as two sub-series for comparison.

6.2 Molecular modelling methods

Molecules were built using the molecular modelling package Chem3D Ultra version 10 (CambridgeSoft, Cambridge, U.K). The minimum energy conformation and most plausible geometry of each compound was searched using the AM1 mathematical algorithm method with a closed shell wavefunction and a eigenvector following (EF) routine geometry optimiser. This was performed in the semi-empirical program, molecular orbital PACkage (MOPAC) 2002 (CambridgeSoft, Cambridge, U.K). The convergence criterion for the energy gradient was <0.1 kcal mol^{-1} and to stimulate physiological conditions optimisation of the geometry was performed in the presence of a 1.4Å layer of water. All calculations were performed on a Viglen desktop PC.

Energy minimised and geometry optimised molecules, where appropriate, were aligned and overlayed. Three sites were chosen as points of alignment and included the amide linker and to a point on each ring system. Molecules were overlaid using Chem3D ultra 'fast overlay' algorithm to a 0.01 kcal mol^{-1} A^{-2} gradient and 0.1 RMS error.

For the optimised geometry of each molecule different molecular electronic properties were calculated. The Wang-Ford parametric electrostatic potential (PMEP) method of MOPAC.was used to calculate the charge distribution accross the molecule.

Also with the MOPAC interface the octanol-water partition coefficient (LogP) was calculated using the clogP driver. In addition, intramolecular hydrogen bonding (H-bond) were searched and their lengths were determined as a measure of validity.

Inter-molecular hydrogen bond (H-bond) sites of interaction were determined using the theoretical hydrogen-bond donor/acceptor (HBDA) calculator plug-in of the Marvin web-tool provided by the Chemaxon Company (Chemaxon, 2007). Those moieties available for H-bonding at at pH 7.2 were calculated.

In addition, this web-tool was also used to calculate theoretical pKa (logarithm of acid dissociation constant) values.

Total polar surface area (TPSA) and molecular volume (Van der Waals volume $Å^3$) was assessed by use of Molinspiration chemoinformatics web-tool (Molinspiration 2007).

Data tables and graphs of selected data were constructed using Graphpad Prism 4 and Microsoft word respectively. Data, where appropriate is displayed as means±SEM. Pictures of molecules were directly exported from Chemdraw Ultra.

6.3 Results

6.3.1 Energy minimisation and alignment

6.3.1.1 BKPr2, BKPr3 and BKPr4

Alignment of energy minimised molecules BKPr2, BKPr3 and BKPr4 was performed to reveal structural differences or commonalities that could be related to differences in efficacy of compounds. BKPr3 and BKPr4 were equally effective in electrophysiology screening whereas BKP2 was significantly less effective (Chapter 4.4.4).

Comparison of structures of the BKPr series indicates that the overall shape of these molecules are generally similar particulary with the N-benzyl ring alignment. However further examination of the linker region and the N-phenyl ring reveal structural differences that could account for the decreased efficacy of BKPr2 (Figure 6.1).

The NH group of the amide linker of BKPr2, BKPr3 and BKPr4 could act as a potential H-bond donor site. The overlay revealed a similar location of this group therefore differences in efficacy between the compounds cannot be attributed to the location of this H-bond site (Figure 6.1; Table 6.2). The carbonyl group of the amide linker could act as an H-bond acceptor site for compounds BKPr2, BKPr3 and BKPr4. The overlay for this group (Figure 6.1) showed that the carbonyl group was orientated differently with each compound and so its positioning is less critical for $BK_{Ca}CO$ properties (Figure 6.1).

The nitrogen atom of the pyridine ring is positioned differently between the BKPr series (Figure 6.1) with BKPr2 the pyridine nitrogen is located closer to the amide NH group of the linker (2.4 Å) compared to that of BKPr3 (3.8 Å) and BKPr4 (4.2 Å) which are located towards the end of the ring. Therefore, with BKPr3 and BKPr4 it could allow an interaction with a H-bonding amino acid in the BK_{Ca} channel protein. The closeness of the pyridine nitrogen of BKPr2 to the amide NH of the linker could promote an interaction so that the NH group would appear less available for hydrogen bond interaction and thus affect the H-bonding ability of the amide linker. There factors may account for the decreased efficacy observed for BKPr2.

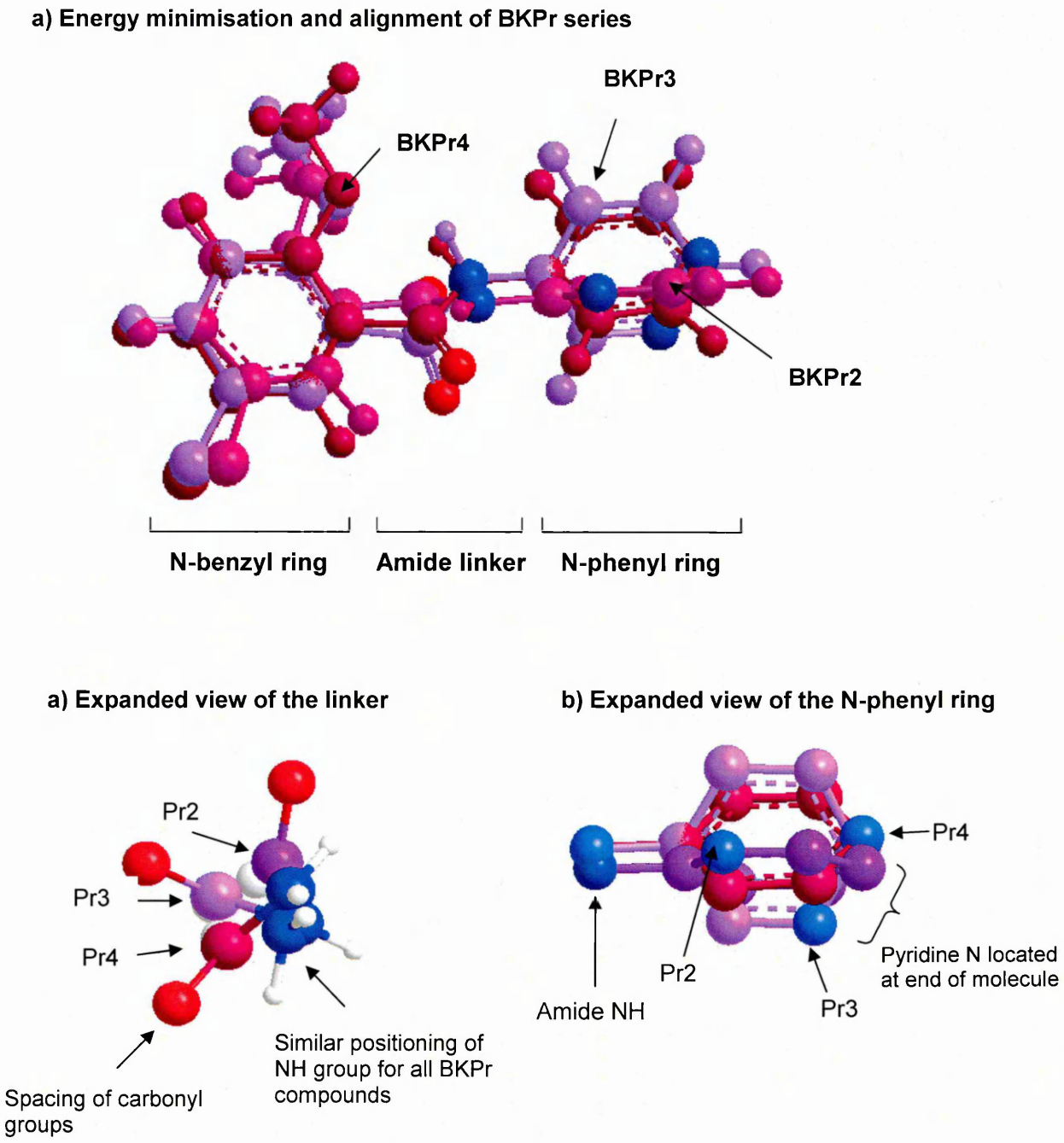


Figure 6.1: Overlaying of molecules BKPr2, BKPr3 and BKPr4

(a) Molecules were built and energy minimised using Chem3D Ultra with MOPAC and overlaid using 'fast overlay' algorithm. Also shown are expanded areas of the amide linker (b) and the N-phenyl ring (c, hydrogen atoms are not shown for clarity) to highlight structural differences. Nitrogen atoms are highlighted in blue and oxygen atoms in red.

6.3.1.2 BKH1, BKMe1 and BKOEt1

BKOEt1 was the most effective benzanilide tested and was 2-fold more effective than any other compound tested in this series. To assess whether any structural differences between BKOEt1, BKH1 and BKMe1 could account for differences in efficacy, energy minimisation using MOPAC and alignment of these molecules using 'fast overlay' algorithm (Figure 6.2) was performed.

All three compounds share an overall similar shape. The major difference in the conformation of these compounds is the extension of an ethoxy group on the benzyl ring of BKOEt1.

All three compounds in this series display a site for intramolecular H-bonding provided by the hydroxyl group of the N-phenyl ring and the amide NH of the linker (Table 6.2). In BKOEt1 a second site of potential intramolecular H-bonding can form between the oxygen of the ethoxy group and the amide NH of the linker. However, the oxygen atom of the ethoxy moiety is located in the same position as the methyl or hydrogen of BKMe1 and BKH1 respectively (Figure 6.2). Consequently, the overall shape of these three molecules at the benzyl ring suggested that the second intramolecular H-bond of BKOEt1 may not be important in the shape or positioning of an ethoxy group.

However, examination of the electrostatic potential profiles demonstrated that the presence of the ethoxy oxygen increases the region of negative charge compared to BKH1 and BKMe1 that may account for a stronger association of BKOEt1 with an area of positive charge on the channel (Figure 6.5). In addition, the presence of the ethoxy oxygen could provide an H-bond acceptor site. Therefore the presence of an oxygen group could be important for an electrostatic or H-bond interaction with the channel that is absent in BKH1 and BKMe1 (Table 6.2).

In addition to the oxygen atom the extension of an ethyl group (CH_2CH_3) on this structure seems to be important for improved efficacy. This contributes by increasing the cLogP value of BKOEt1 and hence hydrophobicity compared to BKH1 and BKMe1 (Figure 6.4c). This may increase the transport of compound (if required) to a potential receptor site within the transmembrane region of the

channel. From electrophysiology studies (Chapter 5) it was proposed that the site of action of BKOEt1 could be within the transmembrane region of the channel. To test whether the oxygen or increased hydrophobicity is important to this pharmacophore compounds bearing either ethyl or propyl or with methoxy or propoxy would provide evidence to which feature is important.

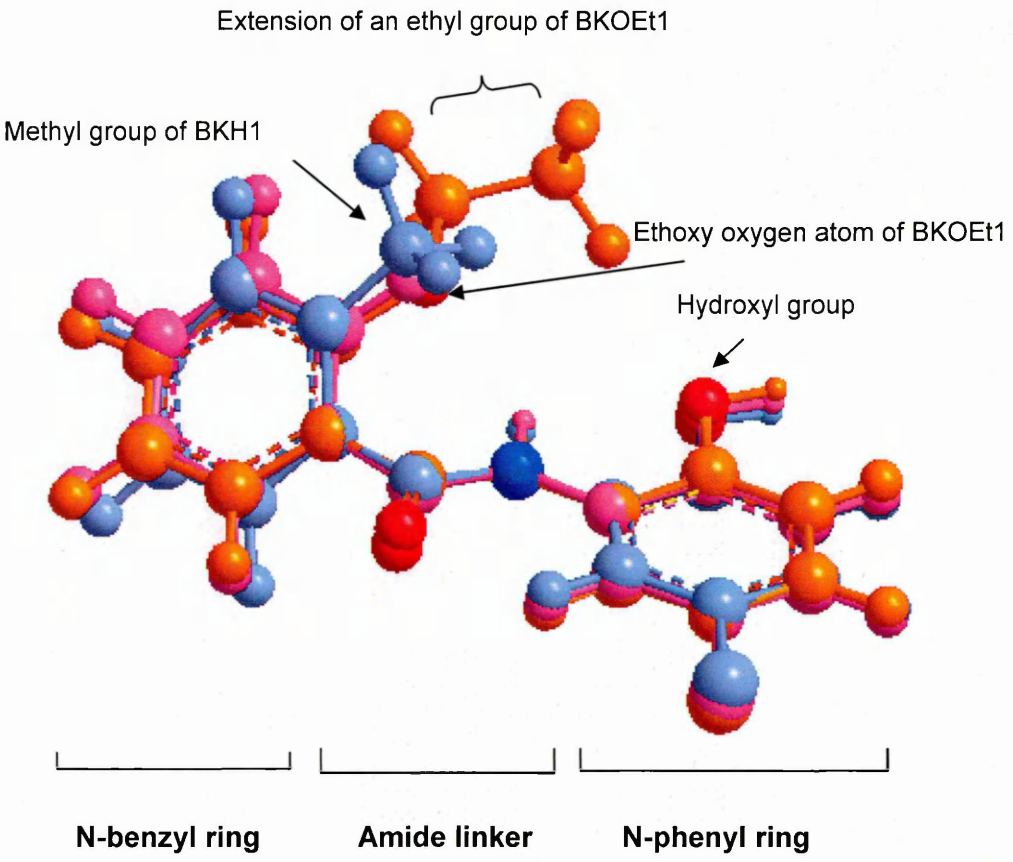


Figure 6.2: Overlaying of molecules BKH1, BKMe1 and BKOEt1

BKH1 (pink), BKMe1 (blue) and BKOEt1 (orange) molecules were built and energy minimised using Chem3D Ultra with MOPAC and overlaid using 'fast overlay' algorithm. Nitrogen atoms are highlighted in blue and oxygen atoms in red.

6.3.1.3 BKOEt1 vs NS1619

An overlay of BKOEt1 and NS1619 (Figure 6.3) was performed to determine if any commonalities or differences exist in the alignment of these two compounds that could account for similarities and differences in $BK_{Ca}CO$ properties determined for these compounds.

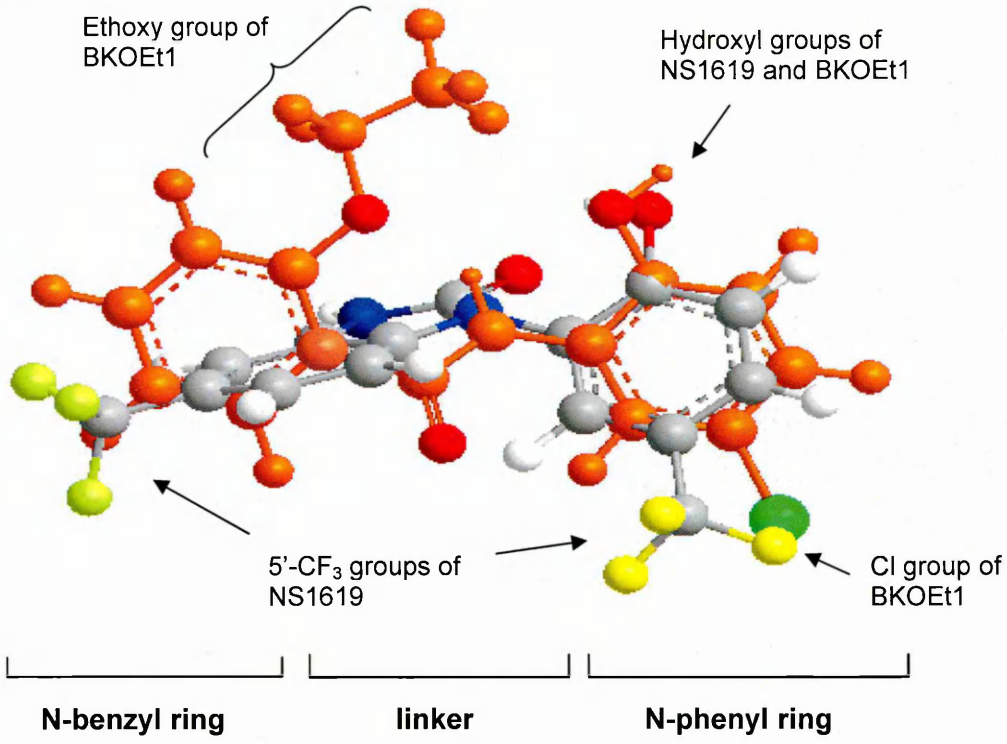
A good overlap in the N-phenyl ring is observed and indicated an essential requirement for a hydroxyl with a halogenated group in the *para* position for both these compounds to display $BK_{Ca}CO$ properties (Figure 6.3). In contrast the N-benzyl ring of BKOEt1 has a dissimilar structure and conformation to NS1619.

The mechanism of BK_{Ca} channel activation by BKOEt1 is different to NS1619 as this reference $BK_{Ca}CO$ has different channel activation properties to BKOEt1. It was demonstrated that BKOEt1 affects the channels voltage-sensitivity (Chapter 5) whereas NS1619 has been shown not to demonstrate this property (Lee *et al* 1995b). In addition, NS1619 had different affects on activation/deactivation kinetics of the channel compared to BKOEt1 assessed using whole cell electrophysiology (Chapter 4).

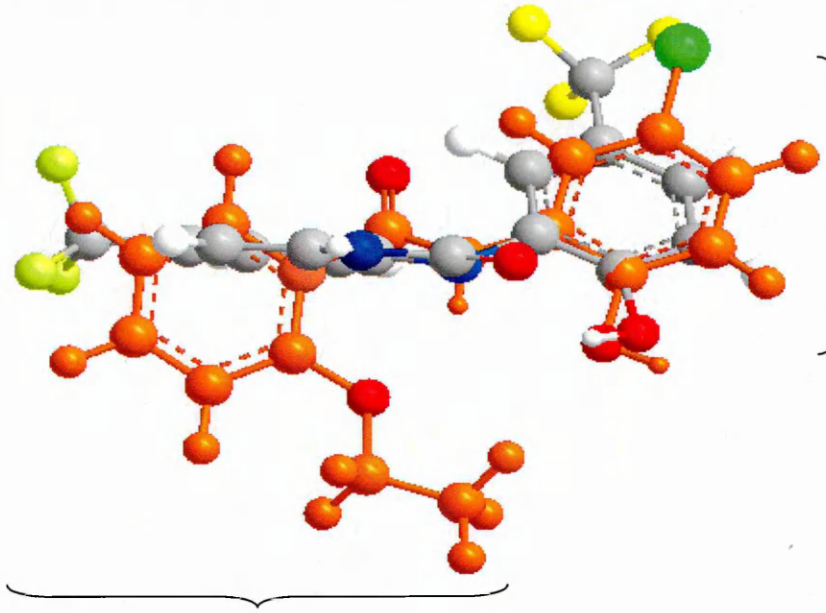
The similarity in the N-phenyl ring may form a 'molecular region' that could be important in affinity and/or specificity of these compounds to a receptor site on the channel. This is supported by experimental data in that BKOEt1 has a similar potency, affinity and co-operativity in activation to NS1619. In addition, both compounds activate the compound equally effectively when applied to either aspect of the channel with similar onset times. Furthermore, both compounds have similar cLogP values (Figure 6.4c).

Therefore, a similar N-phenyl ring arrangement could confer a similar receptor site on the channel for both compounds but with BKOEt1 activating the channel via a different mechanism to NS1619. The difference in the mechanism of activation could be attributed to the structural differences in the benzyl ring (Figure 6.3).

Side view



Flipped view (horizontal by 180°)



The N-phenyl ring of both compounds is located in a similar spatial location. Both rings could interact with the channel at a similar site.

The benzimidazolone ring (grey) of NS1619 and the linker and N-benzyl ring of BKOEt1 (orange) have different orientations and occupy different spaces relative to each other

Figure 6.3: Alignment of NS1619 and BKOEt1

NS1619 (grey) and BKOEt1 (orange) molecules were built and energy minimised using Chem3D Ultra with MOPAC and overlaid using 'fast overlay' algorithm. Shown are the side view orientations. Fluorine atoms are highlighted in green, chlorine in yellow, oxygen is red and nitrogen atoms are blue.

6.3.2 Physiochemical properties

The effect of different physiochemical properties such as cLogP, total polar surface area (TPSA) and molecular volume (Van der Waals volume) was determined for each benzanilide and NS1619 and correlated with efficacy. Efficacy was defined as the shift in V_{50} from control of the G-V curve in the presence of each benzanilide (30μM), (Chapter 4, section 4.4).

Total polar surface area (TPSA) is a measure of the sum of surface contributions of all polar atoms in the molecule and has been shown to be a useful measure in drug transport properties. A TPSA of >140 $Å^2$ are molecules with poor membrane penetration and compounds with a TPSA <60 $Å^2$ are required for blood brain penetration (Ertl *et al* 2000). TPSA values were within a narrow range with values for benzanilides (except BKVV) and NS1619 between 50-60 $Å^2$ and consequently there was no correlation with efficacy. However, a high value of TPSA was not favourable for increased $BK_{Ca}CO$ properties as BKVV (95.2 $Å^2$) was less effective.

Lipophilicity is a major determinant in the biological activity of certain drugs a measure of this is the partition coefficient (logP) which is a ratio of compound in aqueous phase to the concentration in an immiscible solvent (neutral molecule), (Mannhold and Van de Waterbeemd 2001). This was calculated using cLogP program using Chem3D Ultra 10 which is a fragmentation system that generates theoretical values based on octanol/water partition coefficient (Mannhold and Van de Waterbeemd 2001). LogP values provide information of compounds ability to be formulated, dosing and sites of optimal penetration within the body. Medium values of logP, in the range of 2.5 to 3.5, were calculated for the least potent benzanilides; interestingly the most lipophilic compounds were the most potent. BKOEt1 and NS1619 demonstrated high values of clogP (4-5). Therefore, the ability of benzanilides to be available to activate BK_{Ca} channels could be due to the relative differences in lipophilicity.

Molecular volume (Van der Waals volume) is the volume of the union of overlapping spheres generated from the Van der Waals radii for each atom of a molecule. This is employed as a measure of molecular similarity between molecules and is used to help understand the steric requirements of a receptor

site to a particular ligand. The strongest correlation between $BK_{Ca}CO$'s and activity was observed with molecular volume. This suggested that molecular size is a key determinant for activity of benzanilides. BKOEt1 had a similar molecular volume (250.7 $Å^3$) to NS1619 (259.4 $Å^3$) whether this size is important for access to and occupation of the same site for both molecules on the receptor is unknown.

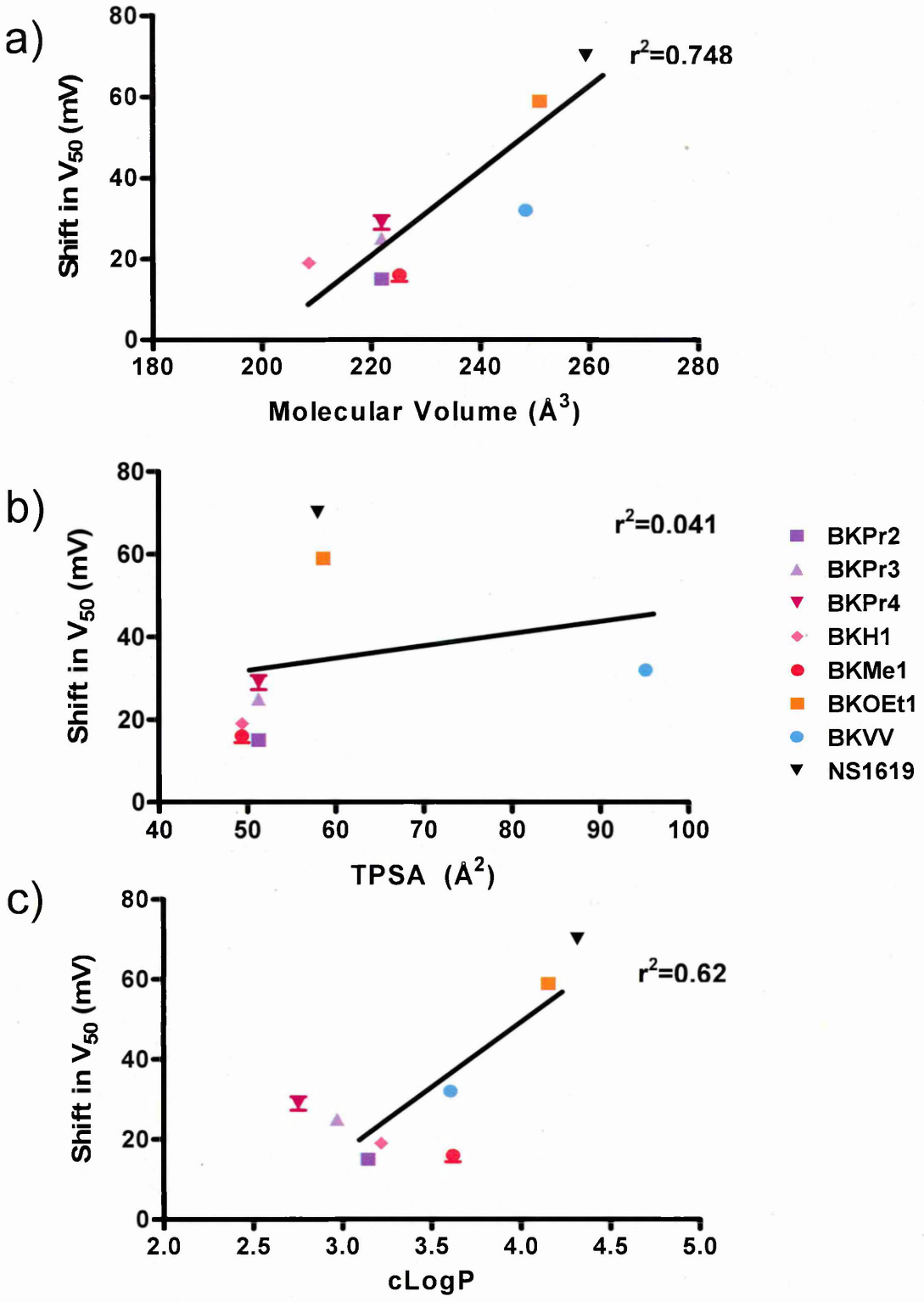


Figure 6.4: The physiochemical properties of benzanilides

The efficacy was determined as the shift in V_{50} relative to control for each compound (at 30μM) from WCR. This was correlated with either molecular volume **(a)**, total polar surface area (TPSA) **(b)** or cLogP **(c)** determined using either ChemDraw 3D or for molecular volume using Molinspiration chemoinformatics web-tool. Data expressed as means±SEM. Data points were fitted by linear regression (y= mx + C) analysis, r^2 values are displayed.

6.3.3 pKa determination

pKa (logarithm of acid dissociation constant) values were determined to indicate whether compounds could act on intracellular sites as ionised molecules are less able to cross biological membranes compared to non-ionised compounds. Therefore, pKa was examined for benzanilides. Although a variety of pKa values were determined there was no correlation between pKa and $BK_{Ca}CO$ ability. For example BKH1, BKMe1 and BKOEt1 had the same pKa values. NS1619 had a similar pKa value in the range of that for BKOEt1, BKH1 and BKMe1. Although there was more variability in pKa for compounds BKPr2, BKPr3 and BKPr4 with BKPr2 from this series having a much lower pKa value. However at pH 7.4, the value for the physiological buffers used in electrophysiology, all compounds (except BKVV) had the same degree of ionisation. Therefore, most compounds exist as neutral molecules and would be available for H-bond interaction with the channel and they could transverse biological membranes. Interestingly, BKVV at pH 7.4 was much more ionised (51.81%); this may afford an ionic or dipole-dipole interaction with the channel.

Compound	pKa values	% molecules ionised at pH 7.4
NS1619	8.31 11.74	7.25
BKPr2	9.83[a]	0.24
BKPr3	9.80[a]	1.17
BKPr4	10.20[a]	1.26
BKH1	8.22[a] 11.54[b]	8.73
BKMe1	8.20[a] 11.66[b]	8.70
BKOEt1	8.20[a] 11.13[b]	9.00
BKVV	7.17[a] 9.70[b]	51.81

Table 6.1: pKa values determined for benzanilides and NS1619 and the % ionisation of compounds at pH 7.4

a= pKa values for phenol; $OH \rightleftharpoons O^- + H^+$

b= pKa values for amide; $N^+H_2 \rightleftharpoons NH + H$

6.3.4 Hydrogen bonding properties of benzanilides

The number of potential H-bond donor and acceptor sites on the compounds was determined (Table 6.2). The locations of intramolecular H-bond were examined and distances were determined as a measure of validity in the strength of bonding. An intramolecular hydrogen bond has a typical distance of between 1.8 Å to 3.5 Å. Intermolecular H-bond distances for benzanilides were between 2.2 to 2.3 Å. Jeffrey (1997) describes H-bonds with donor-acceptor distances of 2.2 to 2.5 Å as strong, mostly covalent interaction, and therefore this can be applied to all H-bonds measured. The most active compounds; NS1619 (10 sites), BKOEt1 (6 sites) and BKVV (6 sites) have more sites of potential H-bond interaction than other benzanilides (<5 H-bond sites). BKOEt1 compared to BKH1 and BKMe1 has one additional H-bond acceptor site provided by the ethoxy group.

Compound	Intra-molecular H-bond (length in Å)	Potential H-bond donor sites	Potential H-bond acceptor sites
NS1619	OH to C=O (2.2Å)	OH and NH	C=O, O-H & 2x (CF_3)
BKPr2	----------	NH	O-CH_3, C=O, -Cl & N of pyridine
BKPr3	NH to O-CH_3 (2.3Å)	NH	O-CH_3, C=O, -Cl & N of pyridine
BKPr4	NH to O-CH_3 (2.3Å)	NH	O-CH_3, C=O, -Cl & N of pyridine
BKH1	NH to OH (2.3Å)	OH and NH	C=O, O-H & -Cl
BKMe1	NH to OH (2.2Å)	OH and NH	C=O, O-H & –Cl
BKOEt1	NH to OH (2.3Å) NH to O-Et (2.2Å)	OH and NH	C=O, O-H, -Cl & O-Et
BKVV	----------	OH and NH	C=O, O-H & 2x(N-O)

Table 6.2: Hydrogen bonding properties of benzanilides

BKPr2, BKPr3 and BKPr4 have the same number of H-bond donors and acceptor sites therefore this is not a distinguishing feature for differences in efficacy. However, as regards intramolecular H-bonds, BKPr3 and BKPr4 have one intramolecular H-bond site where as BKPr2 does not display an interaction.

6.3.5 Molecular electrostatic potential profiles

The electrostatic profile of NS1619 demonstrated three distinct negatively charged areas, Two are formed from the CF_3 groups and a third larger region formed from the carbonyl oxygen of the benzimidazolone ring and the hydroxyl group of the N-phenyl ring (Figure 6.5). This observation has previously been determined for NS1619 (Tricarico *et al* 2004; Calderone *et al* 2007).

Common to all the benzanilides is a large region of negative charge. For the BKPr series it is formed between the carbonyl oxygen of the amide linker and the oxygen of the methoxy group. In addition, for BKH1, BKMe1 and BKOEt1 the region is formed between the amide linker and the hydroxyl group of the N-phenyl ring. In addition, BKVV has one large area of negative charge that envelopes over the amide linker and the nitro group. Therefore an area of negative charge may be a fundamental requirement for compounds to display $BK_{Ca}CO$ properties.

In total BKH1, BKMe1 and BKOEt1 display two regions of negative charge. However, comparable to NS1619; BKPr2, BKPr3 and BKPr4 display three distinct negative charge regions. One as described above and a second associated with the chlorine and a third area whose location is dependent upon the positioning of the pyridine nitrogen. For BKPr2 this charged region occurs nearer to the amide NH group than in BKPr3 and BKPr4 and as suggested this charged area could affect the electronic properties of the amide NH. Due to the commonalities and differences in the electrostatic profiles between the BKPr compounds and NS1619 It would be of interest to perform further electrophysiological studies to evaluate whether these compounds activate the channel with similar or distinct properties to either BKOEt1 or NS1619.

BKH1, BKMe1 and BKOEt1 display a similar negative electrostatic potential profile across the molecules. However, BKOEt1 provides an extension of bulky non-polar ethoxy group in *ortho* position of the ring that occupies an empty space. This could promote a favourable interaction with hydrophobic residues on receptor site of channel. This compared to an absence of this moiety with the less effective BKH1 and BKMe1. Therefore, BKOEt1 has a bulky non-polar group essential for interaction (Figure 6.5). The addition of bulky non-polar

group could be required for hydrophobic interaction with a receptor site on the channel or it could promote an increase in molecular volume that is required for optimum fit to receptor site. The addition of ethoxy group could induce an increase in logP and lipophilicity thus enabling easier access to the receptor site compared to BKH1 and BKMe1 which are less lipophilic.

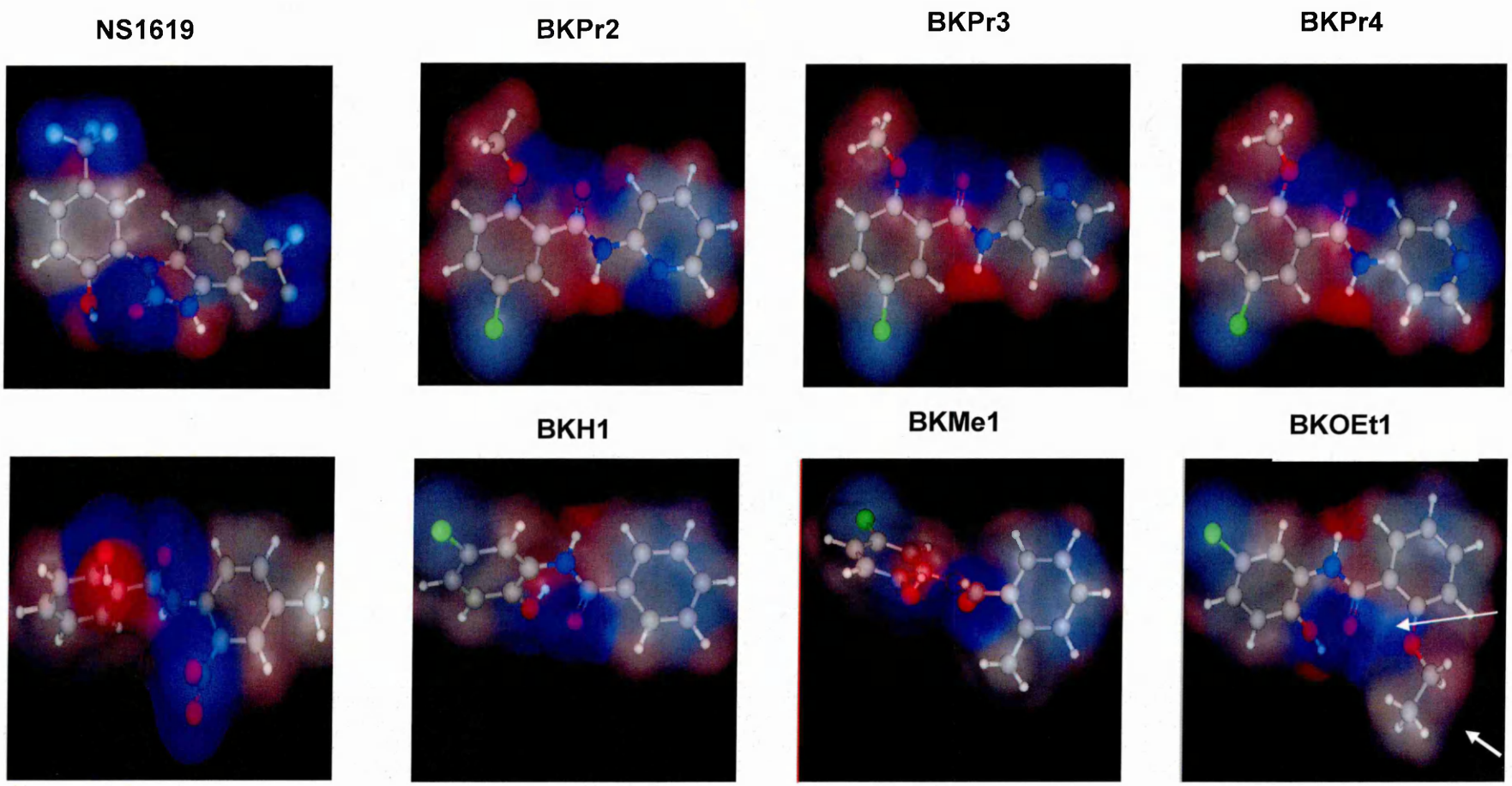


Figure 6.5: Molecular electrostatic potential profile of benzanilides and NS1619

Electrostatic charge profiles of energy minimised compounds were determined using the Wang-Ford PMEP method in MOPAC. Areas of positive charge are highlighted in red, negative in blue and un-charged areas in white.

6.4 A confirmatory pharmacophore for benzanilides?

A generalised pharmacophore pattern has been previously proposed that could form the basis to guide the design of new molecules demonstrating $BK_{Ca}CO$ properties as discussed in chapter 1, section 14 (Figure 1.26). This pattern is represented by two multiple substituted phenyl rings an 'A' and 'B' ring, that are structurally attached by a linker region (Coughlan *et al* 2001; Calderone *et al* 2005a,b; Nardi *et al* 2007).

Benzanilides share a similar pattern to this generalised pharmacophore template and represent the first group of compounds to be reported that are separated by an amide linker introducing a three bond spacer between two phenyl rings.

The presence of hydroxyl groups has been shown to be important in the design of many $BK_{Ca}CO$'s including benzimidazolones (Meanwell *et al* 1996), triazolones (Romine *et al* 2002) and oxadiazolones (Hewawasam *et al* 2003). Further, a carboxylic acid group is also a feature for a number of different $BK_{Ca}CO$'s including acrylamides (Lennox *et al*, Wyeth 1999; Lennox *et al,* Wyeth, 2000), fenamates (Ottolia and Toro 1994; Wu *et al* 2001) and benzofuroindoles (Butera *et al* 2001; Ha *et al* 2006), positioned homologous to the hydroxyl group of the benzimidazolones. Whether the whole carboxylic acid moiety, the hydroxyl group or oxygen atoms are important remains unknown. However, other $BK_{Ca}CO$'s including flavanoids and phenols rely upon the presence of hydroxyl groups for activity.

For benzimidazolones it was proposed that the phenolic OH and carbonyl oxygen could act as a mimic of a carboxylic acid (Meanwell *et al* 1996; Li *et al* 1997). Further, the amide hydrogen and amide carbonyl could also act as a surrogate carboxylic acid. These were essential features for $BK_{Ca}CO$ properties of the benzimidazolones (Meanwell *et al* 1996). The benzanilides; BKH1, BKMe1, BKOEt1 and BKVV have an oxygen containing group provided by a hydroxyl or methoxy group that occupies a similar position to the hydroxyl group of the benzimidazolones. It is unlikely that the hydroxyl and the carbonyl group

of the benzanilides would act as a mimic of a carboxylic acid due to the distance of separation. Furthermore, the NH and carbonyl group within the linker could not mimic a carboxylic acid due to the orientation of these groups relative to each other.

Modelling studies for triazole compounds revealed that the relationship between the carbonyl group and the phenol was not important for $BK_{Ca}CO$ activating properties. However, the relationship and distance between the oxygen of phenol and the amide hydrogen of the triazole ring was important (Romine *et al* 2002). This observation can be made for benzanilides as the presence of an NH group in relation to an oxygen containing moiety on the 'A' ring was important for the introduction of an intramolecular H-bond.

In addition removal of NH from triazoles while retaining the carbonyl group led to inactive compounds; therefore a H-bond donor or protonated N in the heterocycle was thought to be essential for these compounds (Romine *et al* 2002). The presence of this intramolecular H-bond was a feature present for all benzanilides; BKPr2 lacked this feature and consequently was the least effective benzanilide. This could be verified by synthesising a BKOEt1 compound without an NH group in the linker, and this would help elucidate the importance of an NH proton donating group in the linker region.

It has also been suggested that the amide NH of benzimidazolones acts as a weak acid and that this acidity is increased by the attachment of EWG to the heterocyclic nucleus (Li *et al* 1997). Li *et al* (2003) determined that addition of halogens to bisphenols increased the acidity of the hydroxyl groups and those with a pKa value of less than 8 were the most active $BK_{Ca}CO$'s. However there was no correlation between pKa determined for the benzanilides and their effectiveness as BK_{Ca} channel openers.

The compounds BKPr2, BKPr3 and BKPr4 have a methoxy in place of a hydroxyl group in a comparable location with NS1619 and the other benzanilides. The similarity in efficacy between BKH1, BKMe1, and the BKPr series revealed that the hydroxyl function may not be important for activation properties. Substitution of hydroxyl groups with methoxy groups has been

shown to retain $BK_{Ca}CO$ activity, for example with Maxipost (Gribkoff *et al* 2001a), 3-aryloxindoles (Hewawasam *et al* 1997) and triazoles (Biagi *et al* 2000). The presence of an oxygen containing moiety at this position introduced an intramolecular H-bond site which is found as a feature in all benzanilides except BKPr2 which was the least effective. Therefore, the presence of this intramolecular H-bond is a favourable feature for $BK_{Ca}CO$ properties of benzanilides.

Another common feature for $BK_{Ca}CO$ is the presence of a halogen group in the *para* position to the OH or methoxy function. All benzanilides (except BKVV) had Cl atom in the *para* position to the respective function. However, BKVV displayed similar efficacy without Cl in *para* position to hydroxyl group therefore halogenations of this ring are probably less important than the hydroxyl or methoxy function.

Alignment of BKOEt1 and NS1619 showed that there was a degree of overlap between the N-chlorophenol moiety of BKOEt1 and the trifluoromethyl-phenol of NS1619. For both compounds this 'A' ring adopted a planar conformation. However, there was more flexibility in 'B' ring of BKOEt1 than NS1619 which adopted a co-planar alignment across the whole molecule.

Examination of the 'B' rings of BKOEt1 and NS1619 revealed significant differences in structure between the two molecules. Whether this can account for differences in mechanism of action of these compounds remains unknown. However, it does open up the structure of BKOEt1 and induce more flexibility.

Investigation of structural similarity between benzofuroindole and acrylamides by over-lapping of the two structures revealed that the right hand side of the overlap demonstrated good overlay with a common polar feature. In contrast, the left hand side of the rings demonstrated poor overlap showing that these structures had tolerance to different types of subsituents in this side of the molecule; this led to pharmacophore probing of these compounds (Nardi *et al* 2006; Nardi *et al* 2007)

For benzanilides the positioning of an ethoxy group (as with BKOEt1) on the 'B' ring increased hydrophobicity of the molecule and significantly increased its efficacy. Further probing of this ring with other hydrophobic residues such as trifluoromethyl, methoxy, propoxy or the addition of benzyl/phenyl rings could help to further probe the pharmacophore of benzanildes.

The ethoxy group of BKOEt1 does not have steric effects on the molecule as it retains the same shape as BKH1 and BKMe1. Further it does not affect the electronic properties of this ring and the molecules as all three compounds share similar negative electrostatic potential profiles. However, the ethoxy moiety does increase molecular volume and projects out hydrophobic residues to occupy a space not observed with BKH1 and BKMe1.

The addition of non-polar or hydrophobic groups to the 'B' ring such as methyl or benzene rings has shown to increase potency and efficacy. For example, Ohwada *et al* (2003) determined for DHAA derivatives that the addition of hydrophobic groups increased potency and efficacy of the template molecule. Moreover, studies from the same researchers revealed that small differences in the extension and direction of hydrophobic residues at C13 of PiMA derivatives was important for activity and that these groups bind to a common hydrophobic site of the α-subunit. Further, examination of tamoxifen derivatives has shown that addition of hydrophobic groups is critical for activity (Sha *et al* 2005). Moreover, the addition of hydrophobic groups has shown to increase potency of triazolyl -benzotriazoles and -benzimidazolones (Baragatti *et al* 2000; Biagi *et al* 2001) and hydroxyalkyl quinolin-2-ones/BMS223131 (Hewawasam *et al* 2003).

The addition of non-polar groups to $BK_{Ca}CO$ molecules could have potential steric or electronic effects on the molecule; this has been suggested previously for the addition of halogens and hydrophobic groups to phenyl rings of different classes of $BK_{Ca}CO$'s including triazoles (Calderone *et al* 2005b) and 2-amino-4-azaindoles (Turner *et al* 2003) but the effects of these subsituents have not been modelled. In addition, Ottolia and Toro (1994) proposed that the fenamates (niflumic and flufenamic acid) bind to a receptor confined to a hydrophobic pocket within the cytosolic side of the channel.

Duncan (2005) proposed that due to the lipophilic nature of tamoxifen that it could result in channel activation via mechanical stresses it exerts on the membrane bilayer rather than directly binding and activating the channel. Further a similar feature has been shown to occur with cholesterol in that increases in membrane content of cholesterol caused a decrease in BK_{Ca} single channel conductance and mean open time (Chang *et al* 1995). Therefore, lipophilic drugs such as the benzanilides may activate the BK_{Ca} channel through indirect non-specific effects on the properties of the lipid bilayer.

The pKa values determined revealed that the benzanilides remain in neutral form in the pH of buffers used, therefore this coupled to the relatively high clogP values determined indicated that the benzanilides could readily cross the plasma membrane. This supports the finding that BKOEt1 activated, in electrophysiology, the channel with apparent equal affinity when applied to either aspect, so could be concluded that there is a site located in the transmembrane region of the channel.

Further examination of the allosteric sites of interaction on the α-subunit in addition to investigating differential effects in different tissues needs to be investigated to identify specificity of BKOEt1. Further exploration of this could be achieved by expressing mutant BK_{Ca} channel α-subunits and using Rb^+ efflux assay as a screening tool to assess differences in potency and efficacy.

Slight differences in the structure of BKPr2, BKPr3 and BKPr4 had pronounced effects on channel activation. This is a common feature for BK_{Ca}CO as minor variations and structural isomer of PiMA (Imaizumi *et al* 2002), ketoconazole derivatives (Power *et al* 2004) and thymol (Huang *et al* 2005) have shown to have marked effects on the compounds ability to activate the channel. Further, alterations to template structures can also switch specificity of compounds as displayed by 3-fluorooxindoles (BK_{Ca} to K_V7), (Korsgaard *et al* 2005) and acrylamides (BK_{Ca} to KCNQ), cyclobut-3-ene-1,2,-diones (K_{ATP} to BK_{Ca}), (Butera et al 2005) and tetraycle compounds (K_{ATP} to BK_{Ca}) , (Butera *et al* 2001).

Therefore, caution should be taken when making changes to the template structures of benzanilides as small changes in addition to having beneficial

effects on increasing efficacy can also switch specificity. The effect of BKOEt1, when tested on rat bladder myocytes was sensitive to the SK and K_v channel blockers apamin and margatoxin. Genetically and to a certain extent functionally BK_{Ca} channels in some aspects are closer to K_v channels rather than KCNN (SK and IK) channels in particular due to the presence of the voltage-sensor domain (Vergara *et al* 1998). Therefore, as BKOEt1 has a predicted transmembrane binding site and interacts with the channel to increase voltage sensitivity then examination of the K_v channel activating properties of BKOEt1 would be useful.

Unlike that reported for other BK_{Ca} channel openers some compounds of the benzanilide series including BKPr2, BKPr3, BKPr4, and BKH1 displayed specificity to BK_{Ca} channels expressing BK_{Ca} α subunit only. These compounds could form the basis of a pharmacophore for investigating agents with differential selectivity profiles.

Benzanilides display particular physiochemical properties, for example the most effective benzanilides (and NS1619) had a molecular volume >240$Å^3$ and a clogP value of >4.0 with a 50-60 $Å^2$ range in TPSA. In addition, the presence of at least one intramolecular H-bond site seems to be a requirement for all compounds. Further, two intramolecular H-bond sites was also tolerated and could improve efficacy as with BKOEt1 (two sites) compared to BKH1 and BKMe1 (one site). This series of benzanilides contained at least five sites of H-bond interaction the importance of which remains undetermined but it seems at least two H-bond donor sites are required. Therefore, these factors are key to consider in the design of BK_{Ca}CO's based on a benzanilide template.

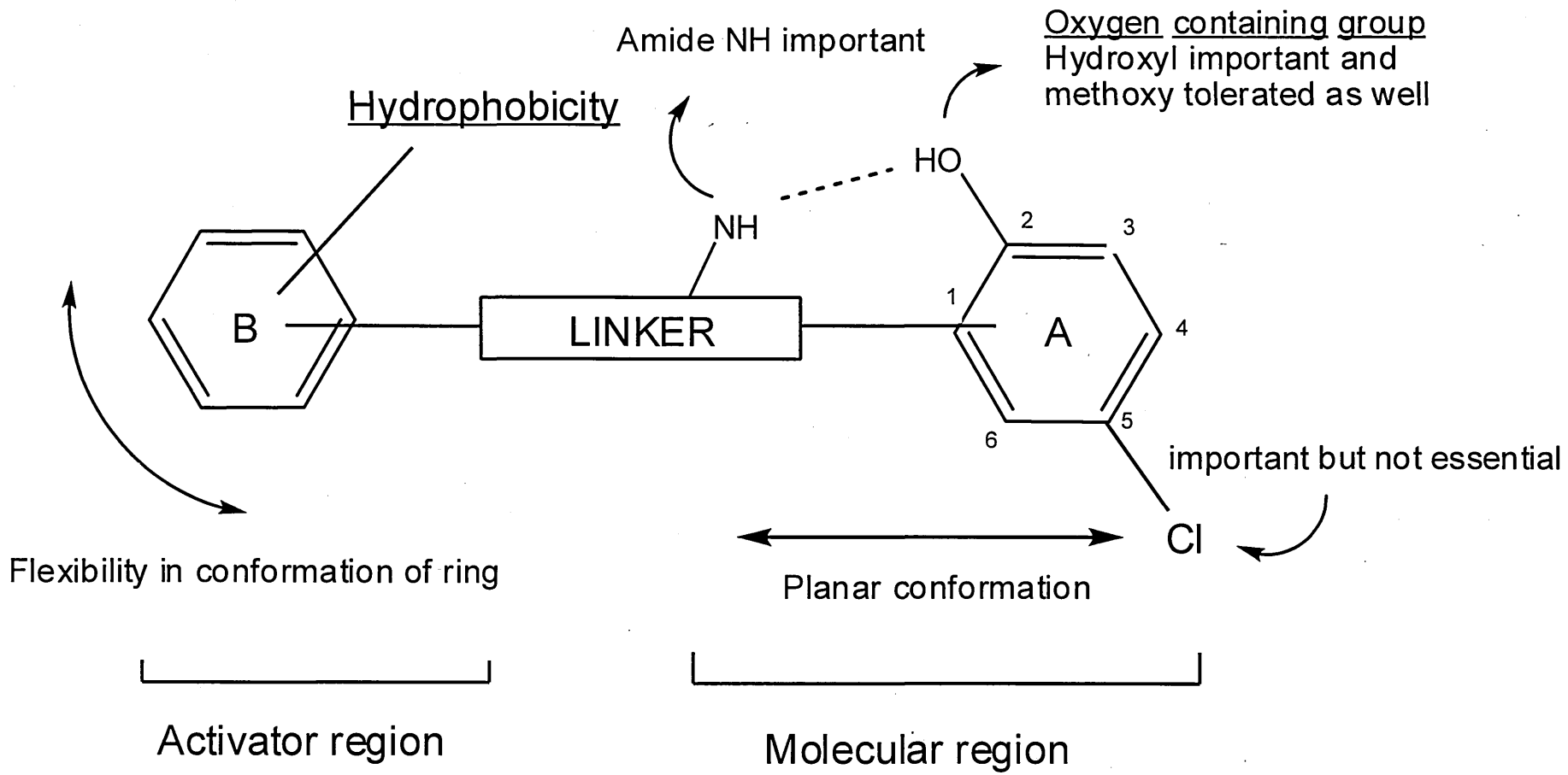
All benzanilides tested fall under the requirements of Lipinski's rule of five which are molecular properties important for drugs to display suitable pharmacokinetic properties *in-vivo* and include no more than 5 H-bond donors, 10 H-bond acceptors and a molecular weight not greater than 500 or clogP greater than 5 (Lipinski *et al* 2001).

Therefore the properties of this series of benzanilides confirm some of the pharmacophore properties suggested for other reported $BK_{Ca}CO$'s. To retain potent $BK_{Ca}CO$ properties compounds require two substituted phenyl rings, the presence of an oxygen containing group, an amide group provided by the linker region and hydrophobic moieties. Further refinement of the generalised pharmacophore model can be achieved by proposing a confirmatory pharmacophore based on the results presented here for the benzanilides (Figure 6.6).

The 2' acidic function is thought to either impart an H-bond donor site with the channel. In addition, the ionic form of this group such as with the hydroxyl subsituent (O^-) could promote a dipole interaction with the channel. Replacement of an acidic moiety with a methyoxy group is also tolerated but in certain compounds resulted in less active openers. Whether these discrepancies are due to the steric 'bulk' effect on the compound or whether it affects the type of interaction with the linker region or the affinity to the channel remain un-resolved.

It can be concluded that the 'A' ring and the linker region could act as the 'molecular region' that is important for binding, affinity and apparent specificity of molecules to BK_{Ca} channels. Where as the 'B' ring is important for the activation properties of compounds. Further that the subsituents of the 'B' ring could also affect the transport properties of drugs across the membrane. In addition, the composition of the 'B' ring could affect the steric conformation of the molecule affecting the type of interaction and mechanism of activation of the channel.

Synthetic molecules that open BK_{Ca} channels offer a novel therapeutic approach for controlling cellular excitability. The benzanilides studied could provide potential therapeutics for un-met clinical conditions and be useful in the understanding of the structure and physiological functions of BK_{Ca} channels.



6.6 A confirmatory pharmacophore model for $BK_{Ca}CO$'s

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