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Molecular Pharmacology of the Capsaicin Receptor (TRPV1) in the Airways

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ABSTRACT
RESPIRATORY MEDICINE
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MOLECULAR PHARMACOLOGY OF THE CAPSAICIN RECEPTOR (TRPV1) IN THE AIRWAYS

By Laura Rachel Sadofsky

The capsaicin receptor (vanilloid receptor 1, transient receptor potential vanilloid 1 or TRPV1) is a member of the transient receptor potential (TRP) family of proteins. This cation channel is sensitive to a range of inflammatory mediators such as some lipoxygenase products, as well as the tussive agents capsaicin, resiniferatoxin and protons. It has been proposed that TRPV1 is a cough receptor and may be important in airways inflammation.

Rat TRPV1 (rTRPV1) and human TRPV1 (hTRPV1) permanently expressing cell lines were generated and successfully characterised by agonist triggered changes in intracellular calcium levels. Thapsigargin and/or removal of extracellular calcium revealed that, both rTRPV1 and hTRPV1 are not only expressed on the cell surface but on thapsigargin sensitive and insensitive intracellular stores respectively.

Citric acid, an agent routinely used in the clinic for inhalation cough challenges, was investigated for its ability to activate TRPV1 permanently expressed in a cell line. rTRPV1 was activated by citric acid in a concentration and pH dependent manner. Citric acid activation of TRPV1 was inhibited by iodoresiniferatoxin but not capsazepine. Mutation of the TRPV1 putative proton binding site (E648 to A648) abolished citric acid activation of the channel without reducing the capsaicin evoked response. Thus, citric acid activates rTRPV1 by a proton dependent mechanism.

The role of *N*-linked glycosylation and sialylation on rTRPV1 and hTRPV1 was investigated. Treatment of rTRPV1 with neuraminidase or tunicamycin dramatically reduced the channels' maximal responses to capsaicin. In addition mutation of the rTRPV1 *N*-linked glycosylation site (N604 to Q604) or expression of rTRPV1 in the glycosylation mutant cell line, Lec2, also resulted in a striking reduction in the receptors' maximal calcium response to capsaicin. Flow cytometry data indicated that these differences in TRPV1 function were unlikely to be linked to differences in receptor cell surface expression. Human TRPV1 also displayed significant reductions in responsiveness to capsaicin following either neuraminidase or tunicamycin treatment. Thus, receptor sialylation regulates TRPV1 activation by capsaicin.

Finally, TRPV1 expression on human primary bronchial fibroblasts (HPBF) was investigated. Negligible endogenous TRPV1 expression was detected in HPBF. Interestingly, the inflammatory mediators tumour necrosis factor (TNF-α), lipopolysaccharide (LPS) and interleukin 1α (IL-1α) all induced TRPV1 expression in HPBF, as assessed by RT-PCR, flow cytometry and calcium signalling. TRPV1 functional expression was observed as early as 6 hrs (for TNF-α) post challenge and remained elevated upto the final time point tested (96 hrs for IL-1α). Thus, TRPV1 may play an important role in the inflammatory process.

In conclusion, TRPV1 may play an important role in conditions where cough and inflammation have been implicated.

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Publications

Papers

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Conference Abstracts

Sadofsky, L. R., Compton, S. J. and Morice, A. H. (2003) Citric acid activates rat Vanilloid Receptor 1 (rVR1). Thorax, 58, supplement III.

Sadofsky, L. R., Compton, S. J. and Morice, A. H. (2004) Molecular investigation of cough receptor (Vanilloid Receptor 1, VR1) stimulation by citric acid and capsaicin. Am J Respir Crit Care Med, 169, A800

Sadofsky, L. R., Compton, S. J. and Morice, A. H. (2004) Could capsaicin act intracellularly?. Thorax, 59, supplement II, ii45.

Sadofsky, L. R., Compton, S. J. and Morice, A. H. (2005)Rat transient receptor potential vanilloid 1 (TRPV1), but not human TRPV1, releases intracellular calcium from thapsigargin sensitive stores. FASEB Journal abstract

Declaration

The work presented in this thesis was done soley by the author except where stated.

Abbreviations

AM	acetoxymethyl ester
ASIC	acid sensitive ion channel
ATP	adenosine 5'-triphosphate
AU	arbitary units
BDB	Brookhaven Protein Data Bank
BK	bradykinin
BK_2	bradykinin receptor
BSA	bovine serum albumin
β-ME	β-mercaptoethanol
bp	base pairs
CAB	calcium assay buffer
CaMKII	calcium-calmodulin dependent protein kinase II
Caps	Capsaicin
CB	Chronic bronchitis
CB_1	canabinoid receptor
cDNA	complementary deoxyribonucleic acid
CGRP	calcitonin gene-related peptide
CHO	Chinese hamster ovary fibroblasts
CMR1	cold and menthol receptor
CNS	central nervous system
COPD	chronic obstructive pulmonary disease
CPZ	Capsazepine
DAG	1,2-diacylglycerol
DMEM	Dulbecco's modified eagle medium

DMF	*N,N*- dimethylformamide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DRG	dorsal root ganglia
ECL	enhanced chemiluminescence
E.coli	*Escherichia coli*
ER	Endoplasmic reticulum
ERp57	thiol-disulphide oxidoreductase
FCS	Foetal calf serum
FITC	fluorescein isothiocyanate
GIT	gastrointestinal tract
GOR	Gastro-oesophageal reflux disease
GPCR	G-protein coupled receptor
HEPES	*N*-2-hydroxyethylpiperazine-N'2-ethanesulphonic acid
HBSS	Hank's Buffered Saline Solution
HEK293	human embryonic kidney cells
hTRPV1	human transient receptor potential vanilloid 1
hTRPV1-HEK	human TRPV1 transfected HEK cells
IBD	inflammatory bowl disease
IL1α	interleukin 1α
Ins(1,4,5)P_3	inositol (1,4,5) trisphosphate
IRK	inward rectifier potassium channel
iRTX	iodoresiniferatoxin
kDa	kilo Daltons
Kv1.1	potassium channel from the *shaker* family

LIC	light induced current
LPS	lipopolysaccharide
MEMα	minimum essential medium α
mRNA	messenger ribonucleic acid
NADA	N-Arachidonoyl-Dopamine
NGF	nerve growth factor
nompC	no mechanoreceptor potential C
OD	optical density
ORF	open reading frame
OST	oligosaccharyltransferase
PALDA	N-palmitoyl-dopamine
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDBu	phorbol 12,13-dibutyrate
PKA	protein kinase A
PKC	protein kinase C_α
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PNDS	Postnasal drip syndrome
PtdIns(4,5)P_2	phosphatidylinositol (4,5)-bisphosphate
PVDF	polyvinylidene fluoride
RAR	rapidly adapting receptors
rTRPV1	rat transient receptor potential vanilloid 1
rTRPV1-HEK	rat TRPV1 transfected HEK cells
RT	reverse transcription

RT-PCR	reverse transcription polymerase chain reaction
RTX	resiniferatoxin
12-(S)-HPETE	12-(S)-hydroperoxyeicosatetraenoic acid
15-(S)-HPETE	15-(S)-hydroperoxyeicosatetraenoic acid
SAR	slowly adapting receptors
SEM	standard error of the mean
SP	substance P
STEARDA	N-stearoyl-dopamine
THC	tetrahydrocannabinol
TNFα	tumour necrosis factor α
TrkA	tyrosine kinase
TRP	transient receptor potential
TRPA1	transient receptor potential A1
TRPC	transient receptor potential canonical
TRPM	transient receptor potential melastatin
TRPML	transient receptor potential mucolipin
TRPN1	transient receptor potential no mechanoreceptor potential C
TRPP	transient receptor potential polycystin
TRPV	transient receptor potential vanilloid
TRPV1	transient receptor potential vanilloid 1
UV	ultraviolet
VRL	vanilloid like receptor
VR-OAC	Vanilloid Receptor-related osmotically active channel

Chapter 1

General Introduction

1.1 Cough

Cough is the most common respiratory symptom and reason for seeking medical help (Morice et al., 2004). In the UK alone, there are approximately 4.5 million primary health care consultations per year for cough (Morice et al., 2001). Cough can be a symptom of numerous conditions and can be an important defence mechanism preventing mucus and potentially harmful agents from entering the lower airways. However, in some circumstances cough can be detrimental and can often be sociably debilitating. Coughing can produce some unwanted side effects including exhaustion, insomnia, muscular pain, hoarseness, excessive perspiration, vomitting and urinary incontinence (Irwin et al., 1998). Cough can be described as acute, with a duration of less than three weeks, subacute lasting three to eight weeks or chronic, lasting longer than eight weeks (Irwin and Madison, 2000).

1.1.1 Main Conditions Causing Cough

The common cold is often followed by a cough and this is the main cause of acute cough (Irwin et al., 1998). Chronic cough can be caused by a number of different conditions, the most prevalent being asthma, gastro-oesophageal reflux (GOR), and post nasal drip syndrome (PNDS) (Palombini et al., 1999). There are a number of other conditions which characteristically cause cough including chronic bronchitis, pulmonary fibrosis and bronchiectosis. Asthma is possibly one of the most common causes of chronic cough and indeed in cough-predominant asthma, cough is the only symptom in some patients (Corrao et al., 1979).

The mechanism by which GOR causes cough is not fully understood yet, however it is thought that aspiration of stomach contents into the larynx and tracheobronchial tree or irritation of fibres of the lower oesophagus may be important (Everett and Morice, 2004). Aspiration of stomach contents into the airways could cause cough in two ways, firstly by mechanical stimulation of airway nerve fibres or by chemical stimulation due to the low pH of stomach acids (Everett and Morice, 2004). Symptoms of this disorder other than cough include heartburn, acid regurgitation and a bitter taste.

PNDS or rhinitis can be characterised by the sensation of 'something running down the back of the throat' (Morice, 2004). It is thought to be caused by secretions from the nose and sinuses dripping down onto the pharynx and mechanically triggering the cough reflex. However it has been hypothesised that it is actually the aspiration of the PNDS secretions from the pharynx to the larynx which triggers the cough reflex because cough cannot be stimulated mechanically or chemically below the larynx (Morice, 2004).

1.1.1 The Cough Reflex.

The cough reflex begins by stimulation of ion channels and receptors expressed on afferent or sensory nerves found in the airway epithelium (Irwin et al., 2000). After stimulation, impulses are passed along these afferent nerves to the vagus nerve and to the cough centre in the brain (see Figure 1.1). There are three different types of afferent nerves that may be involved in mediating cough (Irwin et al., 2000). The first, are the myelinated, rapidly adapting pulmonary stretch receptors (RARs, or

irritant receptors or Aδ fibres), which have been shown to mediate cough by both mechanical and chemical stimulation (Irwin et al., 2000). Unlike the RARs, the second type of afferent nerves, the myelinated, slowly adapting pulmonary stretch receptor (SAR) do not respond to irritants, but instead may be important in increasing the expiratory muscular effect, making the cough more forceful (Irwin et al., 2000). The unmyelinated C-fibres are the third type of nerves. These are subdivided into two groups depending on their location in the airways, either the bronchial C-fibres, found in the airway wall, or the pulmonary C-fibres, located in the alveolar wall (Karlsson and Fuller, 1999). Current understanding suggests that bronchial C-fibres are more likely to be involved in the cough reflex due to their location and the fact that they can be triggered by tussive agents (Karlsson and Fuller, 1999). C-fibres can release neuropeptides such as substance P and calcitonin gene-related peptide in response to irritation or inflammation (Carr and Undem, 2001) (see Figure 1.1).

Responses from the afferent neurons are conducted to the cough centre by the vagus nerve. The exact mechanisms of the cough reflex are poorly understood and it is still unclear whether there is actually a discreet cough centre in the CNS (Karlsson and Fuller, 1999). However it is understood that afferent messages are sent to a region near the nucleus of the tractus solitarius, where a cough response may be co-ordinated in the medulla oblongata (Irwin et al., 2000, Karlsson and Fuller, 1999).

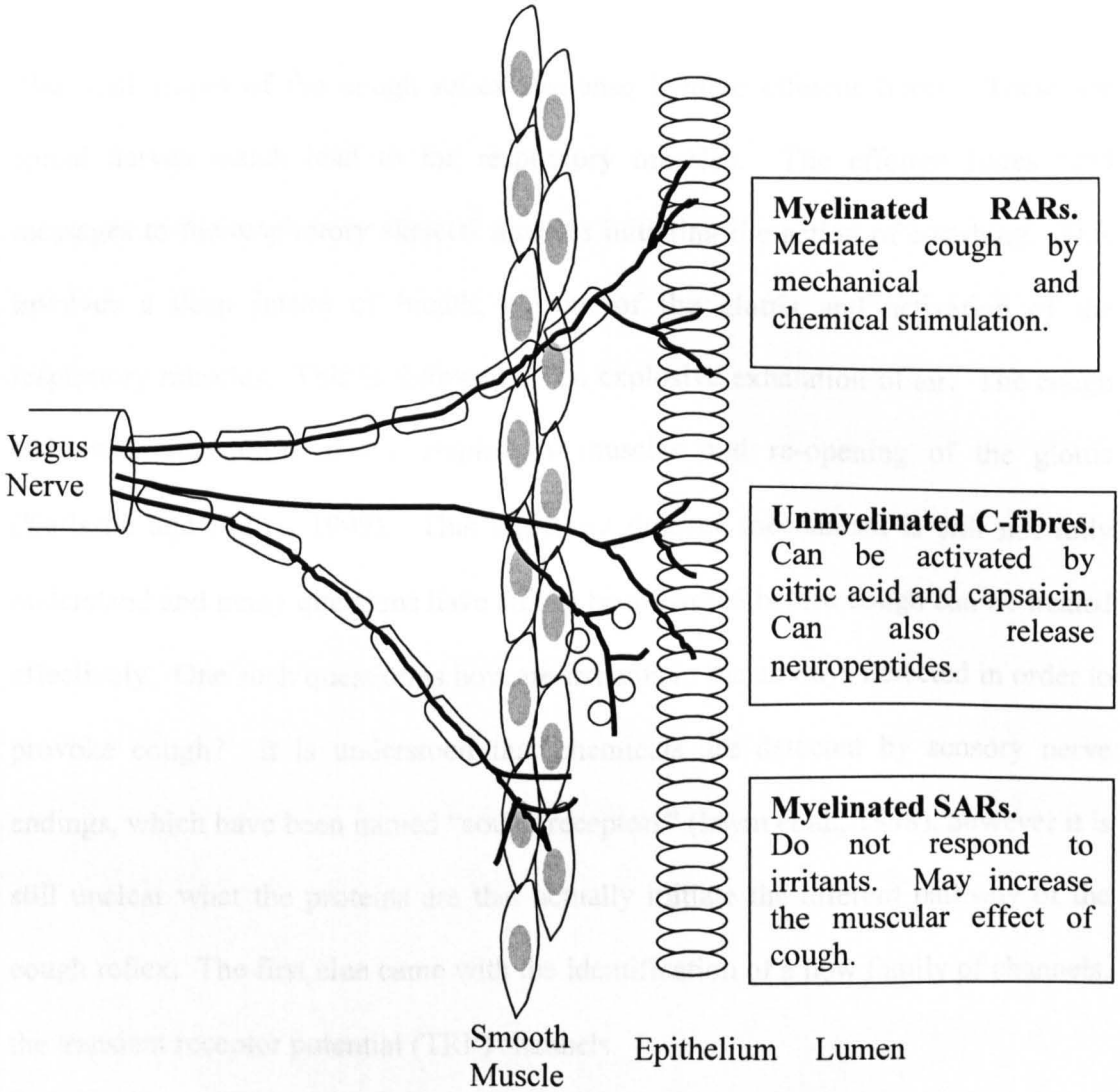


Figure 1.1. Afferent Fibres of the Cough Reflex. Myelinated RARs can be activated by both mechanical and chemical stimulation such as citric acid. Unmyelinated C-fibres can be activated by TRPV1 agonists citric acid and capsaicin. C-fibres can also release neuropeptides such as substance P and calcitonin gene-related peptide (circles). Myelinated SARs do not respond to irritants but may make the cough response more forceful.

The final stages of the cough reflex response involve efferent fibres. These are spinal nerves which lead to the respiratory muscles. The efferent fibres send messages to the respiratory skeletal muscles initiating the action of coughing. This involves a deep intake of breath, closure of the glottis and activation of the respiratory muscles. This is followed by an explosive exhalation of air. The cough ends with relaxation of the respiratory muscles and re-opening of the glottis (Karlsson and Fuller, 1999). This important defence mechanism is still not fully understand and many questions have still to be answered before cough can be treated effectively. One such question is how are irritants in the airways detected in order to provoke cough? It is understood that chemicals are detected by sensory nerve endings, which have been named "cough receptors" (Irwin et al., 1998), however it is still unclear what the proteins are that actually initiate the afferent pathway of the cough reflex. The first clue came with the identification of a new family of channels, the transient receptor potential (TRP) channels.

1.2 The TRP Channels

The first Transient Receptor Potential channel (TRP) was identified in the Drosophila eye (Hardie and Raghu, 2001, Hotta and Benzer, 1969). The protein was identified when mutant flies were shown to lack a calcium entry channel causing a transient light-induced current (LIC) in response to light rather than the continuous LIC seen in wild type drosophila. This calcium entry channel was called 'Transient Receptor Potential'. Since then, many more structurally related TRP channels have been identified in other species including humans (Wes et al., 1995, Zhu et al., 1995,

McKemy et al., 2002, Sun et al., 2000, Di Palma et al., 2002, Story et al., 2003, Sidi et al., 2003, Walker et al., 2000, Gonzalez-Perrett et al., 2001, Mochizuki et al., 1996, Caterina et al., 1997, Hayes et al., 2000). All the TRP channels have six transmembrane spanning domains with a hydrophobic loop between domains five and six (see Figure 1.2). It is believed that up to four of these receptors interact to form heteromeric or homomeric structures (Kuzhikandathil et al., 2001, Kedei et al., 2001, Murakami et al., 2003, Rosenbaum et al., 2002, Strubing et al., 2003, Hellwig et al., 2005) This group of channels has been divided into families on the basis of sequence homology, transient receptor potential canonical (TRPC), transient receptor potential melastatin (TRPM), transient receptor potential vanilloid (TRPV), transient receptor potential mucolipin (TRPML), transient receptor potential polycystin (TRPP), transient receptor potential A1 (TRPA1) and transient receptor potential N1 (where N stands for no mechanoreceptor potential C or nompC, TRPN1).

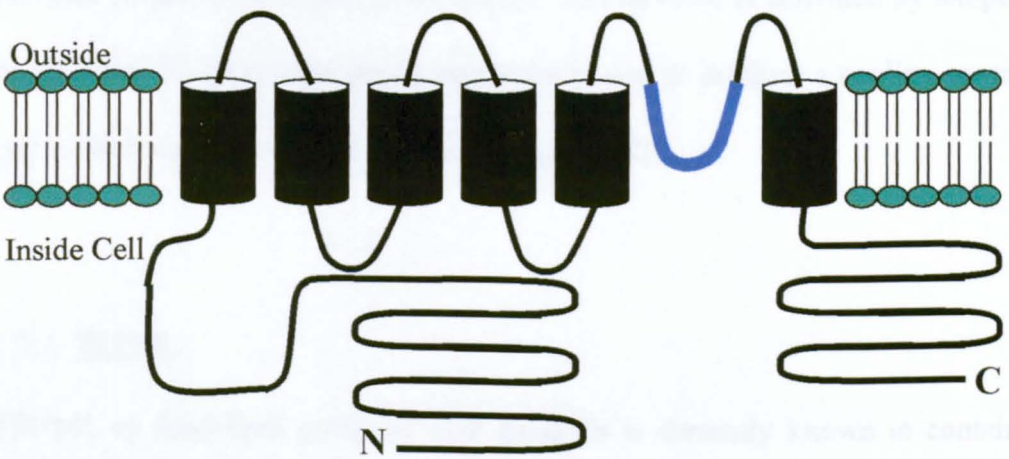


Figure 1.2. Predicted structure of TRP channels. The structure of all the TRP family members is similar however the length of the N- and C-terminal chains can differ greatly. The blue region represents the P-loop (see section 1.4.2).

1.2.1 TRPC

The TRPC or canonical group of TRP proteins is made up of seven mammalian channels, named TRPC1 to 7. TRPC1 shares approximately 40% homology with the first *Drosophila* phototransduction TRP channel found in the *Drosophila* eye, making the TRPC family the most closely related group to the *Drosophila* channel (Wes et al., 1995, Zhu et al., 1995). The TRPC family are thought to be store operated calcium channels (Xu and Beech, 2001, Vazquez et al., 2004).

1.2.2 TRPM

The TRPM or melastatin group contains TRP channels with the longest coding sequences. Currently eight of these channels have been identified and have been named TRPM1 to 8. TRPM8 has recently been identified as the cold and menthol receptor (CMR1) (McKemy et al., 2002). The receptor is activated by temperatures in the range of 19 to 24°C and compounds known to produce a cooling sensation in particular menthol and icilin (McKemy et al., 2002).

1.2.3 TRPML

TRPML or mucolipin group of TRP channels is currently known to contain three members. The first to be identified and cloned was TRPML1 (or mucolipin). The gene encoding this channel was found to be mutated in patients suffering from Mucolipidosis type IV, a developmental disease which can affect the brain, sight and digestive functions (Sun et al., 2000, LaPlante et al., 2002). TRPML1 is thought to be involved in calcium transport and may be involved in exocytosis of late endosomes and lysosomes (LaPlante et al., 2002).

TRPML2 and 3 have been cloned but are not yet well characterised (Di Palma et al., 2002). TRPML3 can be found in hair cells and mutations, as seen in *varitint-waddler* mice, may cause hearing loss and pigmentation abnormalities (Di Palma et al., 2002).

1.2.4 TRPA

Currently the TRPA family consists of just one channel, the non selective cation channel, TRPA1. This channel is activated by temperatures in the range of about 12 to 24°C (Story et al., 2003), allicin (the active component of garlic) (Macpherson et al., 2005), isothiocyanates (the pungent components of mustard, horseradish and wasabi) and tetrahydrocannabinol (THC, the psychoactive component of marijuana) (Jordt et al., 2004). TRPA1 has been found not only on DRGs but also, like TRPML3, on hair cells and thus may have a role in mechanosensitivity and the detection of sound (Story et al., 2003, Corey et al., 2004).

1.2.5 TRPN

TRPN or no mechanoreceptor potential C (nompC) group is currently also composed of just one channel. The channel is also found on hair cells, however to date it has only been detected on *drosophila*, *C. Elegans* and the ear hair cells of zebra fish (Sidi et al., 2003, Walker et al., 2000). Currently, no TRPN channels have been found in mammals. TRPN consists of the classical six transmembrane domains found in TRP channels along with 29 ankyrin repeats (Walker et al., 2000). TRPN is thought to be

involved in hearing and perception of other mechanical stimuli (Walker et al., 2000, Sidi et al., 2003).

1.2.6 TRPP

The TRPP or polycystin family consists of eight structurally related members all grouped because they are believed to be involved in polycystic kidney disease (Pedersen et al., 2005). The exact mechanisms by which TRPPs cause polycystic kidney disease is not yet fully understood (Gonzalez-Perrett et al., 2001). TRPP2, TRPP3 and TRPP5 are all structurally related to the other TRPs in that they all consist of six transmembrane domains, however they do not possess the ankyrin repeat domain (Mochizuki et al., 1996). TRPP1 however does not conform to the classical TRP channel structure as it consists of eleven transmembrane domains. The last six transmembrane domains of TRPP1 do however show similarities to the transmembrane domains of TRPP2 (Sandford et al., 1997). It is thought that TRPP1 and TRPP2 can interact to form heteromers. TRPP1 is necessary for localisation of TRPP2 to the cell surface and TRPP2 affects the ability of TRPP1 to activate G proteins (Delmas et al., 2004)

1.2.7 TRPV

Much has been learnt about the TRPV or vanilloid subfamily of TRP channels in the last few years. There are currently six known TRPV channels named TRPV1 to 6. TRPV1 (Vanilloid Receptor 1, TRPV1), which is the focus of this project, was the first of this family to be discovered and cloned by Caterina and colleagues (Caterina et al., 1997). It responds to vanilloids such as capsaicin, which is known to provoke

cough, as well as resiniferatoxin, pH below 5.9, phorbol esters, some lipoxygenase products and temperatures above 43°C (see Table 1.1). Activators and regulators of TRPV1 are covered in more detail later. In addition to TRPV1, three vanilloid-receptor-like proteins (VRL) have been identified, TRPV2, TRPV3 and TRPV4 (formally known as VRL 1, 3 and 2 respectively). Rat and human TRPV2 share 49% and 66% homology with rat TRPV1 respectively at the amino acid level and does not respond to capsaicin, acid or moderate heat, but is activated by high temperatures of approximately 52°C (Caterina et al., 1999) (see Table 1.1). TRPV3 responds to temperatures in the range of 22 to 40°C (Xu et al., 2002, Smith et al., 2002) and TRPV4 also known as VR-OAC (Vanilloid Receptor-related osmotically active channel or VRL-2) is an osmotically regulated channel (Liedtke et al., 2000). TRPV5 and 6 are both highly selective for calcium and sense intracellular calcium concentrations (Nilius et al., 2000, Peng et al., 1999, Vennekens et al., 2000). The activity of these channels increases at low intracellular calcium concentrations, which suggests they may be important in calcium homeostasis (Vennekens et al., 2000, Yue et al., 2001, Peng et al., 1999, Muller et al., 2000). Table 1.1 summarises the current known activators and inhibitors of the TRPV channels.

Receptor	Activators	Inhibitors	References
TRPV1	Capsaicin and other vanilloids, NADA pH below 5.9, noxious heat (above 43°C), phorbols, lipids including anandamide and some lipoxygenase products.	Capsazepine, ruthenium red, iodoresiniferatoxin	(Hayes et al., 2000, Caterina et al., 1997, Tominaga et al., 1998, Wahl et al., 2001)
TRPV2 (VRL-1 CRC)	Noxious heat (above 53°C) Regulated by IGF-1	Ruthenium red	(Caterina et al., 1999, Kanzaki et al., 1999)
TRPV3 (VRL-3)	Heat ~20-40°C Camphor	-	(Smith et al., 2002, Xu et al., 2002, Moqrich et al., 2005)
TRPV4 (VRL-2 VR-OAC)	Warm temperature ~27°C-34°C Osmotically sensitive, mechanosensitive?	Ruthenium red, Gd^{3+}, La^{3+}	(Wissenbach et al., 2000, Strotmann et al., 2000, Liedtke et al., 2000, Delany et al., 2001)
TRPV5 (ECAC1)	Constitutively active (activity increased by low intracellular $[Ca^{2+}]$)	Ruthenium red, Econozole, Cu^{2+}, Pb^{2+}, Cd^{2+}, Mg^{2+}, Gd^{3+}, La^{3+}	(Hoenderop et al., 2002, Hoenderop et al., 1999, Muller et al., 2000, Peng et al., 2000, Nilius et al., 2001, Nilius et al., 2000, Vennekens et al., 2000, Vennekens et al., 2001)
TRPV6 (ECAC2 CaT1)	Constitutively active (activity increased by low intracellular $[Ca^{2+}]$), Store depletion	Xestospongin, La^{3+}	(Wissenbach et al., 2001, Peng et al., 1999, Yue et al., 2001, Vassilev et al., 2001)

Table 1.1. The current known activators and inhibitors of the TRPV channels.

1.3 TRPV1

1.3.1 Cloning TRPV1.

Caterina and colleagues first cloned rat TRPV1 (rTRPV1) in 1997 (Caterina et al., 1997). cDNA from a rat dorsal root ganglia (DRG) library was expressed in human embryonic kidney cells (HEK293). Screening of the cells to isolate capsaicin sensitive candidates was performed using fluorescent microscopy with the calcium indicator dye, Fura 2 (Caterina et al., 1997). The findings were confirmed using whole-cell voltage-clamp analysis. Figures 1.2 and 1.3 shows the predicted structure of the TRPV1 (Caterina et al., 1997). Rat TRPV1 cDNA contains an open reading frame of 2,514 nucleotides encoding 838 amino acids and has 16 exons and 15 introns. The protein folds to produce six membrane-spanning domains with a hydrophobic loop between domains five and six (Caterina et al., 1997) (Figure 1.2 and Figure 1.3).

1.3.2 Cloning Human TRPV1 (hTRPV1)

Since rat TRPV1 was cloned, human TRPV1 has also been isolated and cloned (Hayes et al., 2000). The human receptor shares 92% homology with the rat receptor at the amino acid level and has an open reading frame of 2,517 base pairs. Human TRPV1 is located on chromosome 17 at position p13 and has 15 exons and 14 introns (Hayes et al., 2000). The receptor responds in a similar way to the rat variant to heat, pH and capsaicin, although the human receptor has a lower acid threshold than rTRPV1 (Hayes et al., 2000, McIntyre et al., 2001, Cortright et al., 2001). Like rTRPV1, anandamide is a full agonist of hTRPV1 and capsazepine and ruthenium

red act as antagonists, however, these two compounds are more potent at antagonising hTRPV1 than rTRPV1 (McIntyre et al., 2001, Smart et al., 2000).

1.3.3 Distribution of TRPV1.

TRPV1 was first identified in dorsal root ganglia and trigeminal ganglia (Caterina et al., 1997). In addition, using immunostaining techniques, TRPV1 expressing neurons have been found in various regions of the brain including all cortical areas (Mezey et al., 2000). High levels of expression of rTRPV1 were located in the kidney, pancreas, placenta and in DRG. Hayes *et al* showed using quantitative PCR, that hTRPV1 was expressed in low levels, uniformly in all the tissues tested but was most highly expressed in DRG (Hayes et al., 2000).

Capsaicin sensitive cells are also present in some non-neuronal tissues. These include mast cells, where stimulation leads to calcium influx and subsequent release of interleukin-4, a proinflammatory cytokine (Biro et al., 1998b), and rat C6 glioma cells (Biro et al., 1998a). TRPV1 has also been detected in cultured normal human epidermal kertainocytes, rat skeletal muscle cells, rat urothelial cells, human airway smooth muscle cells and more recently human dental pulp fibroblasts (Inoue et al., 2002, Denda et al., 2001, Mezey et al., 2000, Birder et al., 2001, Mitchell et al., 2005, Miyamoto et al., 2005, Xin et al., 2005).

TRPV1 was primarily thought to be located on the plasma membrane, however recent findings show TRPV1 is also located on the endoplasmic reticulum (Karai et al., 2004, Olah et al., 2001b, Wisnoskey et al., 2003, Liu et al., 2003, Jahnel et al., 2001). TRPV1 expressed on internal stores are also activated by capsaicin and

resiniferatoxin and can be blocked by capsazepine (Karai et al., 2004, Olah et al., 2001b, Wisnoskey et al., 2003, Liu et al., 2003). Interestingly, it has recently been shown that TRPV1 is expressed on rat skeletal muscle but mainly on the sarcoplasmic reticulum (Xin et al., 2005). Only small amounts were detected on the plasma membrane (Xin et al., 2005).

1.4 Structure of TRPV1

1.4.1 Ligand Binding Site

Locating the actual capsaicin recognition domain of TRPV1 proved difficult due to the lipophilic nature of capsaicin. Capsaicin consists of three reactive groups, a homovanillic acid, an amide bond and an aliphatic chain. The two hydrophilic regions, the homovanillic acid and amide bond are capable of forming hydrogen bonds where as the aliphatic chain reacts by hydrophobic interactions (Jung et al., 2002). Application of capsaicin to the intracellular and extracellular sides of patch excised TRPV1 expressing cells produced identical responses (Caterina et al., 1997). However application of the hydrophilic salt form of a synthetic capsaicin analogue, DA-5018·HCl, to both sides of the membrane provoked a response only from the cytosolic side suggesting that the capsaicin binding site is cytosolic (Jung et al., 1999). The exact location of the capsaicin binding domain was further narrowed down by Jordt and Julius (Jordt and Julius, 2002). By sub domain exchange from rTRPV1 to chicken and other TRPV channels, Jordt and Julius demonstrated capsaicin induced currents in these otherwise capsaicin insensitive channels. They suggested that the vanillyl moiety of capsaicin interacted with an aromatic amino acid on the cytosolic face of the plasma membrane such as tyrosine 511 (see Figure

1.3). They also suggested that polar residues may be necessary to form hydrogen bonds with capsaicin such as serine 512 and arginine 491. All these interactions are necessary for capsaicin interaction and form a vanilloid binding pocket on the loop between transmembrane domains two and three (Jordt and Julius, 2002). Jung *et al* performed further mutagenesis studies again with rTRPV1 and found two more amino acids important in capsaicin evoked responses (Jung et al., 2002). They showed mutation of R114 and E761 on the cytosolic N and C terminals respectively to neutral or oppositely charged amino acids caused the channel to exhibit reduced sensitivity to both capsaicin and resiniferatoxin compared to the wild type. They suggested that these two amino acids constituted binding sites for the hydrophilic portions of capsaicin and the region identified by Jordt *et al* formed the hydrophobic binding site and together these sites form the capsaicin binding pocket (Jordt and Julius, 2002, Jung et al., 2002) (see Figure 1.3).

1.4.2 P-Loop

The P-loop is situated between the fifth and sixth transmembrane spanning segments (see Figure 1.3). This region contains four acidic residues, E636, D646, E648 and E651. Garcia-Martinez and colleagues (2000) neutralised these residues to determine the effect on the channel ion selectivity and blockage by non-competitive antagonists. Neutralisation of D646 with N lowered the receptor's sensitivity to Ruthenium Red blockage and reduced Mg^{2+} permeability (Garcia-Martinez et al., 2000). However further investigation is required to determine the exact function and mechanisms of P-loop regulation.

1.4.3 TRP Domain.

The TRP domain is a conserved region found on many TRP channels following the sixth transmembrane domain (see Figure 1.3). In TRPV1 it incorporates amino acids E684 to R721. The function of this highly conserved region was unknown until recently it was suggested it may play a role in multimerisation of TRP channels (Garcia-Sanz et al., 2004). Deletion of this region resulted in channels which failed to form functional multimers however purified TRPV1 C-terminus was able to form multimers. Therefore the TRP domain may have a role in TRP channel homo or hetero dimerisation (Garcia-Sanz et al., 2004).

1.4.4 Ankyrin Repeats

TRPV1 like many of the other TRP channels contains ankyrin repeats (Wes et al., 1995, Story et al., 2003, Caterina et al., 1997, Walker et al., 2000). Ankyrin repeats were named after 24 of the subunits were discovered in the protein ankyrin (Lux et al., 1990). Around 3000 different ankyrin repeats from more that 400 different proteins have been identified (Sedgwick and Smerdon, 1999). Although the sequence of the approximately 33 amino acids that make up the ankyrin repeat can vary between proteins, several residues are conserved in order to maintain the distinct ankyrin repeat folding. The ankyrin repeat folds to form a β hairpin followed by two α helices running in antiparallel. This is linked directly to the β hairpin of the second ankyrin repeat and it is thought that in order to form a stable structure more than one ankyrin is required (Gorina and Pavletich, 1996). Interestingly, TRPV1 has three ankyrin repeats and it is thought that TRPA1 has 14 ankyrin repeats and TRPN has an unusual 29 ankyrin domains (Caterina et al., 1997, Walker et al., 2000, Story et al., 2003). The exact role of these domains is still undeciphered, however it is

thought that they may be involved in protein-protein interactions (Mosavi et al., 2004).

1.4.5 Glycosylation

N-linked glycosylation is a protein post translational modification whereby a high mannose core oligosaccharide is added to the asparagine residue of the amino acid sequon NXS/T (where X is any amino acid except proline) by an *N* glycosidic bond (see chapter 4) (Kornfeld and Kornfeld, 1985). Both rat and human TRPV1 have one potential glycosylation site at N604 with glycosylation sequons N-N-S and N-D-S respectively (see Figure 1.3). Preliminary studies indicate that rTRPV1 is expressed in both glycosylated and unglycosylated states (Rosenbaum et al., 2002, Wirkner et al., 2005, Jahnel et al., 2001, Kedei et al., 2001). However there are currently conflicting data regarding the effects glycosylation has on channel function. One study by Rosenbaum and colleagues suggested removal of the glycosylation site had no effect on channel function (Rosenbaum et al., 2002) whereas Wirkner and colleagues showed a reduction in the maximum capsaicin evoked response and a reduction in the EC_{50} following loss of the glycosylation site (Wirkner et al., 2005). It is possible that glycosylation of TRPV1 is of importance because of the location near the pore region of the channel and indeed glycosylation has been shown to affect the function of other ion channels such as the voltage-gated sodium channels (Cronin et al., 2005) However a detailed study investigating the role of glycosylation in regulating TRPV1 pharmacology has yet to be performed.

Sialylation may also have an important role in TRPV1 function. In the *trans* golgi, sialic acid can be added to the terminal residue of *N*-linked glycans. Sialic acid has

previously been shown to affect the function of other ion channels including Kv1.1, a potassium channel of the *shaker* family and the voltage gated sodium channel (Thornhill et al., 1996, Johnson et al., 2004). It was thought that sialic acid affected the local electric field detected by Kv1.1 and sialic acid directly appeared to affect channel gating of the voltage gated sodium channel (Johnson et al., 2004, Thornhill et al., 1996). Therefore sialylation may also be important for TRPV1 function, which to date has yet to be investigated.

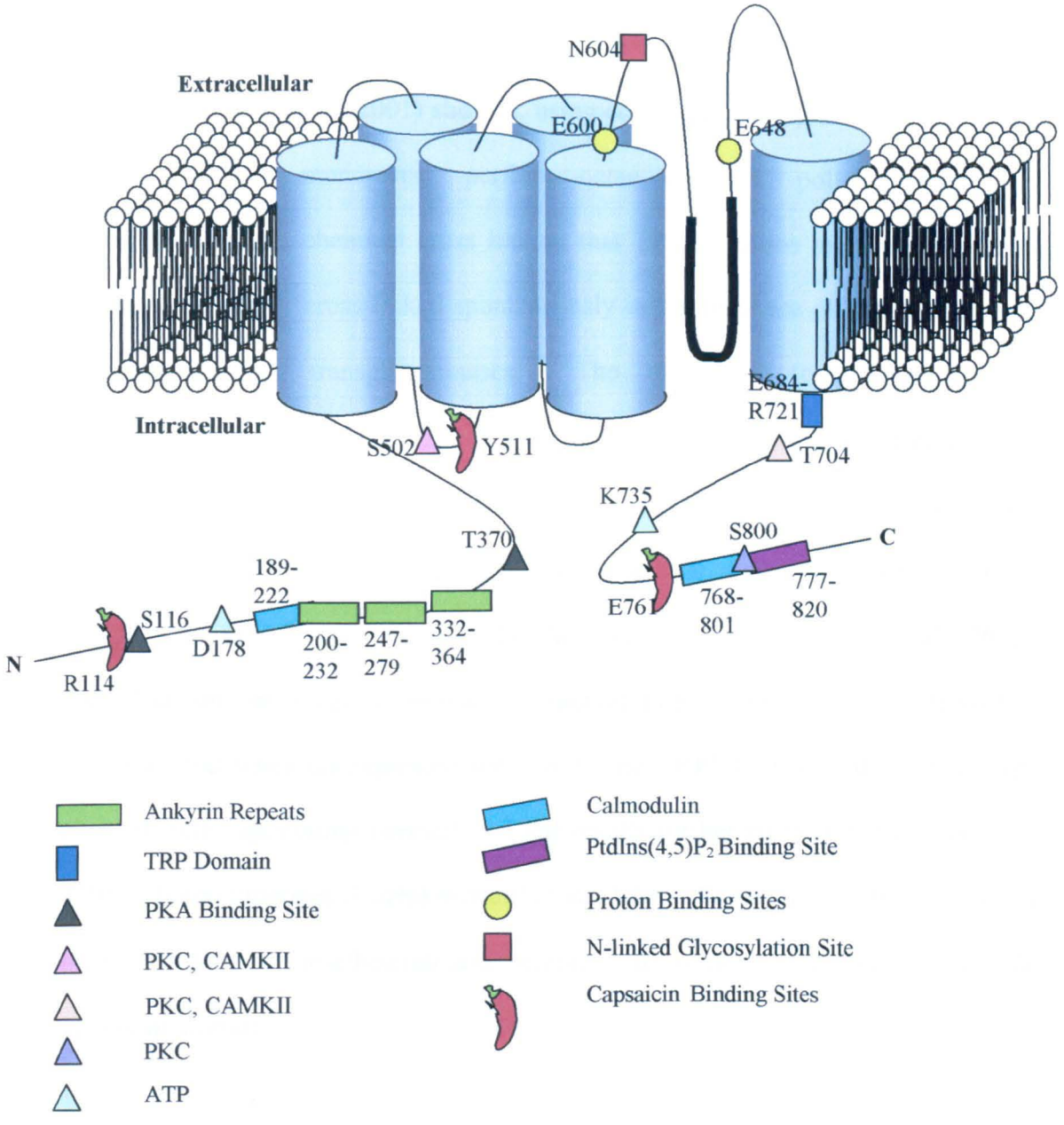


Figure 1.3. Predicted structure of TRPV1 including potential sites of regulation or agonist interaction.

1.4.6 Dimerisation.

It is widely believed now that all the TRP channels are capable of forming multimers. Kedei *et al* (2001) showed, using co-immunoprecipitation of differently tagged TRPV1 monomers, perfluoro-octanoic acid polyacrylamide gel electrophoresis and chemical cross linking that TRPV1 forms multimers. TRPV1 becomes covalently cross-linked spontaneously in the presence of TRPV1 agonists, probably through transglutaminases. The multimers formed are usually homotetrameric (Kedei et al., 2001). This finding has been confirmed recently by Hellwig and colleagues (Hellwig et al., 2005). However, experiments have shown that TRPV3, which responds to temperatures in the range of 20-40°C, but not capsaicin, is able to associate with TRPV1 (Xu et al., 2002, Smith et al., 2002). Kuzhikandathil *et al* (2001) produced a mutant TRPV1 which did not respond to capsaicin and when co-expressed with wild type TRPV1 prevented the wild type channel from functioning normally. Their results further supported the theory that TRPV1 homodimerises (Kuzhikandathil et al., 2001). Sensory neurons may express many variations of these heterologous receptors, allowing them to respond to a wide variety of stimuli.

1.5 Regulation of TRPV1 Activity

1.5.1 Heat.

TRPV1 can be activated directly by temperatures above approximately 43°C (Caterina et al., 1997, Tominaga et al., 1998). The exact mechanism of heat activation is not yet fully understood, however it is thought that the C terminal end of TRPV1 is important (Vlachova et al., 2003). Deletions of up to 42 amino acids from

the C terminal of the rat channel resulted in a lowering of the TRPV1 temperature threshold by up to 9°C without causing much effect on the proton or capsaicin induced responses (Vlachova et al., 2003). Ethanol, between 0.1 and 3% has also been shown to lower temperature threshold for TRPV1 to approximately 34°C (Trevisani et al., 2002). This may explain the warm sensation felt after consuming alcohol.

1.5.2 Protons.

Tominaga *et al* suggested that pH works synergistically to lower the temperature at which TRPV1 is activated (Tominaga et al., 1998). Therefore, at normal body temperature TRPV1 is inactive. When the pH is lowered (below pH5.9), for example during tissue injury and inflammation, TRPV1 can be active at temperatures as low as room temperature. The TRPV1 specific antagonist capsazepine inhibits proton activation of hTRPV1 and guinea pig TRPV1 but not rTRPV1 (Gavva et al., 2005a, McIntyre et al., 2001, Savidge et al., 2002). Phillips and colleagues demonstrated that amino acids in transmembrane domains three and four were important for these species differences and by mutation of rTRPV1 domains I514M, V518L and M547L to the human equivalents, they showed that capsazepine could inhibit rTRPV1 responses to protons (Phillips et al., 2004). However the more potent antagonist iodoresinifertoxin inhibits the proton mediated TRPV1 response of both hTRPV1 and rTRPV1 (Seabrook et al., 2002, Gavva et al., 2005a).

Site directed mutagenesis has provided evidence for proton binding sites. These sites have been identified as glutamic acid residues 600 and 648, which are located either side of the pore forming loop between the fifth and sixth transmembrane domains

(Jordt et al., 2000) (see Figure 1.3). TRPV1 with a mutation at position 600 (E600Q) exhibited increased sensitivity to capsaicin and heat when compared to the wild type channel (Jordt et al., 2000). The responses to capsaicin and heat in E600Q mutants mimic those of the wild type channel under acidic conditions suggesting that this site may be responsible for regulating thresholds to noxious stimuli in response to extracellular proton concentration. Mutations at position 648, (E648A and E648Q) decreased the effects of protons on TRPV1 activation without significantly altering the effects of heat and capsaicin on the channel (Jordt et al., 2000).

1.5.3 Tissue Injury

Bradykinin (BK) and Nerve Growth Factor (NGF) are produced during tissue injury and increase the sense of pain by sensitising sensory nerve endings to noxious stimuli. Bradykinin may elicit its effect in two different ways, firstly by release of arachidonic acid a precursor to 12-(S)-hydroperoxyeicosatetraenoic acid (12-(S)-HPETE) which has been shown to activate TRPV1 (Hwang et al., 2000) (see section 1.5.9) and secondly though the second messenger pathway leading to release of TRPV1 from phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) inhibition.

BK and NGF activate specific receptors, BK_2 a G-protein coupled receptor and tyrosine kinase (TrkA) respectively resulting in PLC stimulation. PLC catalyses the hydrolysis of PtdIns(4,5)P_2 to inositol (1,4,5)trisphosphate (Ins(1,4,5)P_3) and 1,2-diacylglycerol (DAG). Under normal circumstances TRPV1 activity is reduced by bound PtdIns(4,5)P_2 therefore hydrolysis of this molecule releases TRPV1 from this repression leading to receptor sensitisation (Chuang et al., 2001) (see Figure 1.4). A PtdIns(4,5)P_2 binding site has been located in the C-terminal cytoplasmic domain of

TRPV1 (between amino acids 777 and 820) (Prescott and Julius, 2003) (see Figure 1.3). By mutating this region, causing weaker PtdIns(4,5)P_2 binding, the capsaicin and temperature threshold for TRPV1 was reduced, however, by replacing this region with a lipid-binding domain from PtdIns(4,5)P_2-activated inward rectifier potassium (IRK) channel, the resultant mutant TRPV1 channel showed only small currents in response to capsaicin (Prescott and Julius, 2003).

The pathway described above is thought to cause TRPV1 sensitisation due to tissue injury. As mentioned earlier (see section 1.5.2) extracellular protons are generated during tissue injury. Therefore tissue injury can act in two ways in order to sensitise the TRPV1, firstly by releasing NGF and bradykinin, leading to PLC activation and PtdIns(4,5)P_2 hydrolysis and secondly by releasing protons, which lowers the TRPV1 threshold for heat activation (again section 1.5.2) (Tominaga et al., 1998).

1.5.4 Protein Kinase C

Evidence is mounting to suggest that TRPV1 can also be sensitised through phosphorylation by Protein Kinase C_α (PKC) (Olah et al., 2002, Premkumar and Ahern, 2000, Vellani et al., 2001). PKC can be activated by DAG (produced by hydrolysis of PtdIns(4,5)P_2 by PLC, see section 1.5.3). Phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PDBu) are thought to be TRPV1 agonists, however they may not directly cause opening of TRPV1 channels. Instead it is believed that phosphorylation of the channel by PKC causes sensitisation thus lowering the threshold for activation by TRPV1 agonists such as phorbol esters (Vellani et al., 2001). The following potential PKC

phosphorylation sites have been identified: S800, S502 and T704 (Bhave et al., 2003, Numazaki et al., 2002) (see Figure 1.3).

1.5.5 Protein Kinase A

Continuous or repeated exposure to capsaicin (but not resiniferatoxin) causes channel desensitisation or tachyphylaxis possibly by a rapid rise in intracellular calcium levels (Docherty et al., 1996, Piper et al., 1999, Toth et al., 2005). There is now mounting evidence that cyclic AMP-dependent protein kinase A (PKA) can also regulate TRPV1 by preventing desensitisation (Mohapatra and Nau, 2003, Bhave et al., 2002). Therefore dephosphorylation is associated with desensitisation. Phosphorylation of specific sites such as S116 and T370 reduces desensitisation however the full mechanism is only beginning to be understood (Mohapatra and Nau, 2003, Bhave et al., 2002) (see Figures 1.3 and 1.4).

1.5.6 Calmodulin and Calcium-Calmodulin Dependent Protein Kinase II

Calmodulin is a sensor of intracellular calcium levels. When cytosolic calcium levels rise above 500 nM the protein binds up to four calcium ions and then activates various proteins preventing further calcium entry. TRPV1 is desensitised by rapid increases in intracellular calcium following activation (Docherty et al., 1996, Piper et al., 1999, Toth et al., 2005). It has been suggested calmodulin can interact directly with TRPV1 to inactivate the channel in a feedback mechanism, preventing an over-influx of calcium into the cell (Numazaki et al., 2003, Rosenbaum et al., 2004). However, there is dispute over where this interaction occurs (either between amino acids 761 and 801 (Numazaki et al., 2003) or 189 and 222 (Rosenbaum et al., 2004))

if at all. The sites of interaction proposed so far, although disrupt channel function, do not conform to known calmodulin binding sites and may cause inhibition of function by some other mechanism (Numazaki et al., 2003, Rosenbaum et al., 2004).

Jung *et al* have suggested another mechanism of TRPV1 desensitisation (Jung et al., 2004). This involves two enzymes, the protein phosphatase, calcineurin and the protein kinase, Calcium-Calmodulin Dependent Protein Kinase II (CaMKII). Dephosphorylation by calcineurin causes desensitisation, whereas phosphorylation by CaMKII is required for activation. Jung *et al* suggested that phosphorylation was required before ligand binding can occur (Jung et al., 2004). Both enzymes are controlled by calcium signalling and Jung *et al* proposed two mechanisms to explain TRPV1 regulation by the two opposing enzymes. Firstly, once activated, the channel is rapidly dephosphorylated by calcineurin. CaMK11 is activated more slowly and rephosphorylates the channel, returning it to a state ready for ligand binding. Secondly, they suggested the two enzymes possessed different calcium sensitivities or affinities to calmodulin (Jung et al., 2004). Mutagenesis studies provided evidence of potential sites of CaMKII phosphorylation sites, S502 and T704 (Jung et al., 2004).

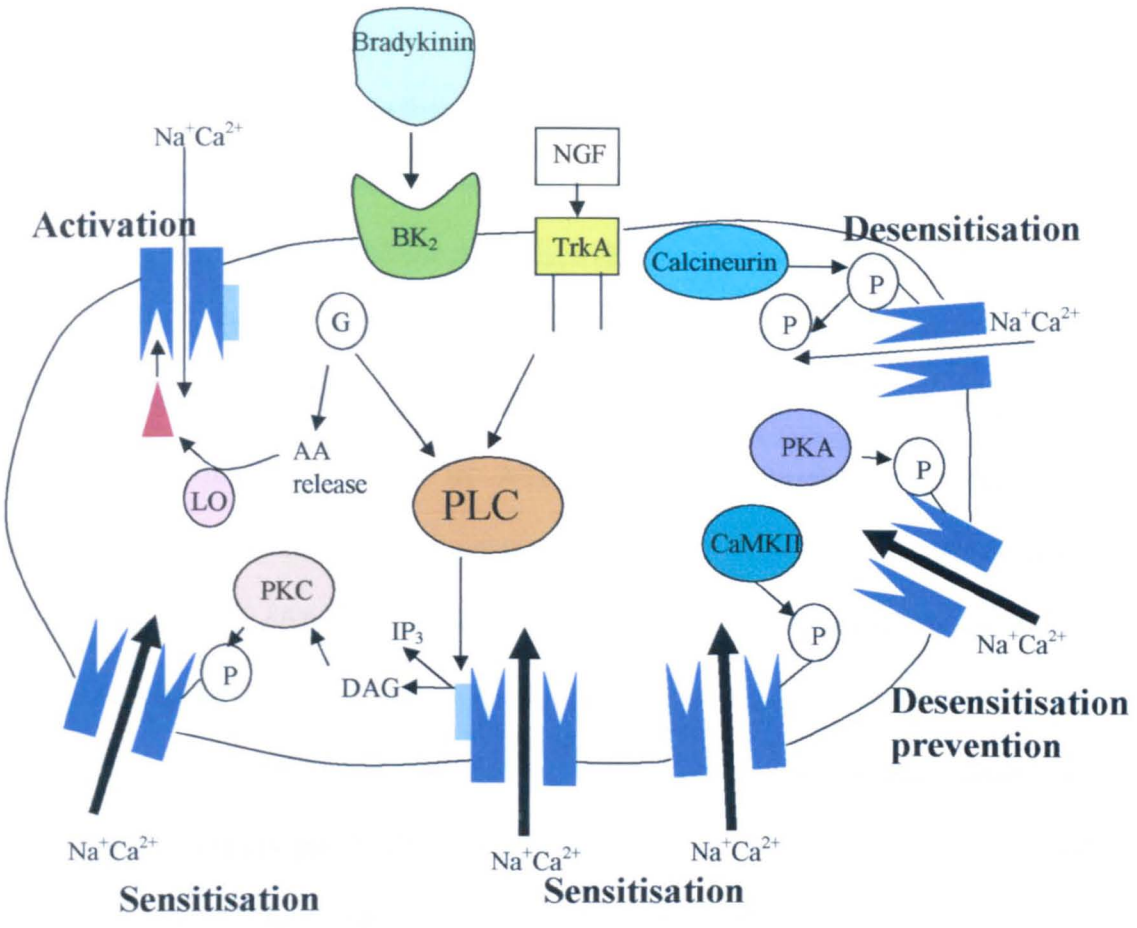


Figure 1.4. The proposed mechanisms of TRPV1 regulation. Bradykinin and NGF activate specific receptors, BK_2 a G-protein coupled receptor (G) and tyrosine kinase (TrkA) respectively resulting in PLC stimulation and arachidonic acid (AA) release. TRPV1 (blue crown) can be sensitised (thick arrow) by PLC hydrolysis of Ins(1,4,5)P_3 (IP_3) or phosphorylation (P) by PKC or activated by lipoxygenase (LO) products (red triangle). Phosphorylation by PKA and CaMKII sensitises the channel whereas dephosphorylation by calcineurin desensitises TRPV1.

1.5.7 ATP

Kwak *et al* (2000) have shown that ATP modulates TRPV1 activity and increases the capsaicin induced activity of the receptor (Kwak et al., 2000). In the absence of magnesium, which is essential for kinase activity, the modulated capsaicin response of TRPV1 to ATP was still observed, therefore Kwak *et al* concluded that this modulation is not mediated by phosphorylation of TRPV1. These findings were confirmed by the replacement of ATP by non-hydrolysable ATP analogues suggesting therefore, that ATP interacts directly with TRPV1. Using single point mutations, potential ATP binding sites were identified as D178 and K735 (see Figure 1.3). Intracellular ATP is normally at a high concentration (4-5 mM) therefore, the receptor is in the ATP bound state and thus under normal physiological conditions the receptor is already positively regulated (Kwak et al., 2000). It is not until ATP levels drop that ATP loss causes a reduced capsaicin response.

1.5.8 Anandamide

Anandamide (Arachidonylethanolamide) is derived from archadonic acid and has been identified in the porcine brain (Devane et al., 1992). It is an agonist for the cannabinoid receptor but it is believed that this compound is also an endogenous mammalian TRPV1 ligand (Zygmunt et al., 1999, Smart et al., 2000, De Petrocellis et al., 2001, Devane et al., 1992). This ligand is thought to mediate vasodilation via the TRPV1 receptor and causes CGRP and SP release from neuronal tissue (Tognetto et al., 2001, Zygmunt et al., 1999). The effect of anandamide on TRPV1 can be blocked by capsazepine and enhanced by PKA activation (De Petrocellis et al., 2001, Smart et al., 2000, Zygmunt et al., 1999)

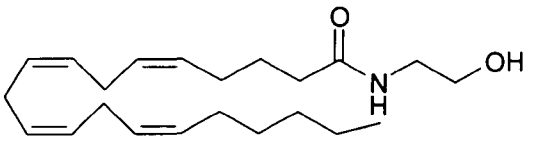


Figure 1.5. Chemical structure of anandamide.

1.5.9 Lipoxygenase Products

Lipoxygenase products are structurally related to anandamide and are derived from arachadonic acid. These compounds are formed during inflammation (Samuelsson, 1983). Hwang *et al* (2000) showed that some lipoxygenase products activated TRPV1, producing currents similar to those produced by capsaicin in both sensory neurons and cloned TRPV1 expressed in HEK cells (Hwang et al., 2000). 12-(S)-HPETE and 15-(S)-HPETE produced the greatest TRPV1 activity and channel activation by these compounds could be inhibited by capsazepine. Hwang *et al* also suggested that the three dimensional structure of 12-(S)-HPETE, in the energy minimised state, superimposes the three dimensional structure of capsaicin (Hwang et al., 2000).

Shin *et al* demonstrated that the 12- lipoxygenase pathway occurs within sensory neurons (Shin et al., 2002) resulting in the production of 12-(S)-HPETE and subsequent TRPV1 activation. BK is known to release arachidonic acid along with other lipids as a direct result of tissue injury in sensory neurons (Shin et al., 2002, Thayer et al., 1988). Lipoxygenases use archidonic acid to produce lipoxygenase products such as 12-(S)-HPETE (see Figure 1.4). Therefore BK can cause TRPV1 activation during tissue injury by release of arachidonic acid and by causing the

hydrolysis of PtdIns(4,5)P_2 reducing TRPV1 inhibition (see section 1.5.3) (Shin et al., 2002, Chuang et al., 2001, Prescott and Julius, 2003).

1.5.10 N-Arachidonoyl-Dopamine (NADA).

NADA is another lipid derived compound, which contains a vanillyl moiety (see Figure 1.6) and is found in the nervous system. This is the first TRPV1 endogenous ligand which shows potency similar to capsaicin in rTRPV1 and hTRPV1 (Huang et al., 2002). The canabinoid receptor, CB_1, is also activated by NADA, however TRPV1 responses to the ligand can be blocked by capsazepine and iodoresiniferatoxin (Huang et al., 2002, Bisogno et al., 2000). The TRPV1 response to NADA can be enhanced by PKC phosphorylation of the channel and Premkumar *et al* suggested that NADA was capable of stimulating PKC because repeated application of the lipid potentiated the channels response to its agonists in a PKC dependent manner. N-palmitoyl- and N-stearoyl-dopamine (PALDA and STEARDA), are structurally related to NADA and are also found in bovine and rat nervous tissue, but show no TRPV1 activating properties. However hTRPV1 overexpressed in HEK cells exhibit enhanced responses to NADA following pretreatement with PALDA or STEARDA (De Petrocellis et al., 2004)

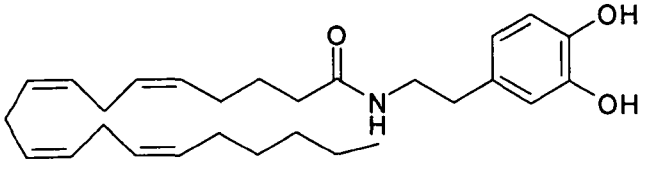


Figure 1.6. Chemical structure of NADA.

1.6 Role of TRPV1

In humans expression of TRPV1 has been seen throughout the body although highest expression appears to be in the DRG (Hayes et al., 2000). TRPV1 may therefore have numerous roles throughout the body.

1.6.1 Pain Perception

It has long been recognised that eating chilli peppers or applying raw chillies or the active component, capsaicin, to the skin causes a burning sensation. Since the channel was first cloned, it is now recognised that capsaicin is acting on the same channel that is activated by temperatures above the physiological range (>43°C) and low pH (Caterina et al., 1997), hence activation of TRPV1 causes a burning sensation, similar to that experienced during inflammation. Repeated application of capsaicin to the skin, although initially causes pain followed by enhanced sensitivity to painful stimuli, ultimately results in pain relief. Pain relief caused by TRPV1 desensitisation is thought to occur by direct regulation of the channel as described in section 1.5 or by depletion of neuropeptides from the nerve terminal or even through loss of capsaicin sensitive afferents (Nolano et al., 1999, Simone et al., 1998). Topical capsaicin is therefore used to treat a variety of painful conditions including arthritis, diabetic neuropathy and even headaches (Mason et al., 2004).

1.6.2 Gastrointestinal Tract (GIT)

The GIT has the highest abundance of nerve cells after the brain (Holzer, 2002). It is therefore not surprising that some of the nerves in the GIT are capsaicin sensitive, TRPV1 expressing fibres, responsible for detecting noxious stimuli (Ward et al.,

2003). TRPV1 in the gut may also be involved in peristalsis (Facer et al., 2001). TRPV1 has been implicated in inflammatory bowl disease (IBD). Immunostaining has indicated TRPV1 may be upregulated in nerve fibres of colonic tissue of IBD patients compared to normal tissue (Yiangou et al., 2001). Thus new therapies for painful inflammatory bowl diseases could be targeted towards TRPV1.

1.6.3 Urinary Tract

TRPV1 mRNA and protein have been identified in the urinary tract of humans and rats along with TRPM8, the cold and menthol receptor (see section 1.2.2) (Avelino et al., 2002, Birder et al., 2001, Stein et al., 2004). The exact role of these channels in the urinary tract is not fully understood but it is postulated that they function to control body temperature by causing excretion of fluid (Stein et al., 2004). Further, using TRPV1 knockout mice it also appears that TRPV1 may be involved in detecting bladder distension (Birder et al., 2002). This theory is further substantiated because capsaicin and resiniferatoxin have successfully been used to treat bladder overactivity. Intravesical capsaicin or resiniferatoxin appears to increase the bladder capacity at which micturition occurs (Lazzeri et al., 1997, Geirsson et al., 1995, Giannantoni et al., 2002, Cruz et al., 1997) suggesting desensitisation of TRPV1 sensitive neurons in the bladder prevents early micturation.

1.6.4 Airways

TRPV1 has been identified in the airways of both humans and guinea pigs by immunostaining (Watanabe et al., 2005, Mitchell et al., 2005). It in now believed that TRPV1 is one of the cough receptors (Anderson, 2004, Morice and Geppetti,

2004). TRPV1 is activated by acids, capsaicin and resiniferatoxin (Caterina et al., 1997) all of which are known to cause cough, indeed, capsaicin and resiniferatoxin are the most potent tussive agents known (Laude et al., 1993). Moreover, in guinea pigs, cough induced by citric acid or capasaicin can be reduced by pre-treatment with the TRPV1 specific antagonists iodoresiniferatoxin and capsazepine (Trevisani et al., 2004). Inflammatory mediators such as bradykinin and nerve growth factor are also known to sensitise TRPV1 (see section 1.5.3). Bradykinin and protons are released into inflamed airways and NGF is elevated in asthmatic lungs (Hwang and Oh, 2002, Bonini et al., 1996) and may therefore play an important role in sensitising TRPV1 in the airways. As discussed earlier these inflammatory mediators all act via specific pathways to sensitise TRPV1, lowering the threshold at which the receptor responds to activators such as lipoxygenase products like 12-(S) HPETE. These lipoxygenase products are known to be released from airway epithelial cells (Holtzman, 1992, Hwang et al., 2000). Activation of TRPV1 in the airways leads to the generation of an action potential causing the release of Substance P and calcitonin gene-related peptide (CGRP) ultimately resulting in smooth-muscle contraction, an important symptom of asthma. It therefore appears that TRPV1 may be important in the airways with roles in the cough reflex and inflammatory disorders such as asthma.

1.7 Working Hypothesis

TRPV1 may be important in asthma and cough stimulation. There is little understanding of how irritants are sensed in the airways provoking cough. TRPV1 is a promising target as it is sensitive to capsaicin and other inflammatory mediators which are known to cause coughing. It is our overall hypothesis that TRPV1 plays a pivotal role in provoking cough in respiratory disease. To this aim, this thesis

focuses on 1) accessing the ability of agents known to induce cough to activate TRPV1, 2) the molecular determinants that may alter TRPV1 susceptibility to activation and 3) the ability of mediators detected in respiratory inflammation to regulate the cellular expression of TRPV1 in human primary bronchial fibroblasts (HPBF).

1.8 Aims

1. Clone and express rat and human TRPV1
2. Pharmacologically characterise the TRPV1 permanently expressing cell lines
3. Determine the ability of citric acid to activate TRPV1
4. Investigate the role of *N*-linked glycosylation in regulating TRPV1 expression and function
5. Determine whether inflammatory mediators can induce functional expression of TRPV1 in HPBF.

Chapter 2

Cloning and Pharmacological Characterisation of rTRPV1 and hTRPV1

2.1 Introduction

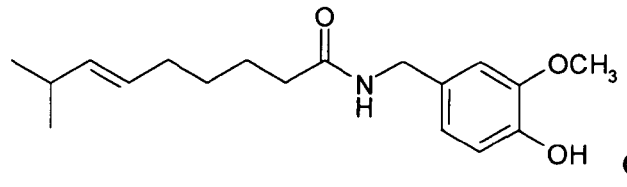
Since TRPV1 was first cloned, there have been a plethora of studies published discussing the various agonists, antagonists and regulators of both rat and human TRPV1 (Caterina et al., 1997, Hayes et al., 2000). It is now well established that TRPV1 is activated by a variety of naturally occurring compounds. The best documented TRPV1 specific agonist and perhaps even the reason for the channels discovery, is capsaicin, the active component of chilli pepers (Caterina et al., 1997) (see Figure 2.1). It is now known that piperine the pungent extract of black pepper is also a TRPV1 specific agonist (McNamara et al., 2005) along with zingerone which is found in garlic (Witte et al., 2002). Resiniferatoxin is the most pungent TRPV1 specific agonist and is found in the latex of the Moroccan cactus-like plant, *Euphorbia Resinifera* (see Figure 2.1).

There are a number of commercially available competitive TRPV1 specific antagonists. The two best characterised are capsazepine and the more potent iodoreiniferatoxin. Iodoresiniferatoxin is structurally similar to resiniferatoxin but is synthesised by iodination of the phenol ring (see Figure 2.1) (Wahl et al., 2001).

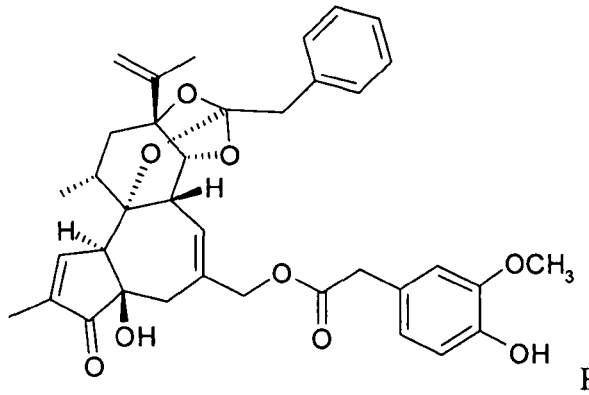
Recently a number of studies have shown that TRPV1 is expressed not only on the plasma membrane but also on the membrane of some internal stores (Karai et al., 2004, Liu et al., 2003, Olah et al., 2001b, Turner et al., 2003, Wisnoskey et al., 2003). Using confocal microscopy Olah and colleagues were able to localize the intracellular staining of rTRPV1 to the endoplasmic reticulum (Olah et al., 2001b). More detailed investigations have revealed that the intracellular rTRPV1 channel signals in response to TRPV1 specific agonists and can be blocked by capsazepine

(Karai et al., 2004, Liu et al., 2003, Turner et al., 2003, Wisnoskey et al., 2003). Reports on the exact location of intracellular rTRPV1 are still confusing. Thapsigargin is an inhibitor of endoplasmic reticulum calcium ATPases (Wictome et al., 1992). This causes depletion of calcium from the endoplasmic reticulum and is therefore a useful tool in determining whether calcium released from intracellular stores is from specific thapsigargin sensitive organelles. Currently, however the literature on whether rTRPV1 is on thapsigargin sensitive stores is conflicting (Karai et al., 2004, Liu et al., 2003, Turner et al., 2003, Wisnoskey et al., 2003). At the moment all work done on intracellular TRPV1 appears to have been carried out on the rat channel and no investigation of intracellular hTRPV1 has been done (Karai et al., 2004, Liu et al., 2003, Turner et al., 2003, Wisnoskey et al., 2003, Olah et al., 2001b).

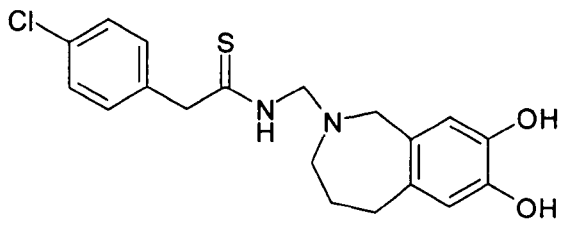
This chapter describes the cloning and characterisation of both rat and human TRPV1. rTRPV1 has been cloned from rat dorsal root ganglia (DRG) and hTRPV1 has been cloned from MRC5 cDNA. Both channels were permanently expressed in HEK293 cells and characterised using known TRPV1 agonists. A comparison between intracellular rTRPV1 and hTRPV1 has also been made.



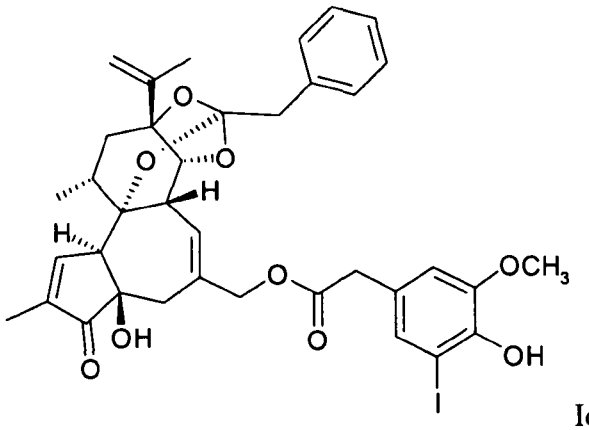
Capsaicin



Resiniferatoxin



Capsazepine



Iodoresiniferatoxin

Figure 2.1. Chemical structures of two of the TRPV1 specific agonists, capsaicin and resiniferatoxin and antagonists capsazepine and iodoresiniferatoxin.

2.2 Materials and Methods

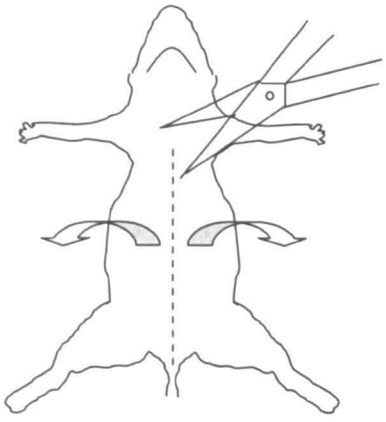
2.2.1 Reagents and Materials

Primers were purchased from either Sigma Genosys, Pampisford, Cambridgeshire, UK or MWG Biotechnology, Ebersberg, Germany. PCR purification kits, gel extraction kits, RNeasy mini kits, plasmid isolation kits, Hot Start Taq DNA polymerase, Omniscript RT PCR kits and Proof Read Taq DNA polymerase were all bought from Qiagen, Crawley, West Sussex, UK. The rapid DNA ligation kit was bought from Roche, Lewes, East Sussex, UK. The Quick Change site directed mutagenesis kit and competent *E.coli* were supplied by Stratagene, Amsterdam, The Netherlands. Random primers (N_6), dNTPs, lipofectAMINE, and the mammalian expression vector pcDNA3 were bought from Invitrogen, Paisley, UK. All restriction enzymes and their respective buffers as well as the NEB DNA polymerase were obtained from New England Biolabs, Hitchin, Hertfordshire, UK. Fluo-3 acetoxymethyl ester was purchased from Cambridge Biolabs, Cambridge UK and later from Invitrogen, Paisley, UK. Penicillin/streptomycin, FCS, DMEM, sodium pyruvate and geneticin (G418) were all bought from Gibco, Paisley, UK. All other chemicals and reagents were purchased from Sigma-Aldrich, Poole, Dorset, UK. Juvenile, male Sprague Dawley rats (Charles River's CD Rats) were supplied by Charles Rivers, Margate, Kent, UK.

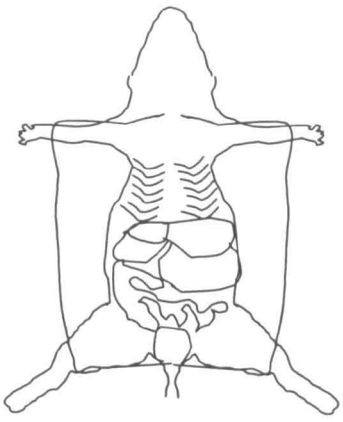
2.2.2 Cloning of Rat TRPV1

2.2.2.1 Rat DRG Isolation

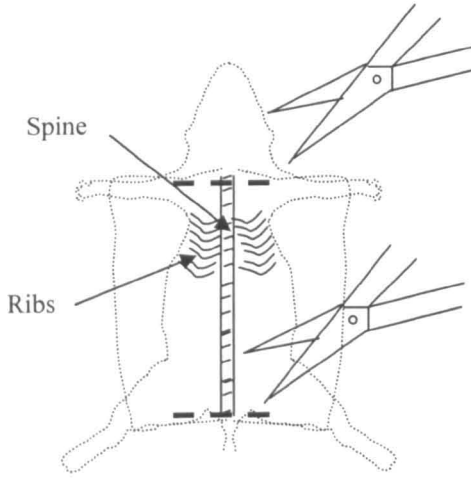
Sprague Dawley Rats were housed and kept in accordance with the UK Home Office code of practice for the housing and care of animals used in scientific procedures. Juvenile, male Sprague Dawley rats (three to five weeks old, Charles River's CD Rats) were sacrificed by a schedule one procedure in accordance with the Home Office Guidance on the Operation of the Animals (scientific procedures) act 1986. An incision was made longitudinally down the front of the animal before the organs of the thorax and abdomen were removed in order to expose the spinal column. The spine was detached from the animal by making an incision along the top of the neck and base of the tail. The skin was then removed, leaving the spinal column. The spinal cord was exposed by removing a strip of spine along the entire spinal column. DRG were accessed by gently pulling on the nerves that branch out from the spinal cord. The exposed DRG were then carefully removed.



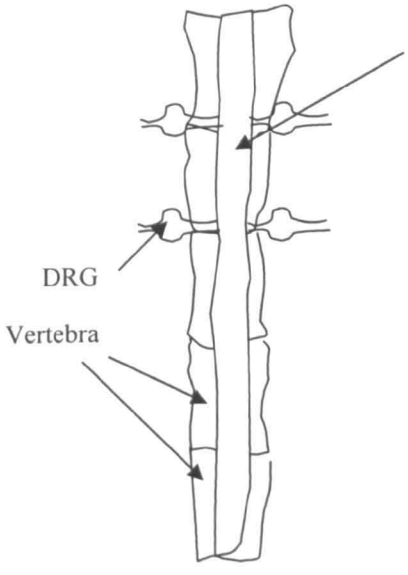
Male rats were killed by concussion and dislocation of the neck. An incision was made longitudinally down the front of the animal.



Organs were revealed by folding back the skin. All organs were removed leaving the spinal column and ribs



The spine was detached from the animal by making an incision along the top of the neck and base of the tail. The skin was then removed, leaving the spinal column.



Spinal Cord

The spinal cord was exposed by removing a strip of spine along the entire spinal column. DRG were accessed by gently pulling on the nerves that branch out from the spinal cord. The exposed DRG were then carefully removed.

Figure 2.2. Dissection of dorsal root ganglia from rat spinal column.

2.2.2.2 mRNA Extraction

Freshly isolated DRG were immediately placed into an eppendorf tube containing 600 µl of lysis buffer (buffer RLT from the Qiagen mRNA isolation kit) containing 1% β-Mercaptoethanol and were kept on ice to maintain mRNA stability. The sample was subsequently shaken before repeatedly passing the suspension through syringe needles. Once full lysis had been achieved, as evident by the transparency of the buffer, 600 µl (one volume) of 70% ethanol was applied to the sample before vortexing. The sample was then applied to a Qiagen RNeasy mini column and the mRNA was eluted following a wash sequence according to the Qiagen RNeasy Kit instructions. Recovered mRNA was then quantified using a Gene Quant Spectrometer (Amersham). mRNA concentration was calculated using the Beer-Lambert Law after measuring absorbtion at 260nm. The mRNA was stored at -80°C until use.

2.2.2.3 Reverse Transcription (RT).

Messenger RNA (mRNA) was reverse transcribed into stable cDNA by reverse transcription (RT). The RT reaction comprised of 2 µg of mRNA, 1 mM dNTPs, 1.8 nM of random primer oligonucleotides (N6), 5 Units (5 U) of RNase inhibitor, 4 U of Omniscript Reverse Transcriptase and buffer RT from the Qiagen omniscript reverse transciptase kit to a total volume of 20 µl. The reaction was performed as described in table 2.1 and cDNA was subsequently stored at -20°C.

The thermal cycler (Techne – Techgene FTGENE2D) was programmed to run the following cycles:

Process	Temperature (°C)	Time (Minutes)
Enzyme Activation	Room Temperature	10
Primer Annealing and Extension	42	50
Enzyme Denaturation	95	10

Table 2.1. RT cycle.

The quality of the cDNA was assessed by amplification of actin.

2.2.2.4 Amplification of TRPV1 from DRG cDNA

rTRPV1 possesses an open reading frame (ORF) of 2,514 nucleotides, accession number AF029310 (Caterina et al., 1997). Primers were designed to amplify the product in four separate but overlapping fragments (see Table 2.2 and Figure 2.3). Primer rTRPV1-1D possessed a HindIII restriction site (AAGCTT) to allow position specific cloning. In addition a Kozak sequence (CCACC) was included prior to the start codon of the TRPV1 sequence (Kozak, 1984). The Kozak sequence is believed to facilitate translation. Primer rTRPV1-1U possessed an ApaI restriction site (GGGCCC) following the stop codon (TGA) to allow positional cloning. The primers were designed using the program Primer 3 on the Internet (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

Fragment	Primer Name	Sequence	**Primer Annealing Temperature (°C)**	**Extension Time (Minutes)**
A	rTRPV1-1D	5'-GGCCGAAGCTTCCACCATGGAA CAACGGGCTAGCTTAGACTC-3'	60	1.5
	rTRPV1-4U	5'-GCCAGGCCTCCCTTTGGTTT-3'		
B	rTRPV1-2D	5'-TGCGGCTAACGGGGACTTCT-3'	62	1.5
	rTRPV1-3U	5'-AGTAGGCAGCCGCGGTGAAG-3'		
C	rTRPV1-3D	5'-CTTCACCGCGGCTGCCTACT-3'	56	1.5
	rTRPV1-2U	5'-TGAACTCCAGGTCGCCCATG-3'		
D	rTRPV1-4D	5'-CATGGGCGACCTGGAGTTCA-3'	56	1
	rTRPV1-1U	5'-GGCCGCGGGCCCTTATTTCT CCCCTGGGACCATGG-3'		
AB	rTRPV1-1D	5'-GGCCGAAGCTTCCACCATGGAA CAACGGGCTAGCTTAGACTC-3'	54	3
	rTRPV1-3U	5'-AGTAGGCAGCCGCGGTGAAG-3		
CD	rTRPV1-3D	5'-CTTCACCGCGGCTGCCTACT-3'	56	3
	rTRPV1-1U	5'-GGCCGCGGGCCCTTATTTCT CCCCTGGGACCATGG-3'		
ABCD	rTRPV1-1D	5'-GGCCGAAGCTTCCACCATGGAA CAACGGGCTAGCTTAGACTC-3'	56	5
	rTRPV1-1U	5'-GGCCGCGGGCCCTTATTTCT CCCCTGGGACCATGG-3'		

Table 2.2. Primers and conditions for rTRPV1 PCR. AB, CD and ABCD were amplified by overlapping PCR.

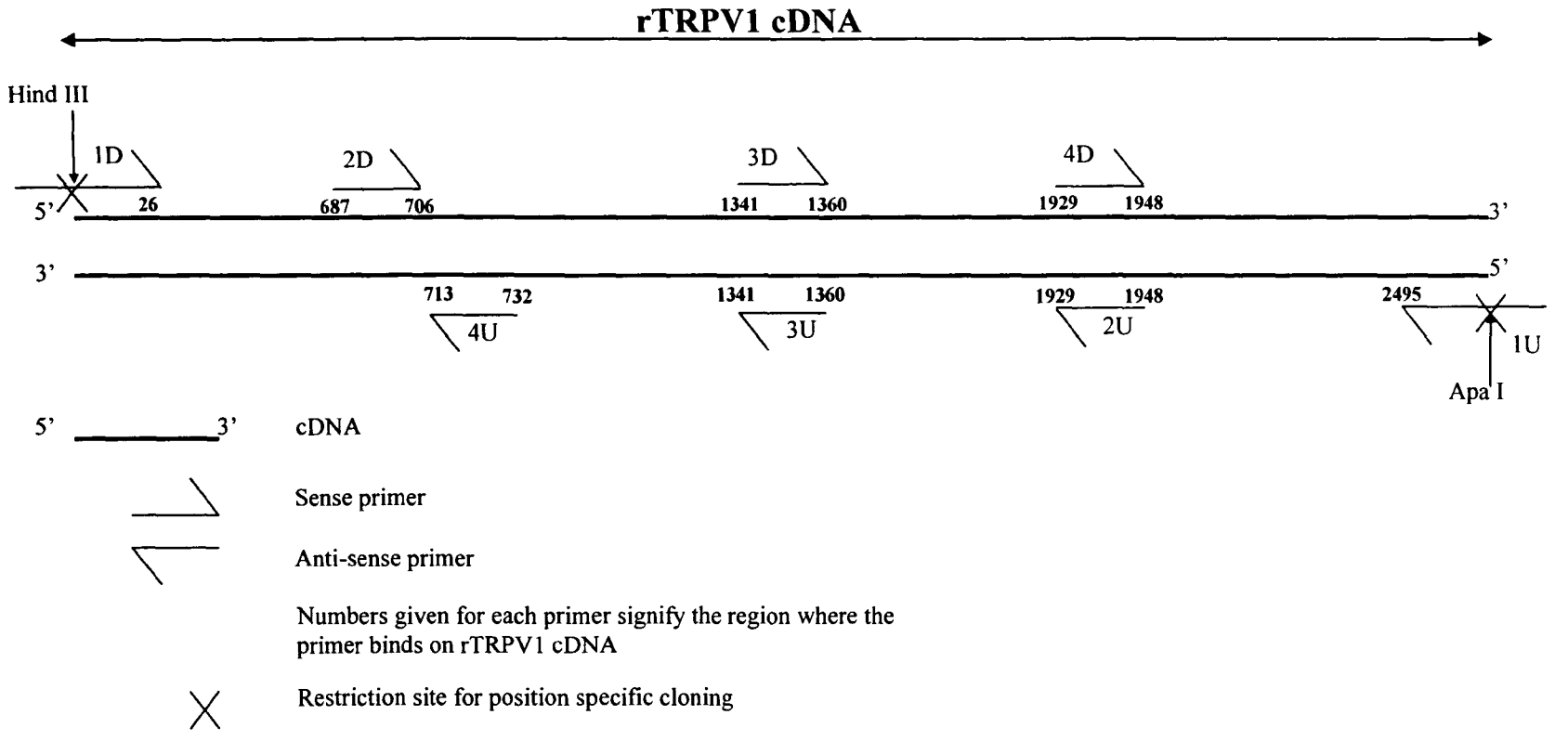


Figure 2.3. Diagramatic representation of the ORF of rTRPV1, showing the primer positions within the sequence.

Each PCR was performed in a Techne Technegene with 1 µl of cDNA (RT reaction mixure containing the newly synthesised cDNA, see section 2.2.2.3), 20 pmol of each primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$ and 1 U of Taq Polymerase (Qiagen) in a 50 µl reaction volume. Specific PCR conditions for each product are given in Table 2.2. The PCR thermal cycler was programmed to run the following cycles:

Number of Cycles	Process	Temperature (°C)	Time (Minutes)
1	Hot Start Taq Initial Activation	95	15
20 – 35	DNA Denaturation	94	1
	Primer Annealing to DNA Template (temperature dependent on specific primer)	50 – 68 (See Table 2.2)	1
	Extension, Temperature optimum for Taq and time dependent on product size (1min per kbase)	72	1 – 3
1	Final Extension	72	10

Table 2.3. Cycle conditions for PCR.

RT-PCR products were separated on a 1.3% agarose gel containing ethidium bromide. The products of the correct size were extracted following visualisation under UV illumination, using a clean scalpel. The DNA fragments were then gel purified using a Qiagen gel extraction kit as per the manufacturers instruction. Once each of the four fragments (A, B, C, and D) had been generated and gel purified, overlapping PCR reactions were performed to link fragment A to B, and C to D, before conducting a final overlapping PCR procedure to link AB to CD (the whole open reading frame of rTRPV1). The PCR product, ABCD, was subsequently gel purified before undergoing restriction digest and then ligation into vector pcDNA3.

2.2.2.5 Restriction Digest of pcDNA3 and PCR Product ABCD.

The vector, pcDNA3 and the PCR product ABCD were digested with HindIII and ApaI restriction enzymes in order to linearise the vector and reveal HindIII and ApaI cohesive ends on each product. HindIII and ApaI exhibit optimum activity at different temperatures. Therefore in a total volume of 20 µl, 5 µl of vector (or 6 µl of ABCD) was incubated with 5 U of ApaI for three hours at room temperature (25°C) in a reaction mixture containing NEB buffer 4, 100 µg/ml BSA and DNase/RNase free water. Following the incubation, 10 µl of HindIII solution containing 1 µl of buffer 4, 1 µl of 1 mg/ml BSA, 15 U of Hind III and 6.5 µl of DNase/RNase free water was added to the original reaction mixture. The total reaction was incubated at 37°C (optimum temperature for HindIII) for a further three hours. The vector and PCR product were then gel purified to remove the unwanted, cleaved portion of DNA, using a Gel Extraction Kit.

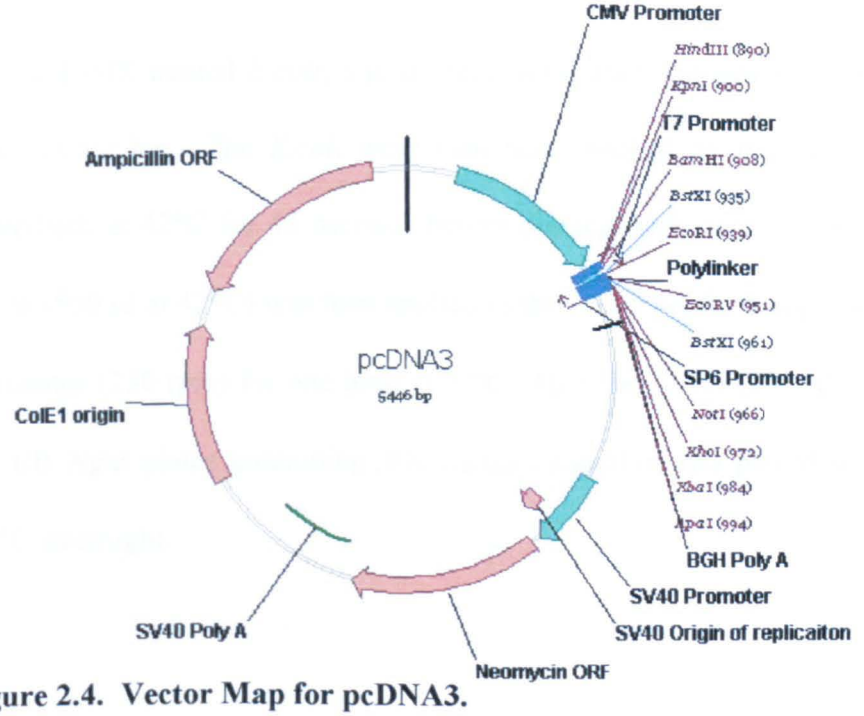


Figure 2.4. Vector Map for pcDNA3.

2.2.2.6 Ligation of PCR Product ABCD into pcDNA3.

The ligation reaction contained 10 µl of 2 x ligation buffer, 1 µl of linearised vector, 6 µl of PCR product, ABCD (with the engineered restriction sites cleaved, see 2.2.2.5), 1 µl of DNA ligase and DNAse/RNAse free water to make a final volume of 21 µl. The reaction was incubated at room temperature for 5 minutes. For the negative control an identical reaction was set up with 9 µl of DNase/RNase free water in place of the PCR product, ABCD.

2.2.2.7 Transformation.

Competent *E.coli* cells (XL-1 Blue, 50 µl) were allowed to thaw on ice before the addition of 1.7 µl of 10% β-Mercaptoethanol to increase transformation efficiency. Cells were then gently agitated every two minutes for a total of 10 minutes.

To the β-ME treated *E.coli*, 3 µl of ligation product was added and incubated for 30 minutes on ice. The *E.coli* were then heat shocked by placing the reaction in a waterbath at 42°C for 45 seconds before placing back onto ice for 2 minutes. LB broth (500 µl at 42°C) was then applied to the *E.coli* before being placed in a shaking incubator (250 rpm) for one hour at 37°C. Half the *E.coli* (250 µl) were then plated on LB Agar plates containing 100 µg/ml ampicillin and placed in an incubator at 37°C overnight.

2.2.2.8 Screening Colonies

Single colonies from the transformed *E.coli* were selected with a 20 µl sterile pipette tip, and placed straight into separate 30 ml universal containers containing 5 ml of LB broth and 100 µg/ml ampicillin. The universal containers were then placed in a shaking incubator at 37°C overnight to allow the colonies to grow.

Plasmid DNA was extracted from the expanded colonies and purified using Miniprep Vector Isolation Kits (as per the manufacturers instructions, Qiagen). From each expanded colony restriction digest analysis (using ApaI and HindIII) was performed to ascertain whether the plasmid contained the PCR product, ABCD. For each digest 5 µl of the plasmid miniprep was used. Following digestion each reaction was run on a 1.3% agarose gel and visualised by UV illumination. Colonies containing the ABCD insert were sent to Qiagen for sequencing.

2.2.2.9 Site-Directed Mutagenesis to Repair Taq Polymerase Errors

cDNA from the cloned ABCD product was compared to the TRPV1 cDNA sequence on PubMed, using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The mistakes identified were then repaired. Briefly, primers were designed between 25 and 45 bases long containing the corrected mutation in the middle of the sequence. The primer also had a minimum GC content of 40% and started and finished with a G or a C. The reaction was set up and run as per the Stratagene Quick Change Site-Directed Mutagenesis Kit instructions. *E.coli* containing the corrected TRPV1 plasmid were kept at -20°C in glycerol stocks (1 ml of 50% glycerol, 50% *E.coli* in LB Broth). In total, 14 errors were identified within the

rTRPV1 sequence and were successfully corrected to the reported sequence by Caterina *et al* 1997. This was confirmed by sequencing by MWG biotech.

2.2.2.10 Generation of a Stable TRPV1 Cell Line.

rTRPV1 was transfected into HEK293, cells using the lipofectAMINE method according to the manufacturer's protocol (Invitrogen). Briefly, the HEK293, cells were plated onto 65 mm diameter petri dishes in Dulbecco's modified Eagle's medium, (DMEM) containing 10% FCS, 1% penicillin/streptomycin and 100 μM sodium pyruvate and allowed to reach 40-80% confluent. To two Falcon 5 ml polystyrene round bottom tubes, labelled "1" and "2", 1 ml of Opti-Mem medium was added. To tube one, 5 μg of the TRPV1 construct was added. In tube two, 20 μl of lipofectAMINE was added. The two tubes were vortexed for four seconds and left to equilibrate for 15 minutes at room temperature. During this time, the DMEM was removed from both the HEK293 and replaced with 4 ml of Opti-Mem. After the incubation period, the contents of tube two were poured directly into the contents of tube one. This was vortexed for four seconds and incubated for a further 15 minutes at RT. The Opti-Mem was removed from the cells and replaced with the Opti-Mem containing the rTRPV1 construct and lipofectAMINE. The cells were incubated at 37°C for 24 hours before the medium was changed for DMEM containing 10% FCS, 1% penicillin/streptomycin, 100 μM sodium pyruvate. Following a further 24 hours the cells were harvested and placed into a 75 cm^2 flask with selection medium (G418 0.6 mg/ml) and propagated for at least two weeks to allow selection.

2.2.2.11 Single Cell Cloning

In order to obtain a single cell line with optimum expression of rTRPV1, transfected cells were counted using a haemocytometer and diluted to 5 cells per ml in culture medium (Dulbecco's modified Eagle's medium, (DMEM) containing 10% FCS, 1% penicillin/streptomycin and 100 μM sodium pyruvate), then 200 μl of this diluted suspension was plated into each well of two 96 well plates. Once colonies of cells became visible in the wells, the contents of wells clearly containing just one colony were transferred to 25 cm^2 flasks containing 5 ml of culture medium. These cells were left to reach confluence before calcium signal measurements were made to identify cells expressing functional rTRPV1. Once a rTRPV1 clone had been identified, this clone was taken through a second and third round of single cell cloning to ensure that the final clone was derived from a single cell.

2.2.2.12 RT-PCR

RT-PCR was performed to verify that the HEK293 cells had been successfully transfected with rTRPV1. mRNA was extracted from mock transfected HEK293 cells, rat dorsal root ganglia and rTRPV1 cells using an RNeasy minikit (Qiagen) according to manufacturer's protocol and quantified using a GeneQuant™ spectrophotometer (Amersham Biosciences). cDNA was synthesised by reverse transcription of 2 μg of mRNA with an omniscript RT kit (Qiagen) and random primers (Invitrogen) as described in section 2.2.2.3. The PCR reaction for rTRPV1 contained 1 μl of cDNA, PCR buffer, 0.2 mM dNTPs and 1 U Taq DNA polymerase (New England Bioscience) with specific oligonucleotide primers (rTRPV13D and rTRPV12U, see Table 2.2). The PCR reactions were carried out for 35 cycles of 94°C for 3 minute, 56°C for 1 minute and 72°C for 1 minute with a final extension

for 10 minutes. The PCR products were subsequently separated on 1.3% agarose gel and visualised under UV illumination.

2.2.3 Cloning of Human TRPV1

2.2.3.1 PCR Amplification of Human TRPV1 from MRC5 cDNA.

Human TRPV1 is 92% identical to rTRPV1 at the amino acid level, with an open reading frame of 2,517 bases (Hayes et al., 2000) (Accession number AY131289). The primer design strategy was similar to that for rTRPV1, however section C (the third quarter) failed to PCR, therefore two new sets of primers were designed (C1D, C1U and C2D, C2U). These primers divided C in two and overlapped B and D by 194 and 153 bases respectively (see Figure 2.5). Primers hTRPV1-1D and hTRPV1-1U were designed to contain HindIII and BamHI restriction sites respectively (see Table 2.4) in order to allow positional cloning of hTRPV1 into pcDNA3 (see Figure 2.5). The primers were again designed using the program Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

Amplification of each hTRPV1 section from MRC5 (ATCC number CCL-171) cDNA was performed according to the PCR conditions described in section 2.2.2.4. The specific conditions for each reaction are detailed in table 2.4. Overlapping PCR was used to link fragments C1 to C2 to make fragment C. Fragments C and D were then linked by overlapping PCR, before CD was then linked to AB, again using overlapping PCR.

As with rTRPV1, the PCR product ABCD was gel purified and digested with HindIII and BamHI, before ligation into vector pcDNA3. The ligation reaction was

transformed into *E.coli* before colonies were screened for pcDNA3 containing the PCR product ABCD (as described in 2.2.2.8). Colonies containing insert ABCD were sent for sequencing to Qiagen.

Fragment	Primer Name	Sequence	Primer Annealing Temperature (°C)	Extension Time (Minutes)
A	hTRPV1-1D	5'-GGCCGAAGCTTCCACCATG AAGAAATGGAGCAGCAC-3'	54	1.5
	hTRPV1-4U	5'-ACAAGCTCCTTCAGGCTGTC-3'		
B	hTRPV1-2D	5'-GACAGCCTGAAGGAGCTTGT-3'	54	1.5
	hTRPV1-3U	5'-CTCCAGCACCGAGTTCTTCT-3'		
C1	hTRPV1-C1D	5'-GGAGCTCACCAACAAGAAGG-3'	54	1.5
	hTRPV1-C1U	5'-GCTGTCCACAAACAGGGTCT-3'		
C2	hTRPV1-C2D	5'-AGACCCTGTTTGTGGACAGC-3'	54	1.5
	hTRPV1-C2U	5'-CCGATGGTGAACTTGAACAG-3'		
D	hTRPV1-4D	5'-TTCTTGTTCGGGTTTTCCAC-3'	54	1.5
	hTRPV1-1U	5'-GGCCGCGGATCCTCA CTTCTCCCCGGAAGCGG-3'		
AB	hTRPV1-1D	5'GGCCGAAGCTTCCACCATG AAGAAATGGAGCAGCAC-3'	54	3
	hTRPV1-3U	5'-CTCCAGCACCGAGTTCTTCT-3'		
CD	hTRPV1-1C1D	5'-GGAGCTCACCAACAAGAAGG-3'	54	3
	hTRPV1-1U	5'-GGCCGCGGATCCTCA CTTCTCCCCGGAAGCGG-3'		
ABCD	hTRPV1-1D	5'-GGCCGAAGCTTCCACCATG AAGAAATGGAGCAGCAC-3'	54	5
	hTRPV1-1U	5'-GGCCGCGGATCCTCA CTTCTCCCCGGAAGCGG-3'		

Table 2.4. Conditions for human TRPV1 PCR. CD and ABCD were amplified by overlapping PCR. Fragment A would not amplify on its own, so AB was amplified straight from MRC5 cDNA.

Once hTRPV1 had been identified within a single *E.coli* clone, site directed mutagenesis was performed to correct Taq polymerase errors within the sequence (see section 2.2.2.9).

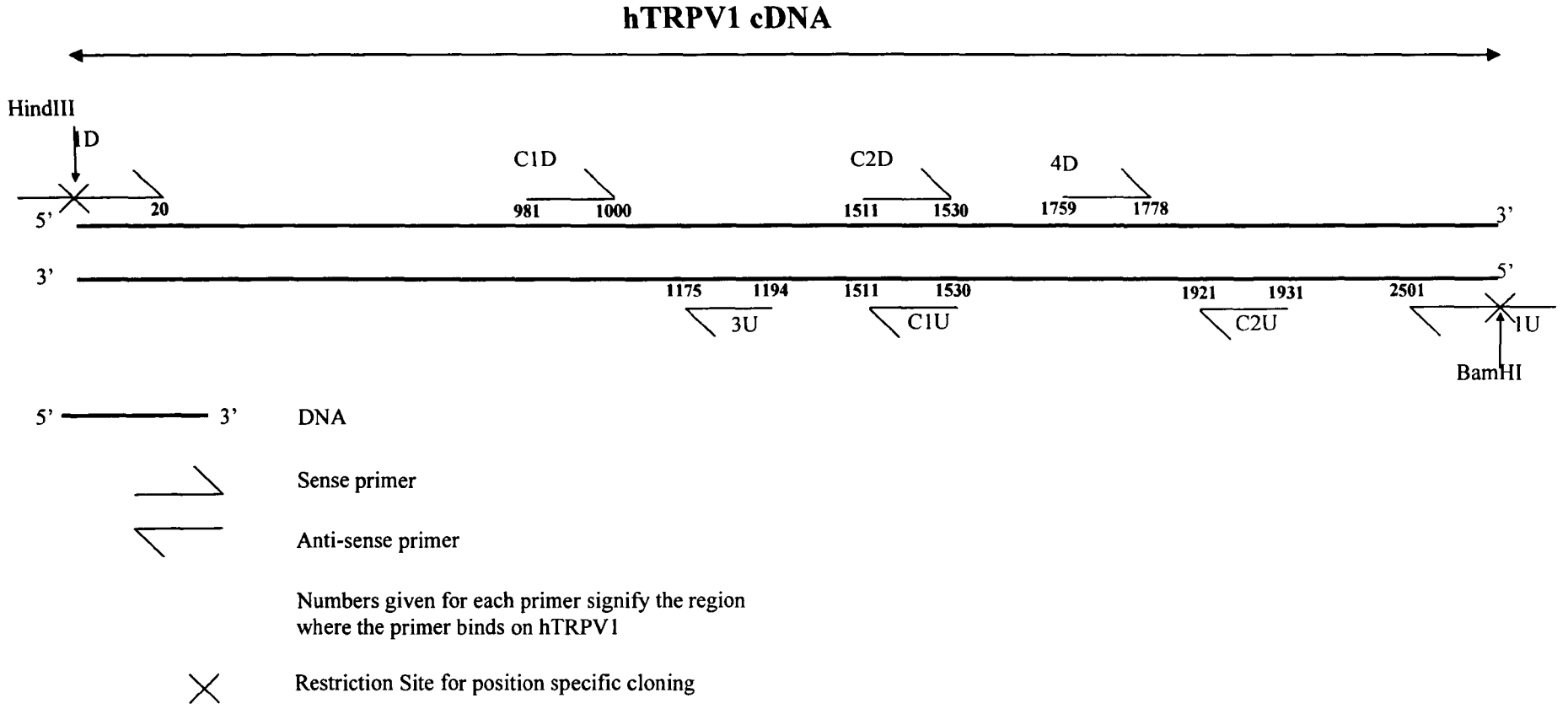


Figure 2.5. Diagramatic representation of the ORF of hTRPV1, showing the primer positions within the sequence.

2.2.4 Intracellular Calcium Measurement using Fluorospectrometry

Calcium signalling was performed using methods based on those by Compton *et al* (Compton et al., 2000). Cells at 90% confluence in 75 cm^2 (approximately $9x10^6$ cells, BD falcon) were washed and harvested with PBS (without calcium or magnesium). Cells were pelleted and resuspended in 1 ml of normal culture medium containing 0.25 mM sulphinpyrazone and 25 μg of Fluo-3 acetoxymethyl ester (Fluo-3 AM) in DMF. The cells were then incubated at room temperature for 25 minutes whilst gently shaking, to allow the fluorescent probe to be taken up by the cells. The cells were then washed by centrifugation to remove excess Fluo-3 AM and resuspended in Calcium Assay Buffer (CAB) (150 mM sodium chloride, 250 μM sulphinpyrazone, 3 mM potassium chloride, 10 mM glucose, 20 mM HEPES and 280 mM calcium chloride ($CaCl_26H_2O$), pH7.4).

Increases in intracellular calcium levels were measured at room temperature using a fluorospectrometer (Photon Technology International). The fluorospectrometer was set to emit an excitation wavelength of 480 nm and record light emitted with a wavelength of 530 nm. Each cuvette contained 2 ml of suspended cells in CAB and a small magnetic flea to keep the cells suspended and mix any reagents added. Concentration-effect curves were constructed for each test agonist by adding increasing concentrations of the agonist to separate cell containing cuvettes in half log increments. The increase in fluorescence measured at 530 nm was expressed as a percentage of the maximum fluorescence signal after the addition of 6 μM calcium ionophore (A23187). For inhibition of capsaicin responses, cells were incubated in

CAB containing 1, 10, or 30 nM iodoresiniferatoxin or 1 or 10 μM capsazepine for 1 minute before the addition of capsaicin.

2.2.4.1 Investigation of the Role of TRPV1 on Intracellular Stores

Calcium signalling was used to determine whether TRPV1 was expressed on the plasma membranes or also on the membranes of internal calcium stores. Capsaicin concentration effect curves were constructed for both rTRPV1 and hTRPV1 in the presence or absence of calcium in the CAB. To determine the dependence of the capsaicin response on thapsigargin sensitive intracellular calcium stores, cells were preincubated for four minutes with 1 μM thapsigargin prior to the addition of capsaicin either in the presence or absence of extracellular calcium.

2.2.5 Flow Cytometry

Flow cytometry was used to compare the density of TRPV1 on cells. Cells were washed and harvested in cold PBS before incubation on ice in PBS with 4% formaldehyde for 30 minutes. The cells were then washed twice by centrifugation at 4°C with cold PBS followed by incubation on ice with rabbit anti-capsaicin receptor polyclonal antibody (1 in 400, Chemicon International) in cold basal DMEM culture medium for 1 hour. The cells were again washed twice by centrifugation at 4°C with cold PBS and incubated on ice with anti-rabbit IgG FITC (1 in 100) conjugated monoclonal antibody (sigma) for 45 minutes. The cells were then washed two more times by centrifugation at 4°C with cold PBS and then analysed using a Becton Dickinson Flow Cytometer.

2.3 Results

2.3.1 rTRPV1 was Cloned from Rat DRG and Permanently Expressed in HEK293 Cells

rTRPV1 was successfully amplified from rat DRG cDNA in four pieces which were linked by overlapping PCR (Figures 2.6 and 2.7). Once the PCR product was ligated into the mammalian expression vector, pcDNA3 (Figure 2.8) and sequenced, 14 single nucleotide DNA polymerase errors were identified in the cloned cDNA, when compared to the published sequence (accession number AF029310). These errors were corrected by site directed mutagenesis. The full rTRPV1 strand in pcDNA3 was then sequenced to confirm the polymerase errors had been corrected. The corrected rTRPV1 in pcDNA3 was then transfected into HEK293 cells and a stably transfected cell line was generated. To ensure this cell line originated from a single cell, the geneticin resistant cells were taken through three rounds of single cell cloning. In the first round 30 single cell clones were tested for capsaicin responsiveness by measuring increases in intracellular calcium (see section 2.2.4). Six and four clones were selected in the second and third rounds respectively, and one clone was chosen, from the latter round for subsequent pharmacological characterisation (rTRPV1-HEK). As has been previously reported (Olah et al., 2001a), we too found that clones displaying the greatest responses to capsaicin (as determined by measuring increases in intracellular calcium) died. Thus, a rTRPV1 cell line was selected which remained stable and simultaneously gave the greatest response to capsaicin.

RT-PCR was performed on cDNA derived from rat dorsal root ganglia, mock transfected HEK and rTRPV1-HEK in order to confirm that mock transfected HEK

cells do not express mRNA for TRPV1 and that our rTRPV1-HEK transfected HEK cell line did express mRNA for rTRPV1 (Figure 2.9). The RT-PCR results confirmed previous studies, that dorsal root ganglia cDNA is positive for rTRPV1 (Jerman et al., 2000, Caterina et al., 1997). No band was observed for mock transfected HEK cDNA, whilst a clear band of the correct size (~607 bp) was observed in cDNA derived from the rTRPV1-HEK cell line.

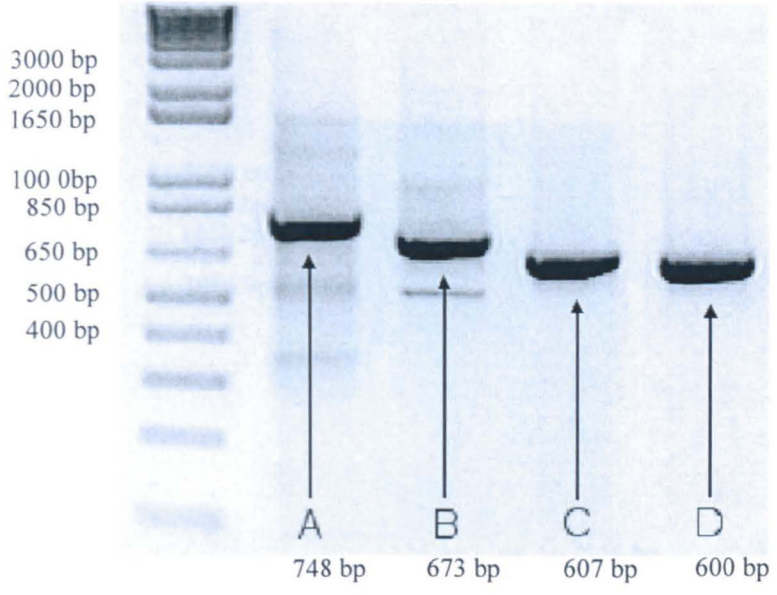


Figure 2.6. PCR of rTRPV1 from rat DRG. rTRPV1 amplified in four separate but overlapping fragments from rat DRG cDNA. The size of the product (in base pairs, bp) is indicated and this includes the Kozak sequence and restriction site HindIII, with A and, ApaI with fragment D.

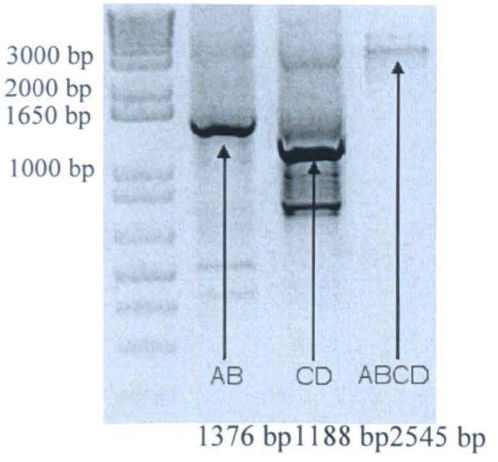


Figure 2.7. Overlapping PCR to join individual fragments. Fragments A and B, and C and D were joined by overlapping PCR to form fragments AB and CD. These were then joined, again by overlapping PCR, to produce the whole rTRPV1 sequence ABCD.

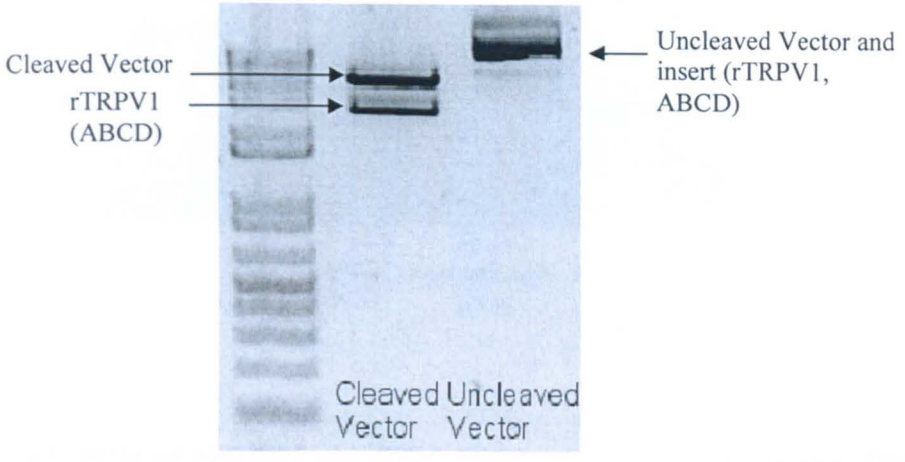


Figure 2.8. Enzymic cleavage of rTRPV1 insert from pcDNA3. ABCD was ligated into pcDNA3, and heat shocked into competent *E.coli*. Vectors were purified from positive colonies and digested with HindIII and ApaI to check for the presence of rTRPV1 containing vectors.

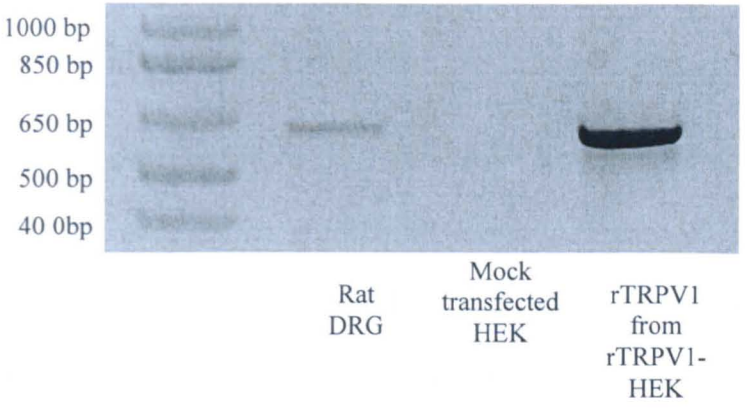


Figure 2.9. RT-PCR of rTRPV1 amplified from rat dorsal root ganglia (DRG), mock transfected HEK and rTRPV1-HEK cDNA. The results are representative of three separate experiments with cDNA derived from three separately grown crops of cells.

2.3.2 Cloned rTRPV1 Responded to known TRPV1 Specific Agonists and was Inhibited by Specific TRPV1 Antagonists

Pharmacological characterisation of rTRPV1-HEK was conducted by measuring agonist triggered increases in intracellular calcium (Figure. 2.10A). In the mock transfected cell line, the two specific TRPV1 agonists, capsaicin and resiniferatoxin had no observable effect on intracellular calcium levels up to the maximum concentration tested (30 μM, open triangle and 30 nM, open circle respectively, Figure 2.10A). In the rTRPV1-HEK cell line, a concentration-effect curve was observed in response to both resiniferatoxin and capsaicin (Figure 2.10A, closed stars and open squares respectively). Resiniferatoxin induced an increase in intracellular calcium from as low as 30 pM, reaching maximal response at 3 nM with an EC_{50} value of ~168 pM. Capsaicin induced an increase in intracellular calcium from as low as 10 nM, reaching maximal response at 30 μM with an EC_{50} value of ~424 nM. As has been previously reported resiniferatoxin is significantly more potent when compared to capsaicin (Caterina et al., 1997, Jerman et al., 2000). A third specific TRPV1 agonist piperine, the active component of black pepper, was also used to characterise rTRPV1. The cells responded from 3 μM, however a maximum response was not achieved at the maximum concentration tested, 100 μM. (Figure 2.10A, open diamonds). Mock transfected HEK cells once again did not respond to piperine at the maximum concentration tested, 100 μM (Figure 2.10A, cross). Comparison of the kinetics of resiniferatoxin (100 pM) and capsaicin (300 nM) evoked calcium responses at concentrations which gave similar maximum responses (when expressed as a percentage of calcium ionophore, A23187), showed

resiniferatoxin appeared to have a slower onset compared to capsaicin (Figure 2.10B).

Next, the ability of the two known TRPV1 antagonists, capsazepine and iodoresiniferatoxin to inhibit capsaicin activation of rTRPV1 was assessed (Figure 2.11 and Figure 2.12 respectively). In the presence of increasing concentrations of capsazepine (1 and 10 μM), the concentration-effect curve for capsaicin exhibited a rightward shift with EC_{50} values rising to approximately 3 μM (closed circles) and 7.5 μM (open circles) respectively (Figure 2.11). Attempts to inhibit capsaicin activation of rTRPV1 using 30 μM capsazepine, were unsuccessful, since precipitation of capsazepine was observed in the CAB (data not shown). In the presence of iodoresiniferatoxin the capsaicin concentration effect curve was again shifted to the right with an EC_{50} value rising to 10.8 μM with 1 nM iodoresiniferatoxin (Figure 2.12, closed stars). Iodoresiniferatoxin at 10 nM resulted in a further rightward shift in the concentration-effect curve for capsaicin, however, an EC_{50} was not obtainable for 10 nM iodoresiniferatoxin, since a complete concentration-effect curve was not achieved (Figure 2.12, open diamonds). iodoresiniferatoxin at 30 nM (Figure 2.12, closed diamond) resulted in apparent complete inhibition of capsaicin responses up to the highest concentration tested (30μM).

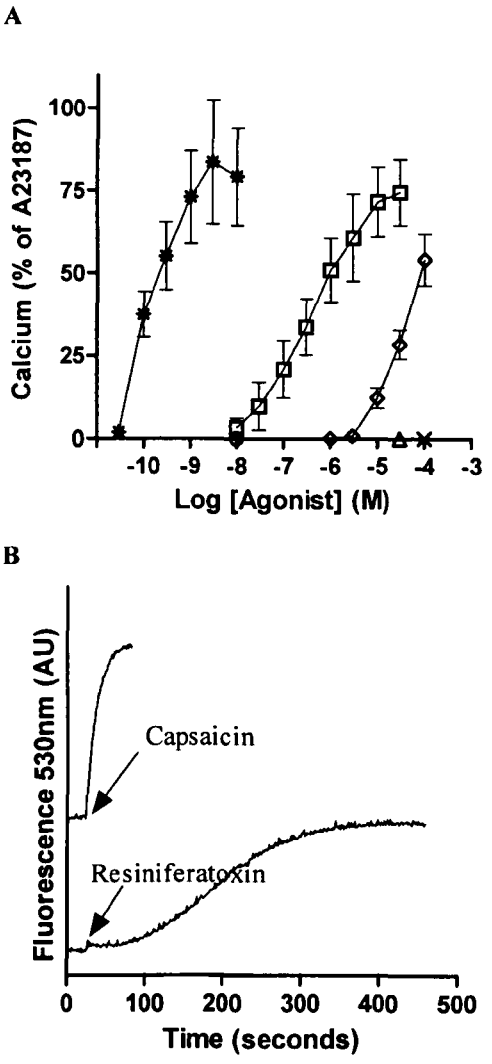


Figure 2.10. Agonist concentration-effect curves for rTRPV1-HEK and comparison of calcium responses following capsaicin and resiniferatoxin challenge. (A) Resiniferatoxin, capsaicin and piperine concentration-effect curves for mock transfected HEK (○, Δ and × respectively) and rTRPV1 cells (*, □ and ◊ respectively). The results are expressed as the mean ± the standard error of the mean of 3 to 6 experiments each performed in duplicate. (B) resiniferatoxin (100 pM) and Capsaicin (300 nM) induced calcium responses showing the different kinetics produced by the two agonists. The concentrations chosen give similar responses as a percentage of calcium ionophore.

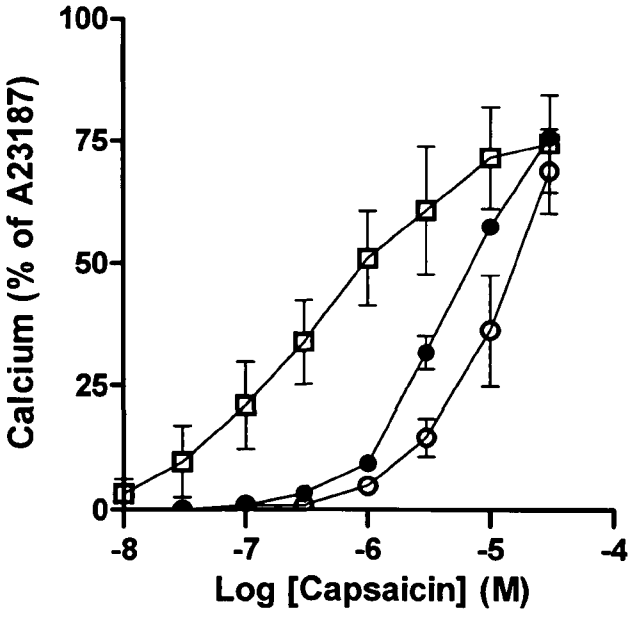


Figure 2.11. Capsaicin concentration effect curves following preincubation with capsazepine. Capsaicin concentration effect curves for rTRPV1-HEK (□) and rTRPV1-HEK cells in the presence of 1 μM capsazepine (●) and 10 μM capsazepine (○). The results are expressed as the mean ± the standard error of the mean of 3 to 6 experiments each performed in duplicate.

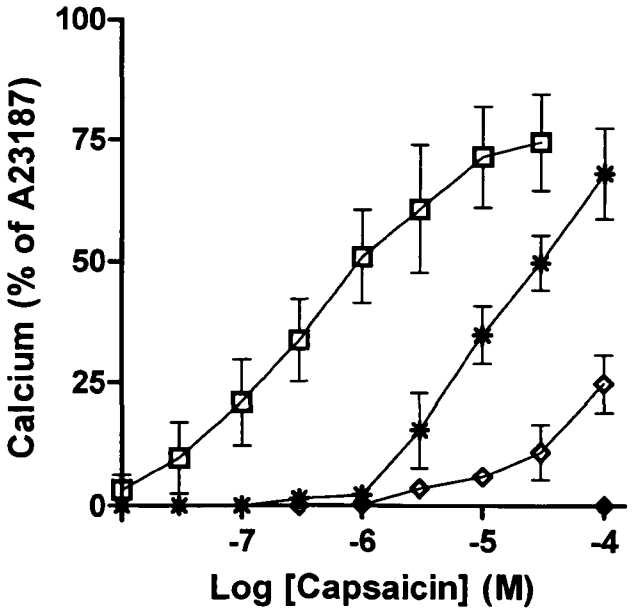


Figure 2.12. Capsaicin concentration effect curves following preincubation with iodoresiniferatoxin. rTRPV1-HEK(□) and rTRPV1-HEK cells in the presence of 1 nM iodoresiniferatoxin (*), 10 nM iodoresiniferatoxin (◊) and 30 nM iodoresiniferatoxin (♦). The results are expressed as the mean ± the standard error of the mean of 3 to 6 experiments each performed in duplicate.

2.3.3 hTRPV1 was Cloned from MRC5 cDNA and Permanently Expressed in HEK293 Cells

hTRPV1 was amplified from MRC5 (foetal human lung fibroblast cell line) cDNA. The sequence failed to amplify in four quarters. Product A could only be obtained when amplified with B (using primers hTRPV1-1D and hTRPV1-3U) (Figure 2.13). All the pieces were joined by overlapping PCR and ligated into pcDNA3. The insert was sequenced and compared to the published sequence (accession number AY131289). The initial sequencing results showed that fragment C was missing and had been replaced by approximately 80 bp of the oncogene, Ras (retrovirus-associated DNA sequence) (data not shown). Hence, section C was amplified in two overlapping sections, C1 and C2 (Figure 2.13). The new PCR product, C1C2, was linked to fragment D and AB by overlapping PCR (AB-C1C2-D) and ligated into pcDNA3 (Figures 2.14 and 2.15). This was again sequenced and compared to the published hTRPV1 sequence, which confirmed the product was hTRPV1 and contained only three DNA polymerase errors. These errors were corrected by site directed mutagenesis.

Human TRPV1

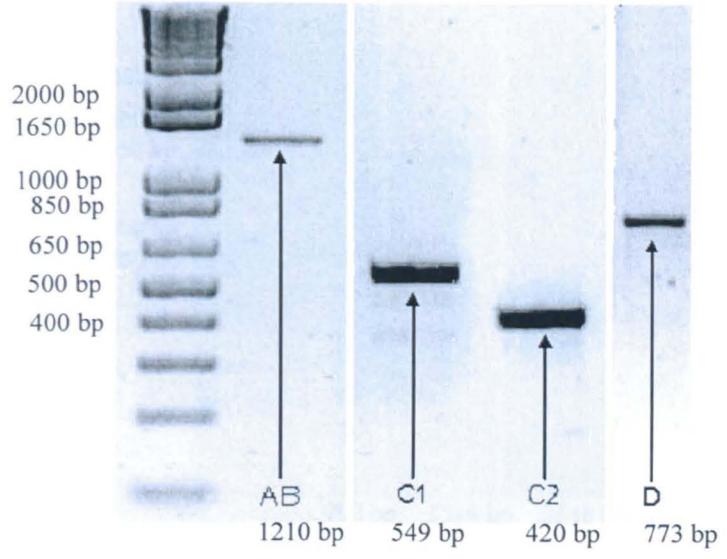


Figure 2.13. RT-PCR of hTRPV1 from the MRC5 cell line. hTRPV1 fragment A failed to amplify, however AB was successfully amplified from MRC5 cDNA. Fragment C was amplified in 2 overlapping sections, C1 and C2, as it would not amplify in one piece using the primers designed. Fragment D, however was amplified from MRC5 cDNA on its own. The sizes of the fragments are indicated in base pairs and these values include the restriction sites which were engineered into the beginning and end of the sequence (HindIII at the 5' end and BamHI at the 3' end) and the Kozak sequence.

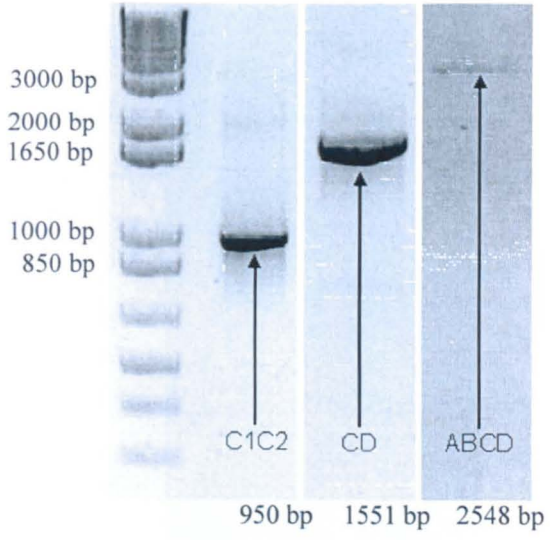


Figure 2.14. Overlapping PCR to join individual fragments. Fragments C1 and C2 were joined by overlapping PCR to form C1C2. Sections C1C2 and D were joined by overlapping PCR to form CD, the second half of the hTRPV1 sequence. AB and CD were then joined by overlapping PCR to form the whole hTRPV1 sequence ABCD.

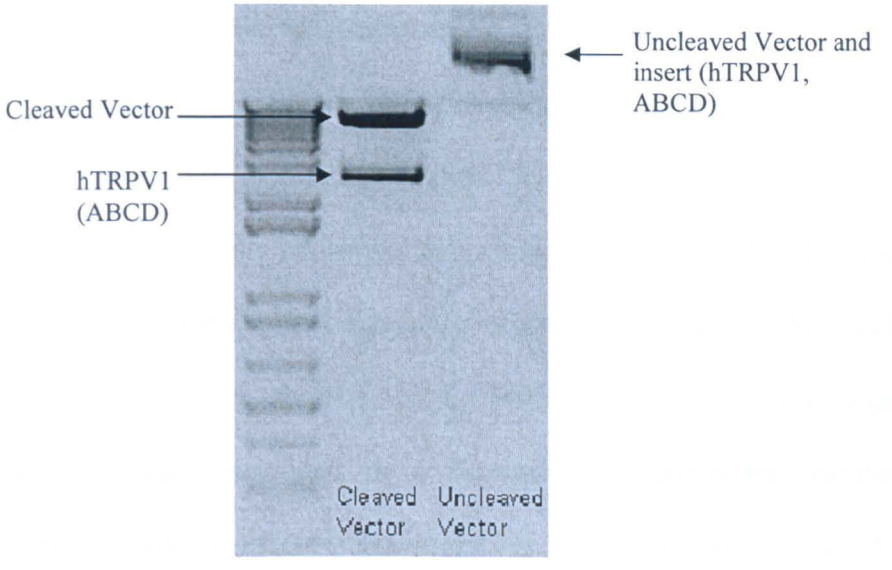


Figure 2.15. Enzymic cleavage of hTRPV1 insert from pcDNA3. ABCD was ligated into pcDNA3, and heat shocked into competent *E.coli*. The vectors were purified from positive colonies and digested with the restriction enzymes HindIII and BamHI to check for the presence of hTRPV1 containing vectors. Cleavage sites for these enzymes had been engineered into the 5' and 3' terminals of the hTRPV1 sequence respectively.

2.3.4 Cloned hTRPV1 Responded to known TRPV1 Specific Agonists

hTRPV1 was permanently expressed in HEK293 cells using the technique employed to permanently express rTRPV1 in HEK293 cells. A stably transfected cell line was selected following three rounds of single cell cloning. The hTRPV1-HEK cells responded in a concentration dependent manner to capsaicin from 300 pM, reaching a maximum by 300 nM with an EC_{50} of 8 nM (Figure 2.16A, open squares). hTRPV1-HEK cells also responded in a concentration dependant manner to resiniferatoxin from 3 pM, reaching a maximum by 3 nM with an EC_{50} of 85 pM (Figure 2.16A, open diamonds).

Comparison of the kinetics of resiniferatoxin (30 pM) and capsaicin (3 nM) evoked calcium responses at concentrations which gave similar maximum responses (when expressed as a percentage of calcium ionophore, A23187), showed resiniferatoxin appears to have a slower onset compared to capsaicin in hTRPV1-HEK (Figure 2.16B).

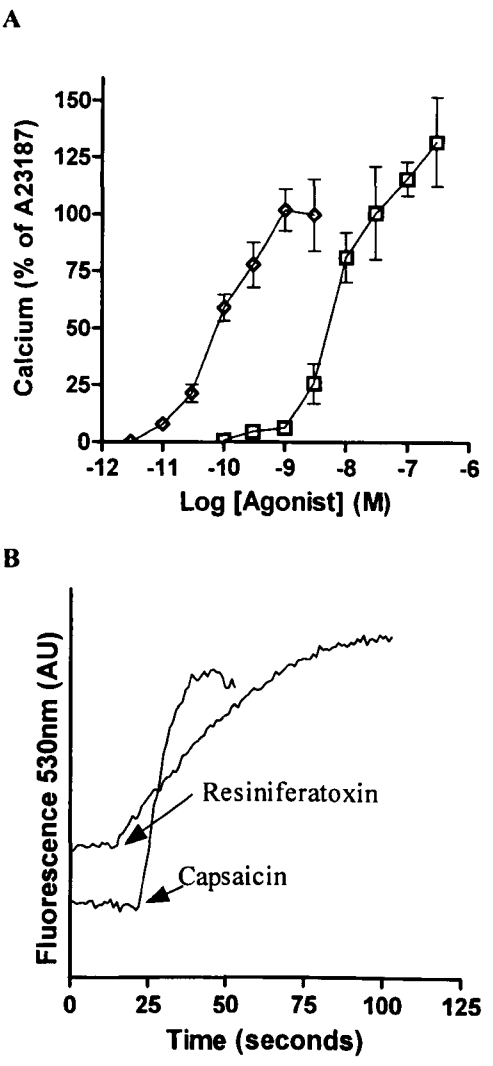


Figure 2.16. Agonist concentration-effect curves for hTRPV1-HEK and comparison of calcium responses following capsaicin and resiniferatoxin challenge. (A) Capsaicin and resiniferatoxin concentration effect curves for hTRPV1-HEK (□ and ◊ respectively). The results are expressed as the mean ± SEM of 3 separate experiments performed in duplicate. Mock transfected HEK cells did not respond to capsaicin or resiniferatoxin at the maximum concentrations tested, 30 μM and 10 nM respectively as shown in Figure 2.9A. (B) resiniferatoxin (30 pM) and Capsaicin (3 nM) induced calcium responses showing the different kinetics produced by the two agonists. The concentrations chosen give similar responses as a percentage of calcium ionophore.

2.3.5 rTRPV1 and hTRPV1 are Expressed in Intracellular Calcium Stores as well as the Plasma Membrane

For rTRPV1, removal of extracellular calcium resulted in a complete loss in the capsaicin response up to 3 μM (Figure 2.17A, closed squares). At 10 μM and 30 μM capsaicin a 79% and 67% reduction in capsaicin response was observed respectively (Figure 2.17A). The rTRPV1 specificity of this remaining response was confirmed by the addition of 10 μM capsazepine (Figure 2.17A, open triangles). In rTRPV1-HEK, in the presence of extracellular calcium, pre-treatment of the cells with 1 μM thapsigargin resulted in a 38% reduction in capsaicin response (30 μM) compared to the controls (Figure 2.17B, open circles), with very little change in EC_{50} (424 nM for untreated and 426 nM for treated rTRPV1-HEK). The combination of extracellular calcium removal and 1 μM thapsigargin pre-treatment resulted in a negligible capsaicin response, even at 30 μM (Figure 2.17C, open diamonds).

The maximal response of hTRPV1 to capsaicin (1 μM) in calcium-free buffer was reduced by 28% (EC_{50} 56 nM, Figure 2.18A, closed squares) compared to the response obtained in calcium-containing buffer (EC_{50} 8 nM, open squares), with no further reduction detected with pre-treatment of 1 μM thapsigargin (EC_{50} 41 nM Figure 2.18C). In the presence of extracellular calcium, pre-treatment of hTRPV1 with thapsaigargin (Figure 2.18B, open circles) caused no change in the capsaicin induced response (EC_{50} values of 8 nM and 9 nM for untreated and treated hTRPV1-HEK respectively).

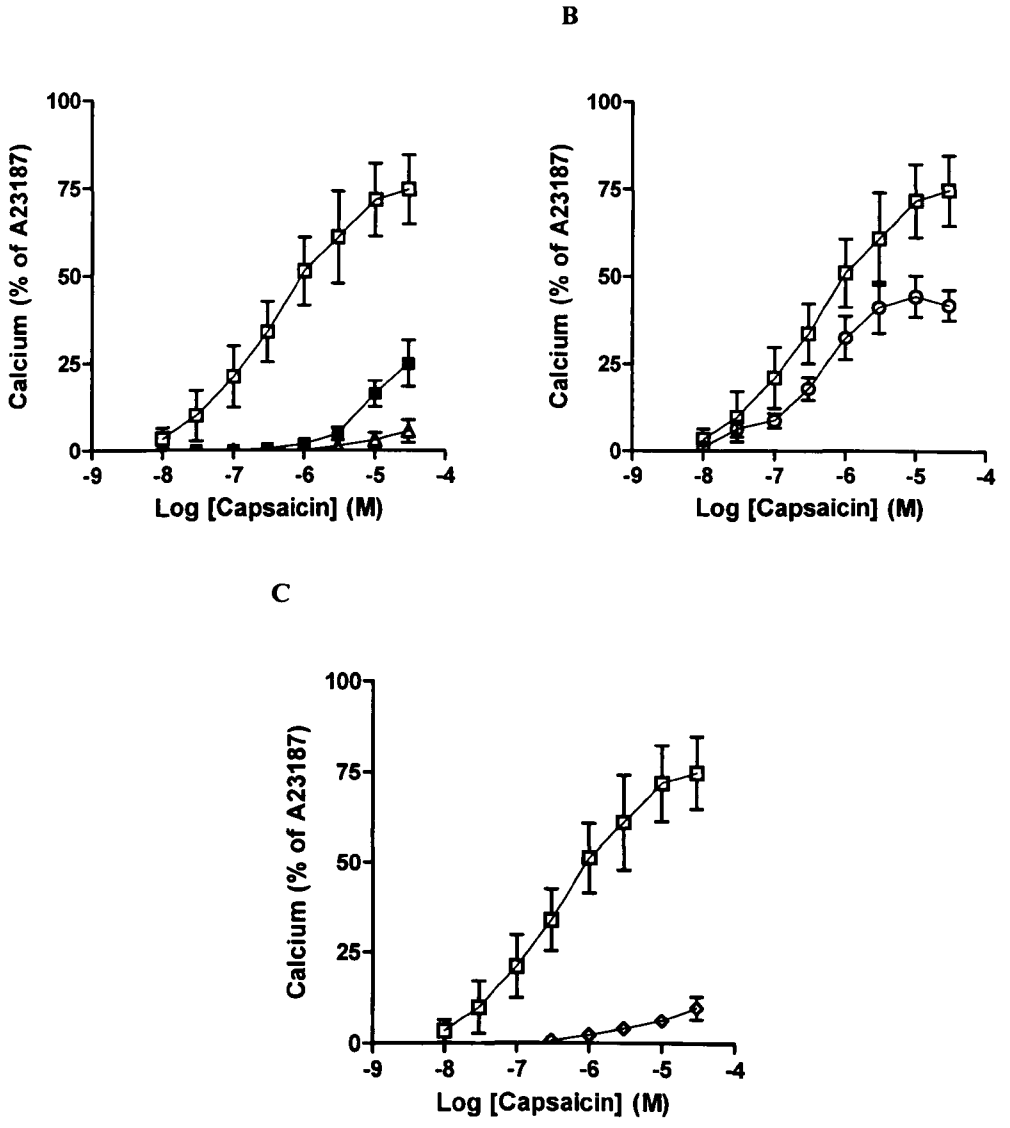


Figure 2.17. Capsaicin concentration effect curves for rTRPV1-HEK. rTRPV1-HEK (A) with and without extracellular calcium (□ and ■ respectively) and without extracellular calcium treated with capsazepine (10 μM, Δ), (B) with extracellular calcium with and without thapsigargin treatment (1 μM, ○ and □ respectively), (C) without extracellular calcium and with thapsigargin (1 μM, ◊). The results are expressed as the mean ± SEM of 3 to 6 experiments each performed in duplicate.

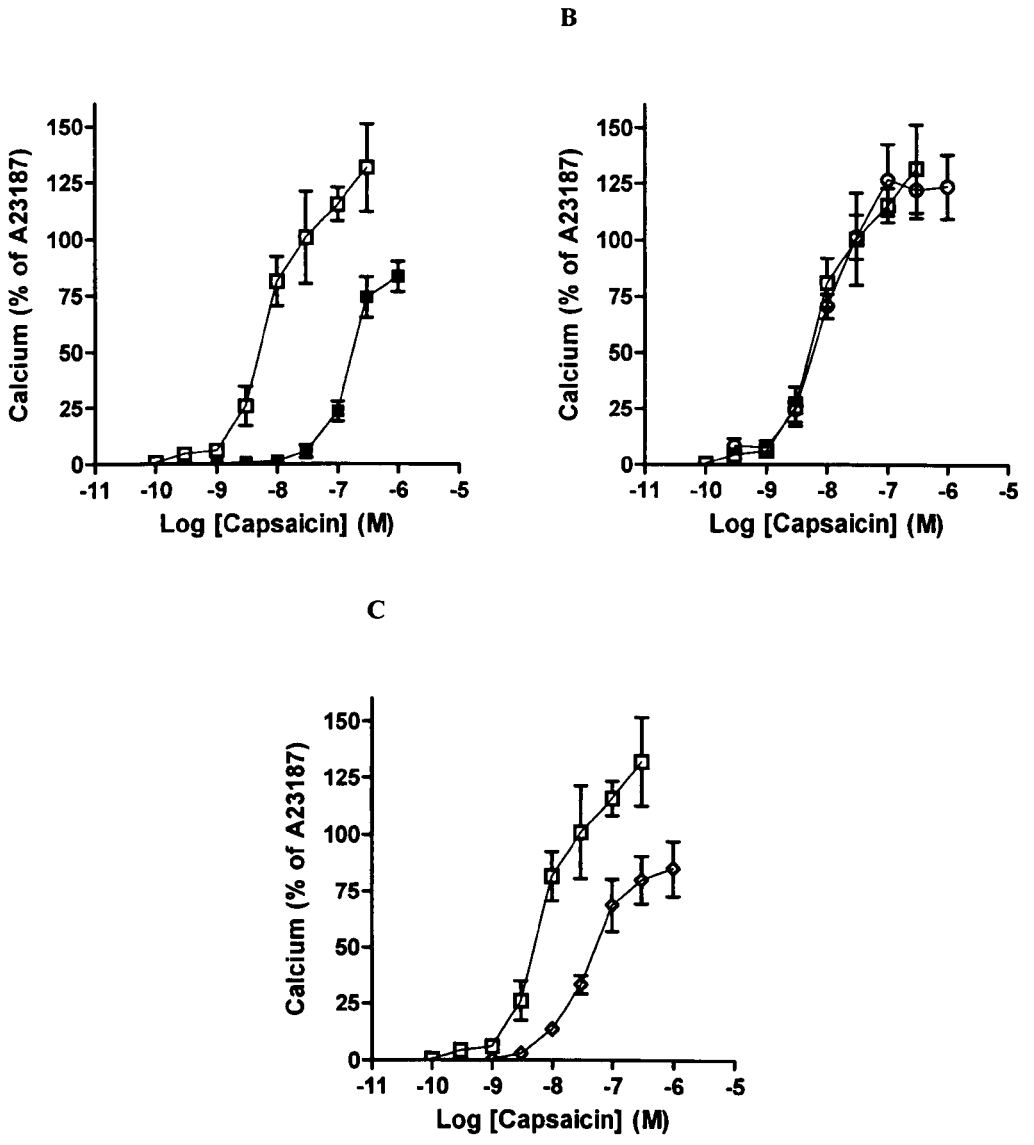


Figure 2.18. Capsaicin concentration effect curves for hTRPV1-HEK. hTRPV1-HEK (A) with and without extracellular calcium (□ and ■ respectively), (B) with extracellular calcium, with and without thapsigargin treatment (○ and □ respectively), and (C) without extracellular calcium and with thapsigargin (1μM, ◊). The results are expressed as the mean ± SEM of 3 to 4 experiments each performed in duplicate.

2.4 Discussion

In order to gain a better understanding of their function and regulation, rTRPV1 was cloned from rat dorsal root ganglia cDNA and hTRPV1 was cloned from MRC5 cDNA and both were permanently expressed in HEK293 cells. Once stable rTRPV1 cell lines were established, we firstly sought to characterise the channels to ensure they responded in a similar fashion to those previously reported (Jerman et al., 2000, Smart et al., 2001, Caterina et al., 1997, Cortright et al., 2001, Hayes et al., 2000). One of our first observations during the process of identifying cell lines positive for rTRPV1, was that cell lines displaying a large calcium response to capsaicin died after some weeks. We noted that this has been reported previously (Olah et al., 2001a) and may be due to the change in the pH of the growth medium as the cells propagate, which subsequently results in the channel remaining continuously open, leading to cell death. Thus, we selected cell lines which generated the highest capsaicin response, in terms of intracellular calcium levels, but remained as a stable cell line. The final TRPV1 cell lines were then characterised.

RT-PCR clearly demonstrated mRNA for rTRPV1 in dorsal root ganglia and rTRPV1-HEK cDNA. However, rTRPV1 could not be amplified from the mock transfected HEK cDNA, clearly indicating that HEK cells do not express rTRPV1.

The pharmacology of our rTRPV1-HEK cell line was clearly compatible with the known pharmacology of the channel. rTRPV1-HEK responded in a concentration dependent manner to three TRPV1 specific agonists, capsaicin, resiniferatoxin and piperine over concentration ranges consistent with previously published studies (Caterina et al., 1997, Jerman et al., 2000, Witte et al., 2002). Mock transfected

HEK cells displayed no increase in intracellular calcium at the highest concentration tested, showing that the calcium uptake in response to capsaicin, resiniferatoxin and piperine are TRPV1 dependent. The relative potencies of the three agonist were resiniferatoxin>capsaicin>piperine. This is again consistent with previous studies (Witte et al., 2002). Resiniferatoxin was approximately 1000 fold more potent than capsaicin in our rTRPV1-HEK cell system. It is well documented that resiniferatoxin is significantly more potent than capsaicin at activating rTRPV1 (Caterina et al., 1997, Jerman et al., 2000, Witte et al., 2002). Capsaicin was approximately 100 fold more potent than piperine at activating rTRPV1-HEK.

The kinetics of the agonists also differed in our rTRPV1-HEK cell systems, again consistent with previously published data (Jerman et al., 2000, Witte et al., 2002, Toth et al., 2005). Capsaicin elicited a rapid increase in intracellular calcium upon addition to rTRPV1-HEK cells. Resiniferatoxin however displayed a much slower onset although the maximum responses were not significantly different.

Capsazepine and iodoresiniferatoxin inhibited capsaicin evoked calcium uptake in a dose dependent manner. Complete inhibition was not achieved with capsazepine due to highest concentrations (30 μM) precipitating out on addition to CAB. However complete inhibition was achieved with iodoresiniferatoxin. Iodoresiniferatoxin appears to be a more potent antagonist than capsazepine in our rTRPV1-HEK cell system.

hTRPV1-HEK responded to both capsaicin and resiniferatoxin in a concentration dependent manner. Again, our hTRPV1-HEK cells responded with pharmacology

which was compatible with the known pharmacology of the human channels (Cortright et al., 2001, Hayes et al., 2000, Smart et al., 2001). In hTRPV1 in our cell system resiniferatoxin is less than 100 fold more potent than capsaicin.

A comparison of rTRPV1-HEK and hTRPV1-HEK pharmacological characterisation revealed that for both channels, in our system resiniferatoxin was significantly more potent than capsaicin. It also shows that in both cell lines resiniferatoxin displayed different kinetics compared to capsaicin. Resiniferatoxin produced a much slower onset following addition to the TRPV1 expressing cells. In our cell system, hTRPV1-HEK required approximately 100 fold less capsaicin and approximately 10 fold less resiniferatoxin than rTRPV1-HEK to see channel activation. The maximal responses were also greater in hTRPV1-HEK than rTRPV1-HEK. No conclusion however can be drawn from these observations as the discrepancies may be due to differences in channel expression between the two cell lines. Unfortunately at present there are no commercially available antibodies specific enough to detect small differences in TRPV1 expression.

Investigation into TRPV1 expression on intracellular stores revealed interesting functional differences between the human and rat channel. Stimulation of both hTRPV1-HEK and rTRPV1-HEK by capsaicin in the absence of extracellular calcium still led to an increase in intracellular calcium signalling, which presumably represents release of calcium from intracellular calcium stores. In the case of rTRPV1-HEK this is consistent with previously published data (Karai et al., 2004, Liu et al., 2003, Olah et al., 2001b, Turner et al., 2003, Wisnoskey et al., 2003). rTRPV1 intracellular signalling appeared to be thapsigargin sensitive, suggesting

rTRPV1 is expressed on the endoplasmic reticulum. The literature regarding rTRPV1 expression on the endoplasmic reticulum is currently confusing and there is debate as to whether the channel is expressed on thapsigargin sensitive (Kanai et al., 2005, Wisnoskey et al., 2003) or insensitive stores (Liu et al., 2003, Turner et al., 2003).

hTRPV1 has not previously been shown to be expressed on intracellular stores and in contrast to rTRPV1-HEK, hTRPV1-HEK was less sensitive to depletion of extracellular calcium and the response was thapsigargin insensitive. All of the organelles of the cell have been shown to be able store calcium, therefore the exact location of intracellular hTRPV1 cannot currently be deduced (Michelangeli et al., 2005).

In conclusion, rTRPV1 and hTRPV1 were successfully cloned and expressed in HEK293 cells. The pharmacology of our permanently expressing cell lines are both clearly compatible with the known pharmacology of the rTRPV1 and hTRPV1. In both cell lines resiniferatoxin is more potent than capsaicin and both agonists display different kinetics. rTRPV1 and hTRPV1 are both expressed on the plasma membrane and the membrane of some internal calcium stores. rTRPV1 is expressed on thapsigargin sensitive intracellular stores but hTRPV1 in our cell system is expressed on thapsigargin insensitive stores.

Chapter 3

Activation of TRPV1 by Citric Acid

3.1 Introduction

Inhalation cough challenge is routinely used to quantify cough, study the cough reflex and investigate the antitussive effects of cough treatments in both humans and animal models (Morice et al., 2001). The method of cough challenge involves the delivery of tussive agents into the airways as aerosols or by ultrasonic nebulizers (Morice et al., 2001). There are three main groups of tussive agents used. These are capsaicin, distilled water and organic acids such as citric acid (Morice et al., 2001). Capsaicin is thought to be a specific agonist for TRPV1 (Caterina et al., 1997). Distilled water is now believed to be an agonist for TRPV4 (Liedtke et al., 2000). Although TRPV1 is known to be activated by protons (Caterina et al., 1997, Tominaga et al., 1998), the molecular mechanism whereby organic acids stimulate cough is not yet fully understood.

A recent study reported citric acid induces cough via a similar mechanism to capsaicin and proposed that both acted via C-fibres (Tanaka and Maruyama, 2005). Citric acid has previously been shown to activate guinea-pig afferent airway nerve fibres via both RAR (rapidly adapting pulmonary stretch receptors) and C-fibres. Activation was presumed to occur via TRPV1 (via C-fibres) and Acid Sensitive Ion Channels (ASIC, through both RAR and C-fibres) (Kollarik and Undem, 2002). However, to date it has not been directly demonstrated whether citric acid can activate TRPV1. Therefore we have explored whether citric acid can activate TRPV1 and by which mechanism, using both a molecular and pharmacological approach.

3.2 Materials and Methods

3.2.1 Materials

The rabbit anti-capsaicin receptor polyclonal antibody was bought from Chemicon International, Hampshire, UK. Citric acid, phosphoric acid and anti-rabbit IgG FITC conjugated monoclonal antibody were purchased from Sigma, Poole, UK. For all other reagents see section 2.2.1.

3.2.2 Cloning and Expression of rTRPV1

The rTRPV1-HEK cell line generated as described in chapter 2 was utilised to investigate acid stimulation of TRPV1. Cloning and expression of rTRPV1 is described in chapter 2 along with characterisation of the permanently expressing rTRPV1-HEK cell line generated.

3.2.3 Generation of rTRPV1E648A

Site directed mutagenesis (QuikChange kit, stratagene) was used according to the manufacturers instructions, to insert a single point mutation to change amino acid E648 to A648 (Jordt et al., 2000) in rTRPV1. Primers were designed as described in section 2.2.2.9 (primers sense, 5'-ATGGGCGACCTGGCGTTCACTGAGAACTAC-3', antisense, 5'-GTAGTTCTCAGTGAACGCCAGGTCGCCCAT-3'). The mutated rTRPV1 in pcDNA3 was heat shocked into competent *E.coli* as per the manufacturers instructions (Stratagene). The mutated vector was amplified in *E.coli* as described in section 2.2.2.8 and the insert was sequenced by MWG biotech to ensure the mutation

was successful and no other mutations had been introduced by the *pfu* turbo DNA polymerase during site directed mutagenesis. HEK293 cells were transfected with the mutant rTRPV1E648A construct and a permanently expressing cell line derived from a single cell was selected as described in sections 2.2.2.10 and 2.2.2.11.

3.2.4 Measurement of Intracellular Calcium

Calcium signalling studies were performed based on the methods described in section 2.2.4. Briefly, cells were loaded with the calcium binding probe Fluo-3 and increases in intracellular calcium were detected using a fluorospectrometer with an excitation wavelength of 480 nm and a detector set to measure at a wavelength of 530 nm. However, phosphoric acid and citric acid triggered increases in intracellular calcium were measured after rTRPV1-HEK cells were added to CAB containing a known concentration of citric acid or phosphoric acid. Increases in fluorescence were measured immediately upon addition of the cells. The pH of the CAB buffer was measured before and after addition of the cells.

3.2.5 Inhibition Studies

For experiments probing potential inhibition of citric acid responses, the cells were added to CAB containing citric acid with 30 nM iodoresiniferatoxin or 10 μM capsazepine before increases in fluorescence were measured.

3.2.6 Flow Cytometry

Flow cytometry was used to compare the expression of cell surface rTRPV1 on mock transfected HEK, rTRPV1-HEK and rTRPV1E648A-HEK cell lines. Due to the low

specificity of commercially available TRPV1 antibodies which are all raised against the intracellular N or C terminals, much technique optimisation was carried out. The primary antibody used (rabbit anti-capsaicin receptor polyclonal antibody, Chemicon International) was raised against the C terminal. Therefore the cells were permeabilised to allow the antibody to bind TRPV1. A number of techniques were tried for permeabilisation, including saponin treatment, methanol treatment and finally formaldehyde treatment at 1, 3 and 4%. The length of time for permeabilisation was also optimised. Primary and secondary antibody titrations were also carried out and different primary and secondary antibody incubation times were tried. Finally comparisons for antibody incubation in PBS and basal medium were compared. The final, optimised protocol used is described.

Cells were washed and harvested in cold PBS before incubation on ice in PBS with 4% formaldehyde for 30 minutes. The cells were then washed twice by centrifugation at 4°C with cold PBS followed by incubation on ice with rabbit anti-capsaicin receptor polyclonal antibody (1 in 400, chemicon international) in cold basal DMEM culture medium for 1 hour. The cells were again washed twice by centrifugation at 4°C with cold PBS and incubated on ice with anti-rabbit IgG FITC conjugated monoclonal antibody (1 in 100 sigma) for 45 minutes. The cells were then washed two more times by centrifugation at 4°C with cold PBS and then analysed using a Becton Dickinson Flow Cytometer.

3.2.7 Statistical analysis

Unless stated otherwise, data are expressed as the mean ± SEM. Data were analysed by the unpaired Student's t test taking $p<0.05$ as statistically significant.

3.3 Results.

3.3.1 rTRPV1 is Activated by Citric and Phosphoric Acid.

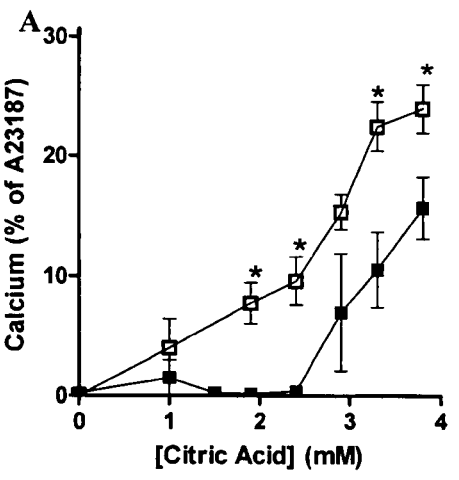
We initially tested whether two acids, namely citric and phosphoric acid used for cough challenge, were capable of activating rTRPV1 in our expression system (Figure 3.1A and 3.2 respectively).

rTRPV1-HEK cells displayed an increase in intracellular calcium in a concentration dependent manner to citric acid from 1 mM to the maximum concentration tested, 3.8 mM (Figure 3.1A, open squares). The mock transfected HEK cells also responded to citric acid in a concentration dependent manner from 2.9 mM to the maximum concentration tested, 3.8 mM (Figure 3.1A, closed squares). Citric acid stimulated a significant increase in intracellular calcium levels in the rTRPV1-HEK cell line above that observed in the mock transfected HEK cell line at all concentrations tested except 1 and 2.9 mM. Addition of a submaximal concentration of capsaicin (30 nM) to citric acid exposed cells had an additive effect when compared to the citric acid response alone (Figure 3.1B, closed triangles).

Phosphoric acid at the same pH values as citric was only found to have a minor effect on intracellular calcium levels in both mock transfected HEK and rTRPV1-HEK (Figure 3.2, closed circles and open circles respectively). Phosphoric acid stimulated an increase in intracellular calcium in rTRPV1-HEK from pH 7.0, reaching maximal response at pH 5.2 (Figure 3.2, open circles). In mock transfected HEK cells phosphoric acid stimulated a lesser, but not significantly different increase in intracellular calcium levels to that observed in rTRPV1-HEK, triggering an increase in intracellular calcium from pH 6.3 to pH 5.2 (Figure 3.2, closed circles).

Since citric acid was found to stimulate a robust increase in intracellular calcium in rTRPV1-HEK cells, compared to phosphoric acid, we decided to investigate the putative mechanism by which citric acid could be activating rTRPV1-HEK. Thus, to ascertain whether the increase in intracellular calcium levels in response to citric acid was TRPV1 dependent, we firstly utilized two specific TRPV1 antagonists, namely iodoresiniferatoxin and capsazepine. Addition of capsazepine (10 μM) had little effect on the ability of citric acid to activate rTRPV1-HEK (Figure 3.3A, open circles). However, addition of 30 nM iodoresiniferatoxin (Figure 3.3B, closed diamonds) significantly inhibited the rTRPV1-HEK response to citric acid at 3.3 mM and 3.8 mM, reducing the responses to that obtained with citric acid in mock transfected HEK cells.

Citric Acid (mM)	pH
0	7.3
1.9	6.6
2.4	6.3
2.9	5.9
3.3	5.6
3.8	5.1



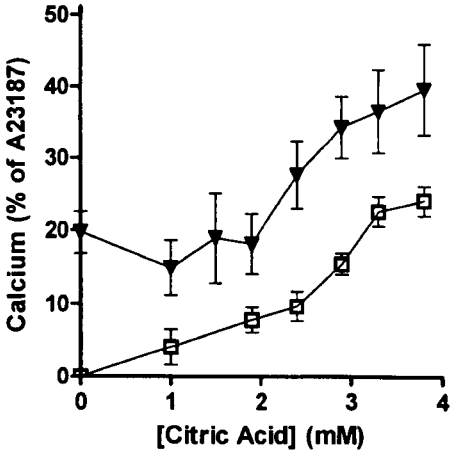


Figure 3.1. Citric acid concentration-effect curves for rTRPV1-HEK and mock transfected HEK with and without a submaximal concentration of capsaicin. (A) Citric acid concentration-effect curve for mock transfected HEK(■) and rTRPV1-HEK cells(□) and (B) effect of citric acid without (□) and with a submaximal concentration of capsaicin (30nM) (▼) on rTRPV1-HEK cells. The results are expressed as the mean ± the standard error of the mean of 4 to 6 experiments each performed in duplicate. * Significantly different compared to the mock transfected HEK ($p < 0.05$).

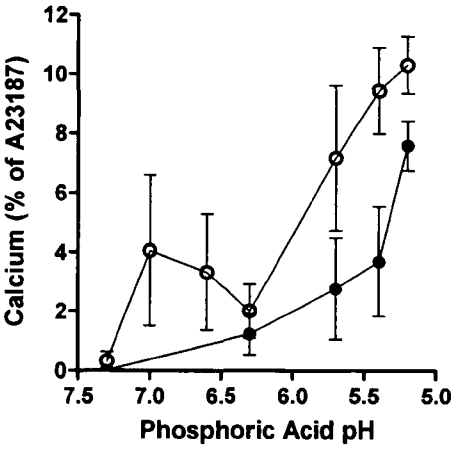
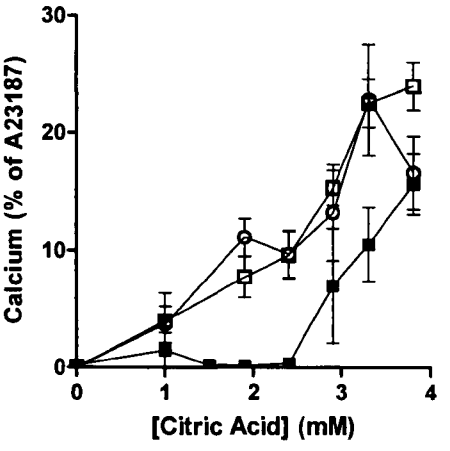


Figure 3.2. Effect of phosphoric acid on mock transfected HEK and rTRPV1-HEK cells. Effect of phosphoric acid on mock transfected HEK (●) and rTRPV1-HEK cells (○). The results are expressed as the mean ± the standard error of the mean of 3 to 4 experiments each performed in duplicate. * Significantly different compared to the mock transfected HEK ($p < 0.05$).

A



B

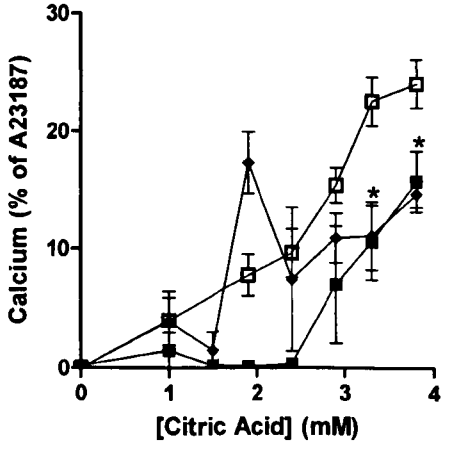


Figure 3.3. Effect of TRPV1 specific antagonists on citric acid evoked responses in rTRPV1-HEK cells. Citric acid concentration-effect curves for (A) mock transfected HEK (■), rTRPV1-HEK cells (□) and rTRPV1-HEK cells exposed to 10 μM capsazepine (○) and (B) mock transfected (■), rTRPV1-HEK (□) and rTRPV1-HEK cells exposed to 30 nM iodoresiniferatoxin (♦). The results are expressed as the mean ± the standard error of the mean of 3 to 4 experiments each performed in duplicate. * Significantly different compared to rTRPV1-HEK not treated with iodoresiniferatoxin ($p < 0.05$).

3.3.2 Mutating the TRPV1 Putative Proton Binding Site E648 to A648 Abolishes the Ability of Citric Acid, But Not Capsaicin, to Activate the Receptor.

rTRPV1E648A-HEK responded to capsaicin from 1 nM, reaching a maximum by 300 nM with an EC_{50} value of 13 nM (Figure 3.4A, closed squares). The magnitude of response was not significantly different from that obtained with the rTRPV1-HEK cells (Figure 3.4A, open squares). However, the capsaicin concentration-effect curve for rTRPV1E648A-HEK cells exhibited a leftward shift of two orders of magnitude (Figure 3.4A, closed squares) when compared to rTRPV1-HEK. rTRPV1E648A-HEK responded to citric acid between 3.3 mM and the maximum concentration tested, 3.8 mM (Figure 3.4B, closed squares). However the responses were of a smaller magnitude than those observed for rTRPV1-HEK and displayed an approximate rightward shift of 2 orders of magnitude (Figure 3.4B, closed squares) when compared to rTRPV1-HEK (Figure 3.4B open squares).

FACS analysis was performed to assess the cell surface expression of rTRPV1-HEK compared to mock transfected HEK and rTRPV1E648A-HEK (Figure 3.5). Surprisingly flow cytometry consistently failed to detect any observable difference in cell surface expression between rTRPV1-HEK and the mock transfected HEK cell line. Nevertheless, rTRPV1E648A-HEK displayed significant receptor cell surface expression compared to the mock transfected HEK cell line. Use of other commercially available TRPV1 polyclonal antibodies, also failed to detect any difference between rTRPV1-HEK and mock transfected HEK (data not shown).

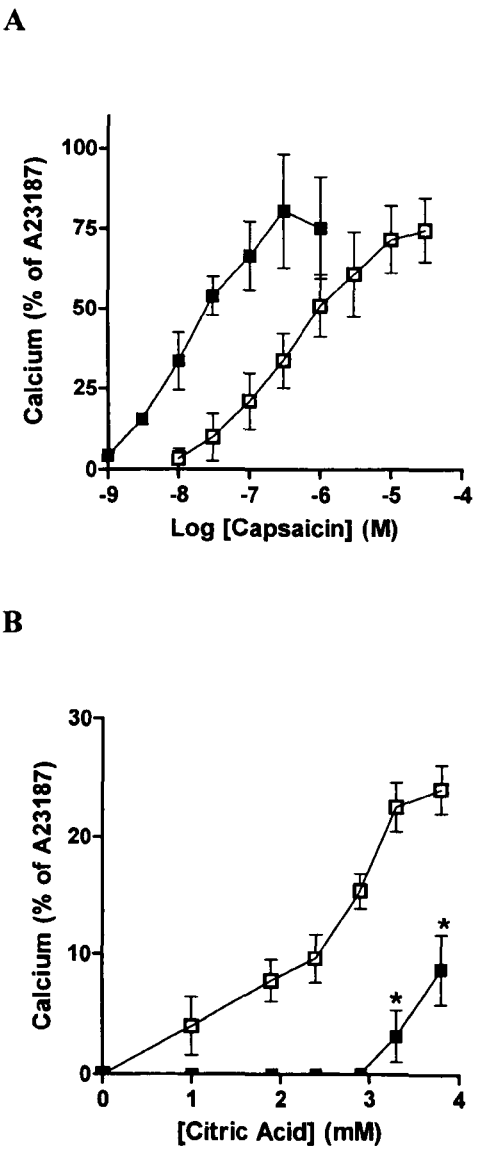


Figure 3.4. Capsaicin and citric acid concentration-effect curves for rTRPV1E648A-HEK compared to rTRPV1-HEK cells. (A) Capsaicin concentration effect curves for rTRPV1-HEK (□) and rTRPV1E648A-HEK cells (■) and (B) citric acid concentration effect curves for rTRPV1-HEK (□) and rTRPV1E648A-HEK cells (■). The results are expressed as the mean ± the standard error of the mean of 3 to 6 experiments each performed in duplicate. * Significantly different compared to rTRPV1-HEK ($p < 0.05$).

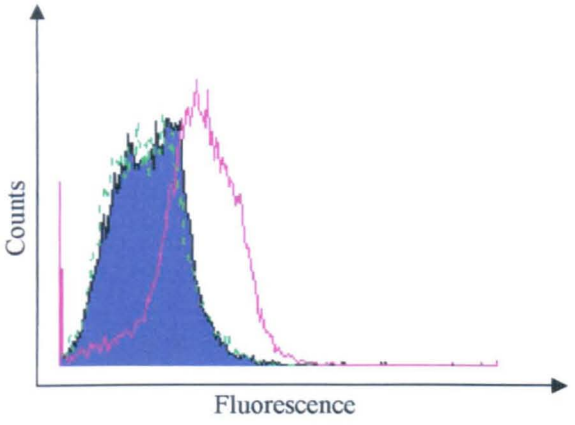


Figure 3.5. Flow cytometry analysis to compare channel densities of rTRPV1 on mock transfected HEK, rTRPV1-HEK and rTRPV1E648A-HEK cells. Flow cytometry analysis for TRPV1 expression on mock transfected (■), rTRPV1-HEK (----) and rTRPV1E648A-HEK cells (——). The results are representative of 3 separate experiments each performed in duplicate.

3.4 Discussion

We have shown here for the first time that citric acid, an agent used routinely for cough challenge tests in the clinic activates TRPV1 in transfected HEK293 cells by a proton dependent mechanism. Activation of TRPV1 by citric acid was specific as the TRPV1 specific antagonist iodoresiniferatoxin completely inhibited the ability of citric acid to activate TRPV1. In addition, by mutating the E648 residue which is known to be important in proton activation of TRPV1 to A648, we clearly demonstrate that citric acid activation of TRPV1 operates by a proton dependent mechanism. Thus, TRPV1 may be an important receptor for initiating the cough reflex.

We sought to determine whether two acids, namely citric acid and phosphoric acid used routinely in the clinic for cough challenge (Wong et al., 1999) could activate rTRPV1 in our cell system. In the rTRPV1-HEK cells, citric acid triggered a concentration dependent increase in intracellular calcium. Interestingly, a small response to citric acid was observed in the mock transfected HEK cell line. We believe that this response is likely to be a result of the endogenous Acid Sensitive Ion Channel (ASIC 1a) expressed in HEK cells as has as previously been reported (Gunthorpe et al., 2001). Surprisingly for phosphoric acid only a negligible increase in intracellular calcium in rTRPV1-HEK cells was observed compared to that in mock transfected HEK challenged with phosphoric acid. The failure of phosphoric acid to activated rTRPV1-HEK to the same magnitude as citric acid may be because phosphoric acid has a lower pKa than citric acid and solutions were made up over the same pH rather than concentration. Therefore, we decided to probe further into the mechanism by which citric acid was activating rTRPV1-HEK.

To assess whether the increase in intracellular calcium in rTRPV1-HEK cells in response to citric acid was rTRPV1 dependent, we employed two TRPV1 antagonists, iodoresiniferatoxin and capsazepine. Addition of 30 nM iodoresiniferatoxin to citric acid exposed cells reduced the response evoked to that obtained with citric acid on mock transfected cells, thus providing evidence that citric acid was indeed activating rTRPV1 in our cell system. Interestingly, addition of 10 μM capsazepine had little effect on the citric acid evoked response in rTRPV1-HEK cells. This is consistent with the current literature, which suggests capsazepine antagonises hTRPV1 and guinea pig TRPV1, but not rTRPV1, proton evoked responses (McIntyre et al., 2001, Savidge et al., 2002, Gavva, 2005 #124, Gavva et al., 2005b). In contrast iodoresiniferatoxin inhibits both proton and capsaicin activation of rTRPV1 and hTRPV1 (Seabrook et al., 2002). Phillips and colleagues carried out site directed mutagenesis studies to investigate this species differences (Phillips et al., 2004). Mutation of three amino acids in transmembrane domains three and four of the rat channel to the corresponding human amino acids (I514M, V518L and M547L), caused capsazepine to significantly inhibit the proton evoked responses of rTRPV1 (Phillips et al., 2004). Addition of a submaximal concentration of capsaicin (30 nM) to citric acid exposed cells caused an increase in intracellular calcium which had an additive effect when compared to the response observed in citric acid exposed rTRPV1-HEK cells alone, suggesting that citric acid activates TRPV1 by a mechanism different to that of capsaicin. Thus, the pharmacological data provides strong evidence that citric acid induced increases in intracellular calcium in rTRPV1-HEK occur via a TRPV1 channel dependent mechanism, possibly by a proton dependent mechanism.

We next sought to determine whether citric acid activation of rTRPV1-HEK was due to proton donation by utilising a molecular approach. The glutamic acid residue (E648) has previously shown to be involved in proton-evoked channel opening (Jordt et al., 2000), therefore we subsequently generated rTRPV1E648A-HEK. In response to citric acid, the rTRPV1E648A-HEK cell line displayed a significantly ablated response when compared to the response observed in rTRPV1-HEK, providing convincing evidence that citric acid activates rTRPV1 by a proton donation dependent mechanism. Interestingly the capsaicin concentration-effect curve for rTRPV1E648A-HEK shifted to the left by two orders of magnitude, whereas the maximal response was not significantly altered compared to rTRPV1-HEK. Jordt *et al* showed that the capsaicin concentration-effect curve for rTRPVE648A was not significantly altered compared to rTRPV1-HEK, however the effect of protons was ablated. The leftward shift in the concentration-effect curve for capsaicin with TRPV1E648A can be explained by our flow cytometry data which suggests that rTRPV1E648A-HEK is expressed to a higher degree than our rTRPV1-HEK cell line. Our ability to achieve a highly expressing rTRPV1E648A-HEK cell line is probably due to the fact that the receptor does not respond to protons, and thus is not susceptible to low pH, which results in receptor activation and cell death (Olah et al., 2001a). Thus, using a molecular approach we demonstrate that citric acid activates TRPV1 by a proton dependent mechanism.

Characterisation by flow cytometry was more problematic. No observable difference between mock transfected HEK and rTRPV1-HEK cell lines could be detected by flow cytometry. However, rTRPV1E648A-HEK displayed a clear shift in

fluorescence. We believe that the lack of ability to observe cell surface expression of rTRPV1-HEK was due to the low affinity of the polyclonal antibody employed. The fact that rTRPV1E648A-HEK was clearly observed by flow cytometry is explainable by the fact that a higher TRPV1 expressing cell line could be selected and thus observed by flow cytometry analysis, since this receptor is insensitive to activation by the presence of protons (Olah et al., 2001a).

In conclusion we have shown that citric acid, like capsaicin, which are both employed in the clinic for cough challenge experiments activates TRPV1, by a proton dependent mechanism and may provide a novel target for conditions where cough is a major factor.

Chapter 4

Investigation into the Role of *N*-linked Glycosylation and Sialylation on TRPV1 function

4.1 Introduction

Glycosylation is a protein post translational modification which occurs in the endoplasmic reticulum (ER) and golgi apparatus (Kornfeld and Kornfeld, 1985). It has been predicted that more than half of all proteins which occur in nature are glycosylated (Apweiler et al., 1999). Addition of a glycan side chain to a protein can have important consequences including affecting protein trafficking, folding, maturation, function and solubility (Lodish and Kong, 1984, Leavitt et al., 1977, Compton et al., 2002, Bergh et al., 1987, Gala and Morrison, 2002). Glycosylation can occur via *N*, or *O* linkages. *O*-linked glycosylation, unlike *N*-linked glycosylation, appears to have no consensus sequence (Apweiler et al., 1999). The carbohydrate links to the hydroxyl of serine or threonine by an *N*-acetylgalactosamine. The most common form of glycosylation, however, is *N*-linked (Apweiler et al., 1999), which is the focus of this chapter.

4.1.1 *N*-Linked Glycosylation

Using the SWISS-PROT database, Apweiler predicted that three quarters of all glycoproteins would be *N*-link glycosylated and a further one in ten would be *N*-,*O* link glycosylated (Apweiler et al., 1999). Addition of the oligosaccharide chain to proteins is a complex process, which occurs in the ER and golgi. *N*-linked glycosylation occurs via an *N*-glycosidic bond to the nitrogen of the side chain of asparagines in the sequon N-X-S/T where X is any amino acid except proline (Bause, 1983). A presynthesised oligosaccharide chain is transferred to the growing protein by oligosaccharyltransferase from the lipid carrier dolichol-pyrophosphate in the lumen of the ER. This core oligosaccharide remains fairly consistent among

eukaryotes and consists of three glucoses, nine mannoses and two *N*-acetyl-glucosamines (Figure 4.1) (Kornfeld and Kornfeld, 1985).

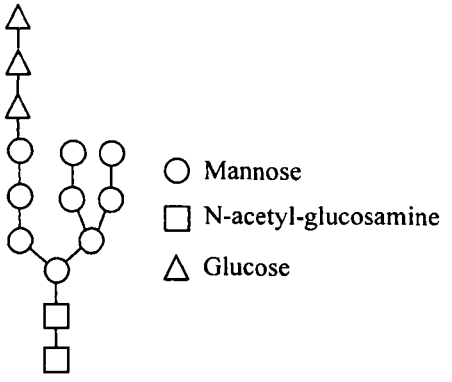


Figure 4.1. *N*-linked core oligosaccharide.

Synthesis of the oligosaccharide commences on the cytosolic surface of the ER (see Figure 4.2). The first seven sugars are added to the dolichol pyrophosphate lipid carrier by specific glycosyltransferases (Burda et al., 1999). The antibiotic, tunicamycin can inhibit this first step of the pathway, the transfer of *N*-acetyl-glucosamine to dolichol pyrophosphate thereby blocking the whole *N*-glycosylation process (Tkacz and Lampen, 1975). Once the seven sugar chain has been added to dolichol pyrophosphate, the chain is then translocated to the lumen side of the ER by heptasaccharide flippase enzymes (Helenius and Aebi, 2002). Here the remaining seven oligosaccharides are added, the last being a glucose residue. This terminal sugar acts as an indicator to oligosaccharyltransferase (OST) that the core oligosaccharide is complete (Burda and Aebi, 1998), and OST attaches the glycan to the asparagine residue of the glycosylation sequon of the polypeptide by an *N*-glycosidic bond. Asparagine has an uncharged, polar amide side chain which is fairly inert. Therefore the hydroxyl side groups of threonine or serine are required

(Bause and Legler, 1981, Silberstein and Gilmore, 1996). Due to the unique cyclic structure of proline, where the side chain is bonded to both the α carbon atom and nitrogen, proline cannot be amino acid X in the glycosylation sequon. Proline prevents folding of the growing peptide into a loop thus preventing the third amino acid in the sequon (serine or threonine) contacting the asparagines to cause it to become more hydrophilic and thus more reactive (Helenius and Aebi, 2004).

Once the core glycan has been attached to the peptide the two terminal glucose residues are cleaved by glycosydases I and II. This structure is recognised by the lectin molecular chaperones, calnexin or calreticulum, which associate with the glycoprotein and aid protein folding (Peterson et al., 1995, Nauseef et al., 1995, Hammond et al., 1994). This is achieved by exposing the newly formed protein to the thiol-disulphide oxidoreductase, ERp57, which forms disulphide bridges by oxidation. Although proteins being newly synthesised begin folding immediately upon entry to the ER, before detachment from the ribosomes, folding still continues after dissociation. By binding to the chaperone proteins, the glycoproteins cannot be prematurely exported from the ER, and are allowed to complete their correct folding process (Hammond et al., 1994). Binding to the chaperones may also prevent degradation. Correctly folded proteins are released from calnexin or calreticulum when glycosidase II cleaves the third and final glucose. If the protein is still incorrectly configured, UDP-Glc:glycoprotein glucosyltransferase (GT) prevents the protein being exported by reglucosylating the protein causing it to bind again with one of the lectins (Hammond et al., 1994). This cycle continues until the protein is correctly folded, oligomerised or degraded (see figure 4.2).

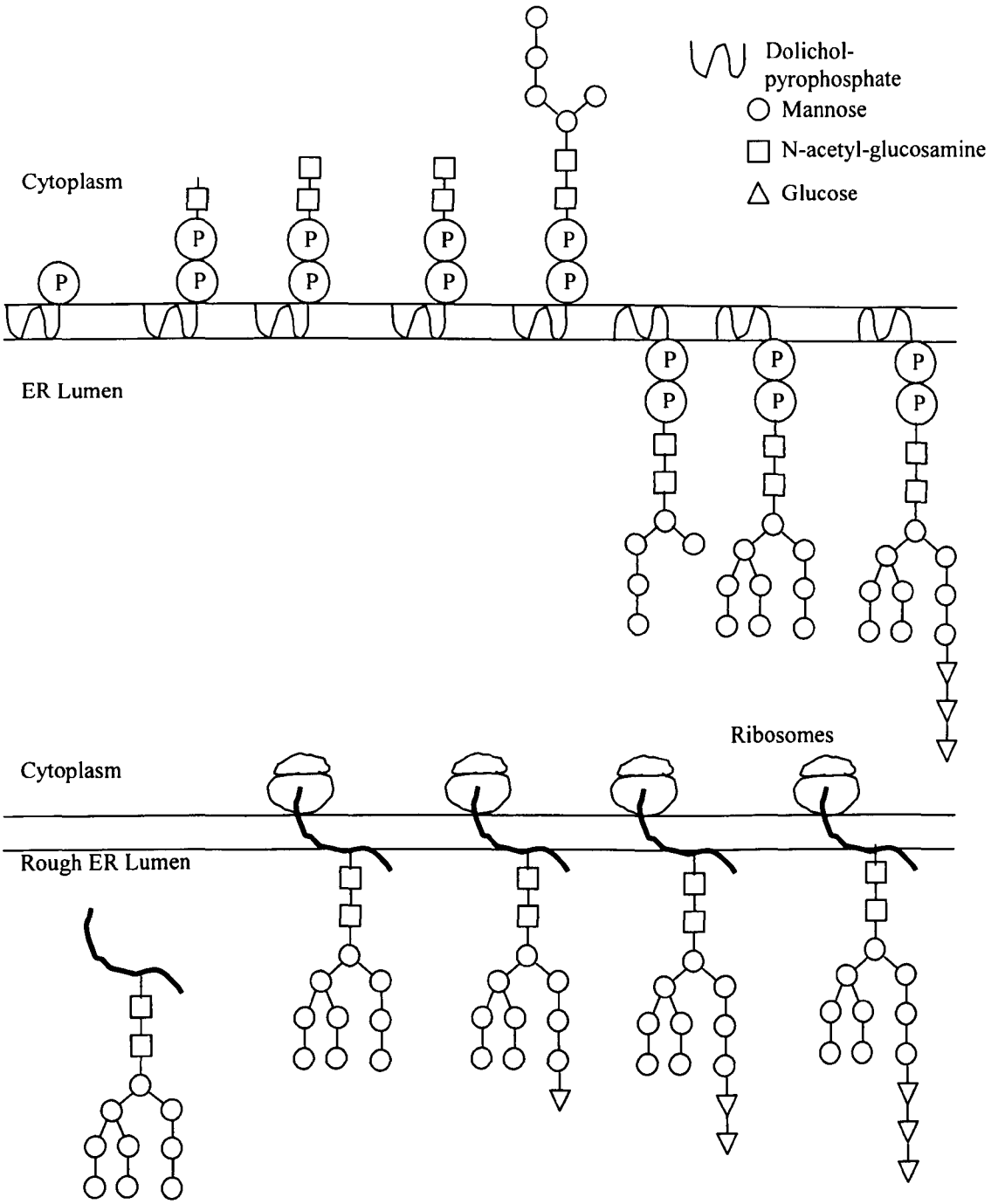


Figure 4.2. Formation of the N-linked glycan in the ER. The first seven sugars are added to the dolichol-pyrophosphate by glycosyltransferases. The glycan is then translocated to the ER lumen by heptasaccharide flippase. The remaining oligosaccharides are added including a terminal glucose which indicates to OST that the glycan is complete and the sugar is attached to the growing protein. The next two glucose residues are cleaved by glycosydases I and II before the protein detaches from the ribosome.

Glycosylation may be important in protein folding not only as a recognition site for the chaperone proteins. Current understanding suggests that the presence of a glycan can induce rigidity in a protein structure possibly by restricting freedom of mobility of the peptide chain. For proteins whose structure has been determined by X-ray chrystallography in the Brookhaven Protein Data Bank (BDB), Petrescu *et al* 2004 showed that *N*-link glycosylation tends to occur at regions of protein where there is a change in the secondary structure, more regularly bends or turns (Petrescu et al., 2004). This high incidence of glycosylation occurring at crucial locations on the protein could provide evidence in favour of a role for *N*-linked glycosylation in protein folding (Petrescu et al., 2004). If proteins are still misfolded after glycans are added, they are not allowed to leave the ER, instead they are degraded.

Once the protein has folded correctly, the final glucose residue is removed, indicating the protein is ready for the next step (Hammond et al., 1994). A series of mannosidases then cleave one or all of the α1,2 bound mannoses (Kornfeld and Kornfeld, 1985). From here, the diversity of glycans begins to develop. Complex glycoproteins are transported to the golgi apparatus for the next steps of processing in vesicles which bud off from the ER membrane (Palmer et al., 2005). These vesicles fuse with the membrane of the *cis* golgi. The glycoproteins then move systematically through the golgi. The golgi contains a diverse range of glycosyltransferases, which can add a wide range of saccharide moieties to the developing *N*-linked glycan. So after the glycoprotein enters the *cis* golgi lumen it then proceeds through the golgi apparatus passing through the *medial* golgi and into the *trans* golgi. On its journey the addition of various oligosaccharides is catalysed

by different glycosyltransferases (Kornfeld and Kornfeld, 1985). Most glycans consist of bi-antennary structures, however tri-antennary and tetra antennary occur often, and five or more branches do occur (Eklund and Freeze, 2005). The final step which occurs in the *trans* golgi is the addition of a galactose and sialic acid residue to the terminal sugar of each branch in reactions catalysed by galactosyltransferase and sialyltransferase respectively (see figure 4.3).

4.1.2 Sialic Acid.

Sialic acid has been implicated in a number of disease states including cystic fibrosis and tumours (Malykh et al., 2001, Kube et al., 2001) and may be involved in release of the influenza virus from host cells (Nayak et al., 2004). Sialic acid residues are added to the terminal sugar of the glycan of glycosylated proteins by sialyltransferases in the *trans* golgi. These residues can be linked via α2,3, or α2,6 to D-galactose or α2,6 to D-N-acetlygalactosamine or D-N-acetylglucosamine. α2,8 linkages also occur (Angata and Fukuda, 2003). There are over 50 different sialic acids all derived from either neuraminic acid or deaminoneuraminic (Munster-Kuhnel et al., 2004). In the nucleus, sialic acids for example N-acetylneuraminic acid (Neu5Ac) are converted to their CMP diester form (e.g. CMP-Neu5Ac) by CMP-sialic acid synthetase (e.g. CMP-Neu5Ac synthetase). From here the CMP-sialic acid is transported to the golgi where it becomes a substrate for the sialyltransferases, which subsequently attach it to the oligosaccharide chain (Munster-Kuhnel et al., 2004). There are currently around 18 different known human sialyltransferases (Harduin-Lepers et al., 2001).

A mutant Pro-5 cell line derived from Chinese Hamster Ovary cell (CHO) has been developed, Lec2 cells (ATCC Number: CRL-1736) which have impaired CMP-sialic acid transport to the goli and therefore glycoproteins synthesised in these cells are not sialylidated. This cell line is therefore a useful tool to determine whether sialic acid is important in glycoprotein function or trafficking (Compton et al., 2002). Another useful tool for studying the effect of sialic acid on proteins is the enzyme neuraminidase. Neuraminidase is a carbohydrase or glycosidase enzyme which was first discovered in the influenza virus. It cleaves the sialic acid residues from the terminal of glycans on glycoproteins (Gottschalk, 1958).

Sialic acid is known to affect the functioning of other ion channels including the potassium channel Kv1.1 and the voltage-gated sodium channel (Johnson et al., 2004, Thornhill et al., 1996). Sialic acid is thought to alter the local electric field of Kv1.1 (Thornhill et al., 1996). Sialylation of the β_1 subunit of the voltage-gated sodium channel is thought to regulate the channel by an electrostatic mechanism (Johnson et al., 2004). It is currently unknown what role sialic acid plays in the TRP family of ion channels.

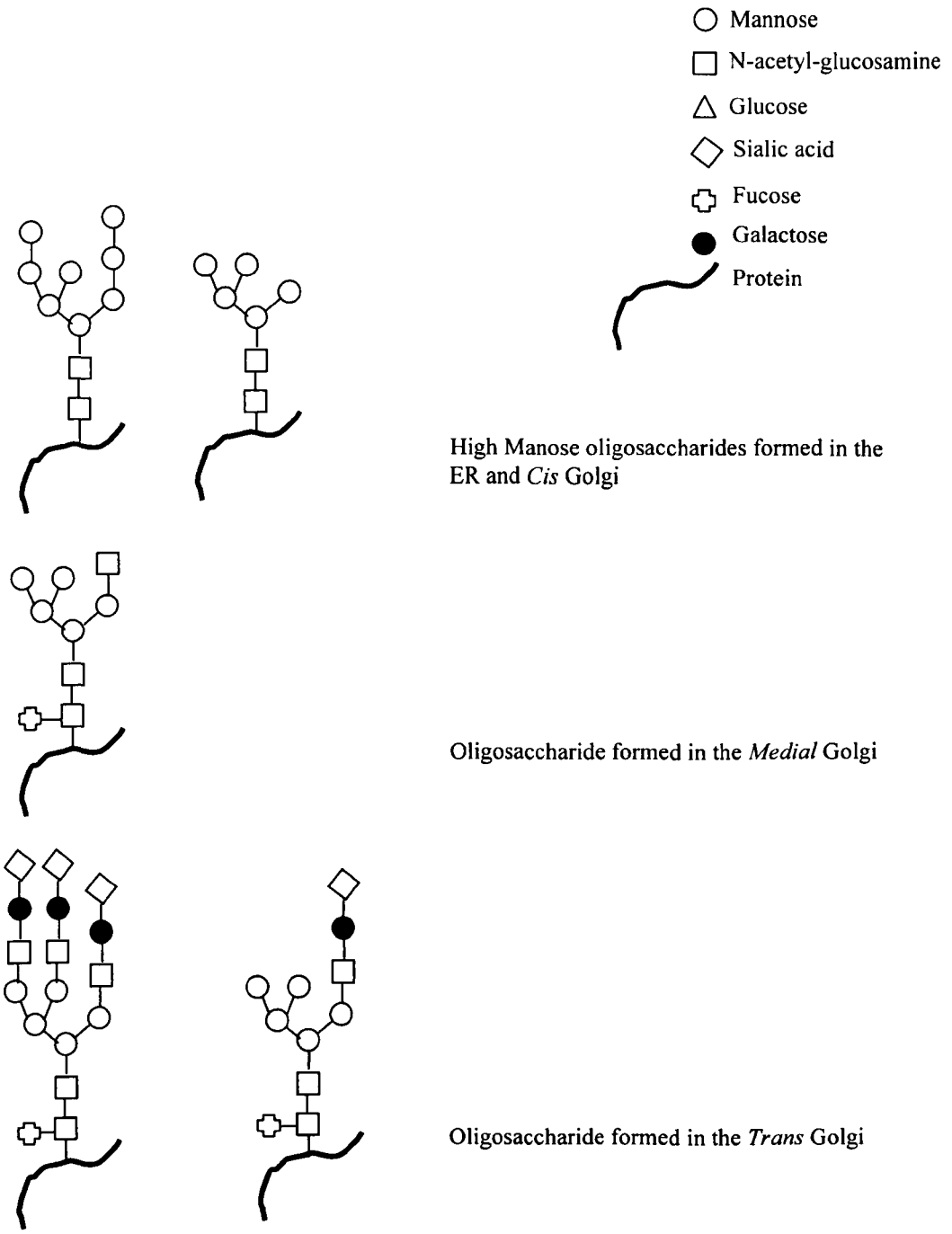


Figure 4.3. *N*-Linked Glycan Processing in the Golgi. The glycosylated protein moves systematically through the Golgi, which contains a diverse range of glycosyltansferases. The glycosyltransferases catalyses the addition of various oligosaccharides to the glycan. The final step, which occurs in the *Trans* Golgi, is the addition of a galactose and sialic acid residue to the terminal sugar catalysed by galactosyltransferase and sialyltransferase respectively.

4.1.3 Glycosylation and rTRPV1

rTRPV1 has one potential *N*-linked glycosylation sequon (NNS) situated at amino acid 604 on the extracellular region of transmembrane spanning domain 5 in close proximity to the P-loop (see Figure 1.3). Two previous studies have provided preliminary but conflicting data on the role of glycosylation in regulating TRPV1 function. A study by Rosenbaum *et al* 2002 where they mutated the asparagines to serine (N604S) detected no change in function of the mutant TRPV1 lacking the glycosylation site compared to the wild type channel (Rosenbaum et al., 2002). In contrast, Wirkner *et al* (2005), mutated the asparagines of the glycosylation sequon to a threonine (N604T), and reported a reduction in the maximum of the capsaicin concentration effect curve and a reduced EC_{50} (Wirkner et al., 2005). The results of both studies are conflicting and although the asparagine was mutated to amino acids with uncharged polar side chains, serine and threonine, the most widely used amino acid in replacement of asparagine and closely related structurally is glutamine (Q). Glutamine, like asparagine, threonine and serine has an uncharged polar side chain, however threonine and serine contain aliphatic hydroxyl groups where as asparagine and glutamine have a terminal amide group. Altering the amino acid can affect folding of the protein, so in order to minimise disruption of the channel and to see the effects of removing the glycosylation site alone, the amino acid exhibiting the closest properties to asparagine should have been selected.

In this investigation in order to give a more in depth study of the role of potential *N*-linked glycosylation site 604, the asparagine was mutated to glutamine (N604Q) and both the N604Q mutant and wild type TRPV1 were expressed in Pro5 and the mutant cell line Lec2.

4.2 Materials and Methods

4.2.1 Materials

Neuraminidase was supplied by Calbiochem, UK. Tunicamycin was obtained from Sigma, Poole, UK. Protein assay was purchase from BioRad. The murine anti-HA11 monoclonal antibody was bought from Covance, UK. The ProFound HA11 immunoprecipitation Kit was purchase from Pierce, Northumberland, UK. The enhanced chemiluminescence (ECL) western blotting detection reagents and analysis system and anti-murine horse radish peroxidase conjugated secondary antibody was supplied by Amersham Biosciences, Buckinghamshire, UK and the polyvinylidene fluoride (PVDF) transfer membrane was purchased from Amersham Pharmacia Biotech. For all other reagents see section 2.2.1.

4.2.2 Mutation of the Potential Glycosylation Site N604

Site directed mutagenesis was performed as described in section 2.2.2.9. Briefly, a mutation was inserted at amino acid position 604 to change N604 to Q604. The primers were designed as described in section 2.2.2.9 (sense, 5'-GGATGGGAAGCAGAACTCTCTGC-3', antisense, 5'-GCAGAGAGTTCTGCTTCCCATCC-3') and the site directed mutagenesis reaction was carried out as per the manufacturers protocol (Stratagene). The mutated rTRPV1 construct was amplified as described in section 2.2.2.8 and rTRPV1 and rTRPV1N604Q permanently expressing cell lines were created in both Lec2 and Pro5 cells as described in section 2.2.2.10 and 2.2.2.11 as well as mock transfected Pro5 and Lec2 cell lines. Cells were grown in MEM α, containing 10 % FCS, 100

U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B and 0.6 mg/ml G418.

4.2.3 Comparing Expression by Flow Cytometry

To ensure any functional differences in responses seen between rTRPV1-Pro5, rTRPV1N604Q-Pro5, rTRPV1-Lec2 and rTRPV1N604Q-Lec2 were due to mutation or lack of sialic acid, all four cell lines were analysed by flow cytometry. This was performed based on the methods described in section 3.2.6. Briefly, cells were grown to 70% confluence and harvested with cell dissociation buffer (Invitrogen). Cells were permeabilised in PBS containing 4% formaldehyde before incubation with the primary antibody, mouse anti-HA11 monoclonal antibody (1 in 1000, Covance) followed by incubation with the secondary antibody, anti-mouse IgG FITC polyclonal antibody (1 in 100, Sigma). Optimisation was again carried out for this protocol involving both primary and secondary antibody titrations.

4.2.4 RT-PCR

RT-PCR was performed as one of four separate approaches to verify that the Pro5 and Lec2 cell lines had been successfully transfected with rTRPV1 or rTRPV1N604Q. mRNA was extracted from mock transfected Pro5 and Lec2 cells, rTRPV1 expressing Pro5 and Lec2 cells and rTRPV1N604Q expressing Pro5 and Lec2 cells using an RNeasy minikit (Qiagen) according to manufacturer's protocol and quantified using a GeneQuant™ spectrophotometer (Amersham Biosciences). cDNA was synthesised by reverse transcription of 2 μg of mRNA with an omniscript RT kit (Qiagen) and random primers (Invitrogen). The reverse transcription reaction

took place at 24°C for 10 minutes, 42°C for 50 minutes and 95°C for 10 minutes (as described in 2.2.2.3). The PCR reaction for rTRPV1 contained 1 µl of cDNA, PCR buffer, 0.2 mM dNTPs and 1U Taq DNA polymerase (New England Bioscience) with specific oligonucleotide primers (primers sense, 5'-CTTCACCGCGGCTGCCTACT-3', antisense, 5'-TGAACTCCAGGTCGCCCATG-3'). The PCR reactions were carried out for 35 cycles at 94°C for 3 minutes, 56°C for 1 minute and 72°C for 1.5 minutes with a final extension for 10 minutes. The PCR products were subsequently separated on 1.3% agarose gel and visualised under UV illumination using a UVP Epichemi system (UVP Laboratory Products, Cambridge, UK). The PCR products were sent for sequencing to ensure each cell line was expressing the correct rTRPV1 mRNA varient, either wild type rTRPV1 or rTRPV1N604Q.

4.2.5 Western Blot Analysis.

4.2.5.1 Membrane Preparations

Cells were plated into 175 cm^2 flasks and grown to 70% confluence. The cells were washed with PBS (4°C) then incubated for 30 seconds in deionised water (4°C). This was replaced by membrane buffer (tris base 5 mM, EDTA 0.5 mM, leupeptin 1 µg/ml, soybean trypsin inhibitor (STI) 1 µg/ml and orthovanadate 1 mM, pH 7.5) at 4°C. The swollen cells were harvested using a cell scraper and transferred to a 15 ml centrifuge tube before incubation on ice for 30 minutes with vortexing every 10 minutes to lyse the cells. The cell suspension was then centrifuged at 500 g for 10 minutes at 4°C. The supernatant was transferred to 1.5 ml centrifuge tubes and the pellet containing the nuclear fraction was discarded. The supernatant was

centrifuged at 20,000 g for 45 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 100 µl of membrane buffer. Crude membrane preparations were stored at -80°C until required.

4.2.5.2 Immunoprecipitation

HA11 tagged rTRPV1 was immunoprecipitated using a ProFound HA11 immunoprecipitation kit as per the manufacturer's protocol. Protein was quantified using the BioRad Protein assay, again as per the manufacturer's protocol.

4.2.5.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

For reducing blots, 10 µg of protein was incubated with sample buffer and 1 µl of β-mercaptoethanol for 1 minute at 100°C to denature the proteins. β-mercaptoethanol is a reducing agent and was used to break any protein covalent bonds. Reduced or non-reduced protein (10 µg) in sample buffer were then run on a 10% SDS polyacrylamide gel using a Scie-Plus modular electrophoresis system (Jencons, Leighton Buzzard, UK) as shown in table 4.1. The gel was run in a vertical gel electrophoresis unit in electrode buffer consisting of 29 mM tris base, 192 mM glycine and 0.1% SDS.

Stacking Gel	10% SDS polyacrylamide gel
5.5% Acrylamide	10% Acrylamide
12.6% Stacking gel buffer 0.5 M tris HCl pH 6.8	25% Resolving gel buffer 1.5 M tris HCl pH 8.8
0.1% SDS	0.1% SDS
0.06% Amonia persulphate	0.05% Amonia persulphate
0.14% TEMED	0.05% TEMED
Deionised water	Deionised water

Table 4.1. Chemicals used to make the SDS polyacrylamide gel.

4.2.5.4 Blotting

Protein from the gel was transferred to a polyvinylidene fluoride (PVDF) transfer membrane (Amersham Pharmacia Biotech) by overnight electro transfer using an electro transfer module in a Scie-Plus modular electrophoresis system. The membrane was then incubated in PBS-tween 20 (0.1%) containing non fat milk (2%) for at least 1 hour. The membrane was then washed for 15 minutes four times with PBS-tween 20 (0.1%) before incubation overnight with the primary antibody, murine anti-HA11 monoclonal antibody (1 in 1000) in PBS-tween 20 (0.1%) containing non fat milk (2%). The membrane was washed for 15 minutes four times with PBS-tween 20 (0.1%) followed by incubation with the secondary antibody, in PBS-tween 20 (0.1%) containing non fat milk (2%) for 2 hours. The protein was visualised in a UVP gel doc system following treatment with an enhanced chemiluminescence solution (ECL western blotting detection reagents and analysis system, Amershan Biosciences) as per the manufacturer's instructions. Pictures were taken every 5 minutes for 1 hour, the picture depicting bands most clearly was chosen.

4.2.5.5 Optimisation

Due to low levels of cell surface expression of the protein, up to ten 175 cm^2 flasks of cells were used per membrane preparation. Western blots were initially tried with crude membrane preparations, however, multiple bands were detected due to the low specificity of the secondary antibody and therefore membrane preparations were HA11 immunoprecipitated. A secondary antibody titration was also performed in order to maximise protein visualisation and eliminate non-specific antibody binding.

4.2.6 Confocal Microscopy

Confocal microscopy was performed to determine whether glycosylation had an effect on cell surface expression of rTRPV1. Cells were plated into Petri dishes (60 mm) containing coverslips. The cells were grown in normal growth media for 48 hours or until they reached 70% confluence before washing two times with PBS. The cells were then fixed by incubation in formaldehyde (3%) in PBS for 15 minutes, this was followed by washing twice with PBS and permeabiling in PBS containing 0.2% triton X-100 for 10 minutes. The cells were washed another two times with PBS, then incubated in the primary antibody, murine anti-HA11 monoclonal antibody (1 in 1000, Covance) for 1 hour at room temperature (rTRPV1E648A-HEK cells) or overnight at 4°C (rTRPV1-Pro5 cells). The differences in primary antibody incubation times for rTRPV1E648A-HEK and rTRPV1-Pro5 cells were due to differences in rTRPV1 expression levels. The cells were washed again two times with PBS before incubation with the secondary antibody, anti-mouse IgG FITC polyclonal antibody (1 in 500, sigma) for 1 hour at room temperature. The cells were washed a final two times with PBS before the coverslips were fixed to slides. The cells were visualised using a Nikon Eclipse (TE2000-E) microscope with a BioRad Radiance 2100 scanning system and lasers.

4.2.7 Calcium Signalling

Activation of TRPV1 by capsaicin was assessed by measuring increases in intracellular calcium levels following agonist challenge. This was carried out as described in section 2.2.4. Briefly, cells were loaded with the calcium binding probe Fluo-3 and increases in intracellular calcium were detected using a PTI fluorospectrometer with an excitation wavelength of 480 nm and a detector set to

measure at a wavelength of 530 nm. Responses to capsaicin were expressed as a percentage of the maximum fluorescence signal after the addition of 6 μM calcium ionophore (A23187) and used to construct concentration-effect curves.

4.2.8 Neuraminidase Treatment

rTRPV1-Pro5 cells (one 75 cm^2 flask at 70% confluence) were lifted with cell dissociation buffer and washed by centrifugation with PBS. hTRPV1-HEK cells and rTRPV1E648A-HEK cells (75 cm^2 flask at 90% confluence) were lifted with PBS and centrifuged. The cells were resuspended in 200 μL of normal growth media (MEM α growth media for the Pro5 cell lines and DMEM containing 10 % FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B and 0.6 mg/ml G418 for the HEK cell lines) and 0.15 U/ml V*ibrio cholerae* neuraminidase (Calbiochem). The cells were incubated at 37°C for 45 minutes with gentle shaking. The cells were then washed twice by centrifugation with PBS before responses to capsaicin were investigated by calcium signalling (see section 2.2.4).

4.2.9 Tunicamycin Treatment.

24 hours after cells had been plated in 75 cm^2 flasks in normal growth media, tunicamycin (sigma) was added to give a final concentration of 1 μg/ml as described previously (Compton et al., 2001). Pro5 cell lines were allowed to propagate for 48 hours and HEK cell lines were allowed to propagate for 72 hours before responses to capsaicin were investigated by calcium signalling (see section 2.2.4). rTRPV1E648A-HEK cells grow slower than rTRPV1-Pro5 cells and as tunicamycin only inhibits *N*-glycosylation of newly synthesised proteins, the rTRPV1E648A-

HEK cells were allowed to propagate for a further 24 hours (compared to rTRPV1-Pro5 cells) to allow enough newly synthesised protein to be produced.

4.3 Results

4.3.1 Tunicamycin and Neuraminidase Severely Reduce TRPV1 Function

A pharmacological approach was initially employed to determine whether the glycosylation of rTRPV1 was important for the correct functioning of TRPV1. rTRPV1-Pro5 displayed a concentration dependent increase in intracellular calcium in response to capsaicin from as low as 3 nM reaching a maximum by 300 nM with an EC_{50} of 20 nM (Figure 4.4A, open squares). For rTRPV1-Pro5 cells pretreated with tunicamycin (1 μg/ml, 48 hours), a response was observed at 30nM capsaicin which reached a maximum by 1 μM capsaicin with a 79% reduction in the maximal response and rightward shift of 1 order of magnitude with an EC_{50} value of 21 nM (Figure 4.4A, open triangles). No difference in activation kinetics was observed between treated and non treated rTRPV1-Pro5 cells (Figures 4.4B and C). Mock transfected Pro5 cells failed to respond to capsaicin at the maximum concentration tested (3 μM, closed square). To determine more specifically if sialic acid was involved in this loss of response, rTRPV1-Pro5 cells were treated with neuraminidase. Pretreatment of rTRPV1-Pro5 cells with 0.15 U/ml neuraminidase resulted in capsaicin evoked response from 10nM, reaching a maximum by 100nM with a 57% reduction in maximal response compare to untreated cells with an EC_{50} 21 nM (Figure 4.5A, open circles). No difference in activation kinetics was observed between treated and non treated rTRPV1-Pro5 cells (Figure 4.5B).

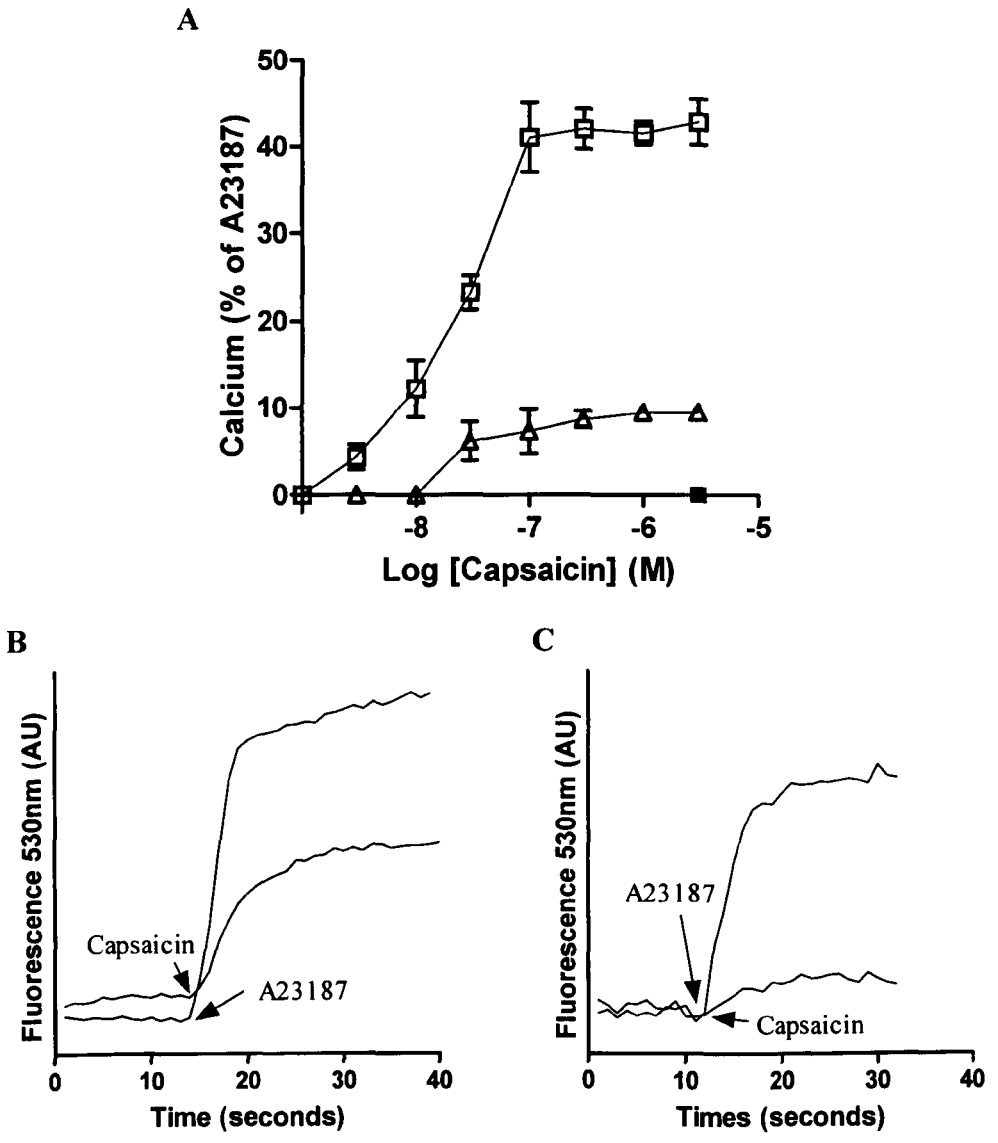


Figure 4.4. Capsaicin concentration-effect curves for rTRPV1-Pro5 cells with and without tunicamycin treatment. Capsaicin concentration-effect curve for mock transfected Pro5 (■), rTRPV1-Pro5 (□) and tunicamycin treated rTRPV1-Pro5 (Δ). Results are expressed as the mean ± SEM of 3 to 4 experiments each performed in duplicate. (B) Representative traces for rTRPV1-Pro5 for capsaicin (3μM) and A23187 (6 μM) evoked calcium responses. (C) Representative traces for tunicamycin treated rTRPV1-Pro5 for capsaicin (3μM) and A23187 (6 μM) evoked calcium responses.

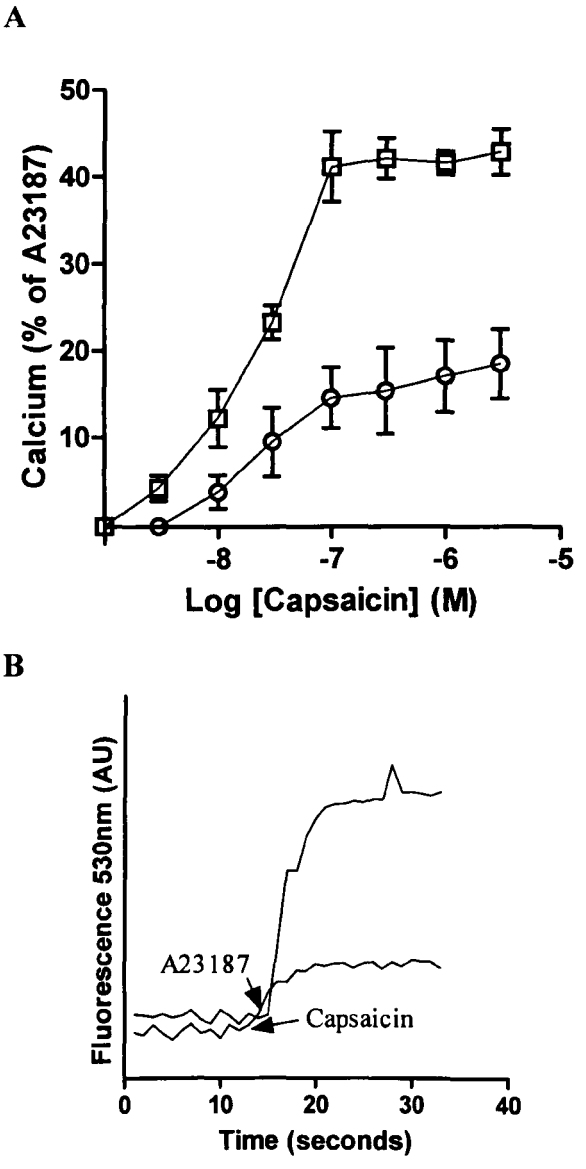


Figure 4.5. Capsaicin concentration-effect curves for rTRPV1-Pro5 cells with and without neuraminidase treatment. Capsaicin concentration-effect curve for rTRPV1-Pro5 (□) and neuraminidase treated rTRPV1-Pro5 (○). Results are expressed as the mean ± SEM of 4 experiments each performed in duplicate. (B) Representative traces for neuraminidase treated rTRPV1-Pro5 for capsaicin (3μM) and A23187 (6 μM) evoked calcium responses.

4.3.2 The Glycosylation Mutant Channel rTRPV1N604Q-Pro5 and rTRPV1-Lec2 Display Dramatic Reductions in Channel Activation by Capsaicin

We next sought to determine on a molecular level the importance of the glycosylation site and sialylation for rTRPV1 function. To do this the potential site of glycosylation (N604) was mutated in rTRPV1 producing rTRPV1N604Q. Pro5 and Lec2 cells were successfully transfected with either rTRPV1 or rTRPV1N604Q to create three further cell lines, rTRPV1N604Q-Pro5, rTRPV1-Lec2 and rTRPV1N604Q-Lec2. RT-PCR confirmed transfection had been successful in each cell line (Figure 4.6). Clear bands at ~607 bp were observed for rTRPV1-Pro5, rTRPV1N604Q-Pro5, rTRPV1-Lec2 and rTRPV1N604Q-Lec2. No bands were detected for mock transfected Pro5 or Lec2 cells.

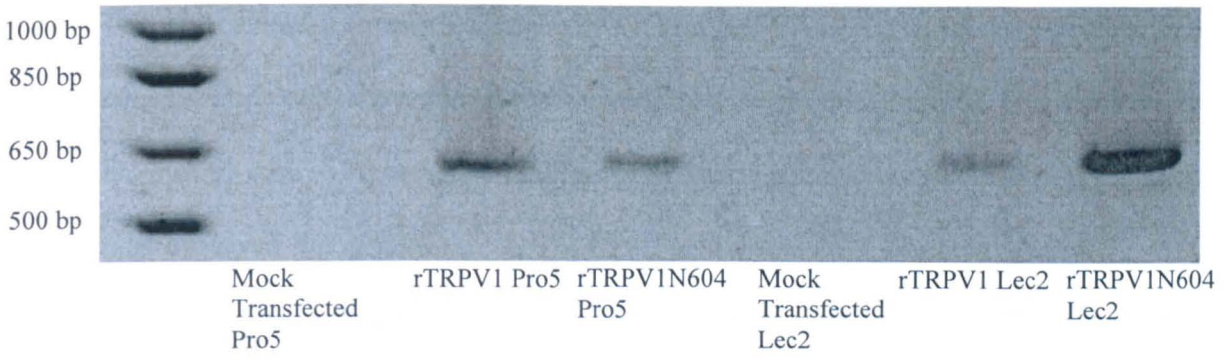


Figure 4.6. RT-PCR of rTRPV1 amplified from mock transfected Pro5 and Lec2, rTRPV1-Pro5, rTRPV1-Lec2, rTRPV1N604Q-Pro5 and rTRPV1N604Q-Lec2 cDNA. The products were separated on a 1.3% agarose gel containing ethidium bromide and visualised under UV light. The size of the product is ~607 bp.

The glycosylation mutant receptor rTRPV1N604Q-Pro5 responded to capsaicin in a concentration dependent manner from 3 nM reaching a maximum by 300 nM with an EC_{50} of 38 nM. The maximal obtainable response for N604Q was ~76% lower than that observed for capsaicin in rTRPV1-Pro5 (Figure 4.7, closed triangles). No difference in activation kinetics was observed between rTRPV1-Pro5 and rTRPV1N604Q-Pro5 cells (Figures 4.4B and 4.7B). rTRPV1-Lec2 and rTRPV1N604Q-Lec2 both responded in a concentration dependent manner to capsaicin from 3 nM and 10 nM respectively, again reaching a maximum by 300 nM (Figure 4.8A, EC_{50} values of 20 nM, open circles and 21 nM, closed circles respectively). The maximal responses of rTRPV1-Lec2 and rTRPV1N604Q-Lec2 cells to capsaicin were ~74% and ~69% lower than that observed for capsaicin in rTRPV1-Pro5 cells respectively. Mock transfected Lec2 cells failed to respond to capsaicin at the highest concentration tested (3 μM, closed square). Little difference in activation kinetics was observed between rTRPV1-Pro5, rTRPV1-Lec2 and rTRPV1N604Q-Lec2 cells (Figures 4.4B, 4.8B and 4.8C).

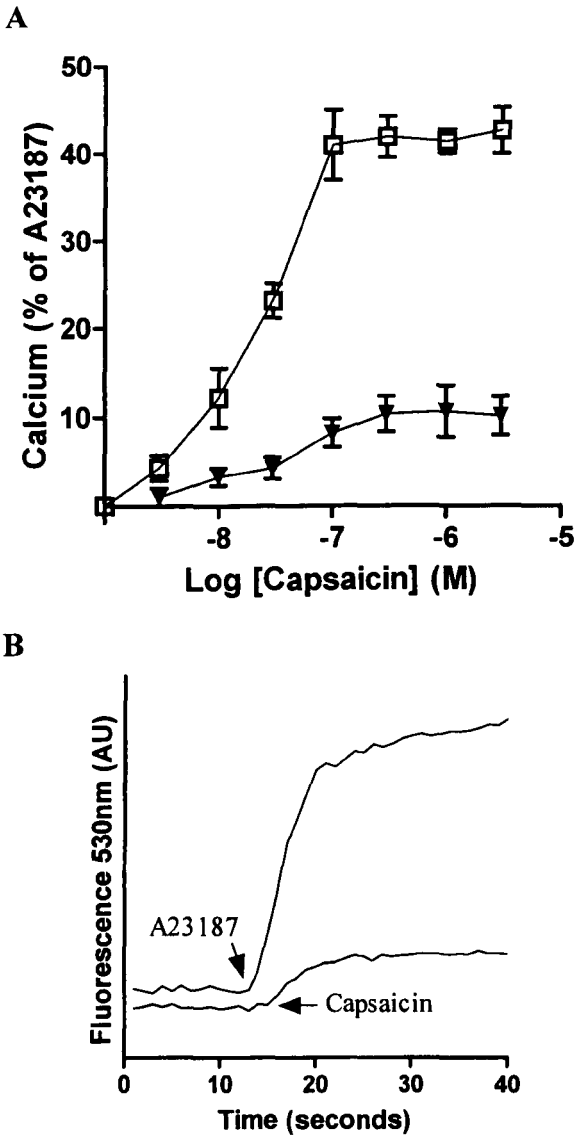


Figure 4.7. Capsaicin concentration-effect curves for rTRPV1-Pro5 and rTRPV1N604Q-Pro5 cells. Capsaicin concentration-effect curve for rTRPV1-Pro5 (□) and rTRPV1N604Q-Pro5 (▼). Results are expressed as the mean ± SEM of 4 to 6 experiments each performed in duplicate. (B) Representative traces for rTRPV1N604Q-Pro5 for capsaicin (3μM) and A23187 (6 μM) evoked calcium responses.

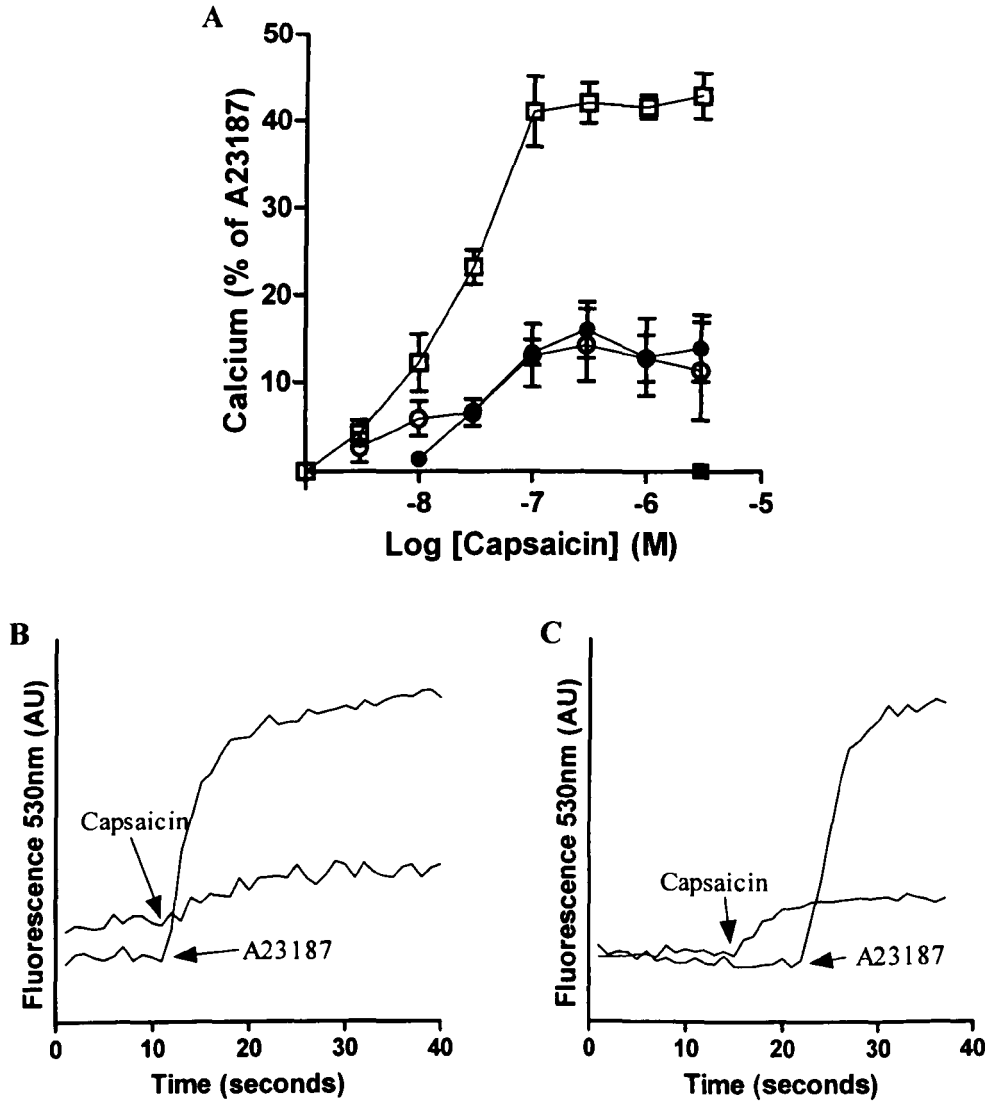


Figure 4.8. Capsaicin concentration-effect curves for rTRPV1-Pro5 and rTRPV1-Lec2 and rTRPV1N604Q-Lec2. (A) Capsaicin concentration-effect curve for mock transfected Lec2 (■) rTRPV1-Pro5 (□), rTRPV1-Lec2 (○) and rTRPV1N604Q-Lec2 (●). Results are expressed as the mean ± SEM of 3 to 7 experiments each performed in duplicate. (B) Representative traces for capsaicin (3μM) and A23187 (6 μM) evoked calcium responses for rTRPV1-Lec2 cells. (C) Representative traces for capsaicin (3μM) and A23187 (6 μM) evoked calcium responses for rTRPV1N604Q-Lec2 cells.

4.3.3 Glycosylation has little Effect on TRPV1 Expression.

Confocal microscopy and flow cytometry were employed to show whether glycosylation or sialylation affected cell surface expression of the channel.

4.3.3.1 Confocal Microscopy

Due to the high expression of rTRPV1E648AHEK cells, this cell line was used as a positive control and clearly displays TRPV1 expression throughout the cell except for in the nucleus (Figure 4.9A). No staining was seen in cells treated with only the secondary antibody (Figure 4.9C and 4.9D). The same cellular staining was observable in rTRPV1-Pro5 cells (Figure 4.9B).

4.3.3.2 Flow Cytometry

Flow cytometry was employed to compare the receptor densities of the individual cell lines to ensure that the differences in responses were not due to differences in expression levels. Due to the unreliability of TRPV1 antibodies, rTRPV1 and rTRPV1N604Q were tagged with an influenza haemagglutinin epitope (YPYDVPDYA) at the C terminus. The commercially available antibody, murine anti-HA11 (Covance) could then be used to visualise the protein. Despite the epitope tagging, the expression of TRPV1 was still hard to detect and no significant TRPV1 expression could be detected in the rTRPV1-Pro5 cell lines (Figure 4.10A). However clear rightward shifts could be detected in both the rTRPV1N604Q-Lec2 and rTRPV1-Lec2 cell lines, when compared to mock transfected cells (Figure 4.10B).

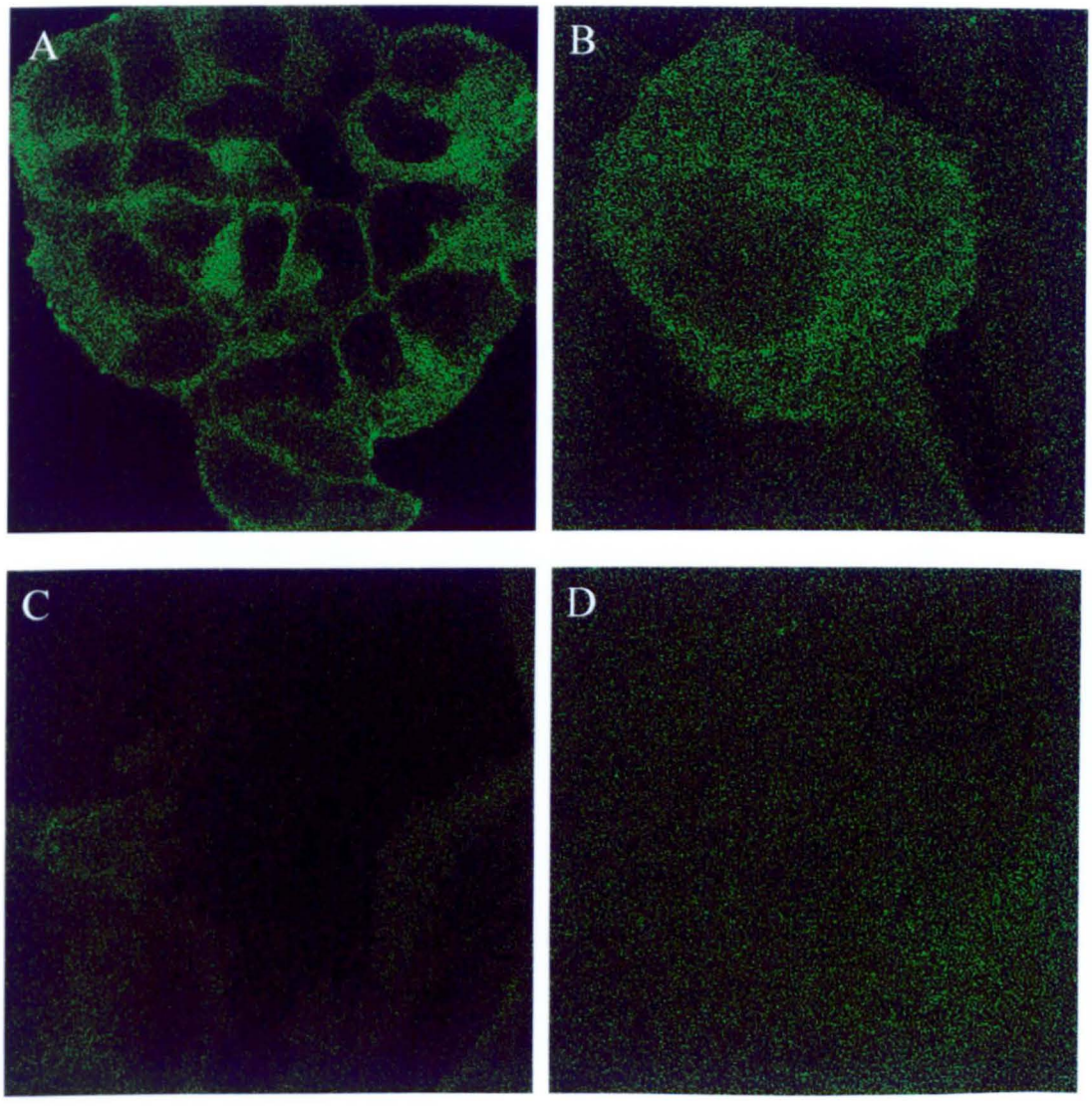


Figure 4.9. Confocal Microscopy showing rTRPV1 cellular distribution. (A) rTRPV1E648A-HEK and (B) rTRPV1-Pro5 stained with murine anti-HA11 antibody and anti-mouse FITC conjugated antibody. (C) rTRPV1E648A-HEK and (D) rTRPV1-Pro5 stained with secondary antibody only, anti-mouse FITC conjugated antibody.

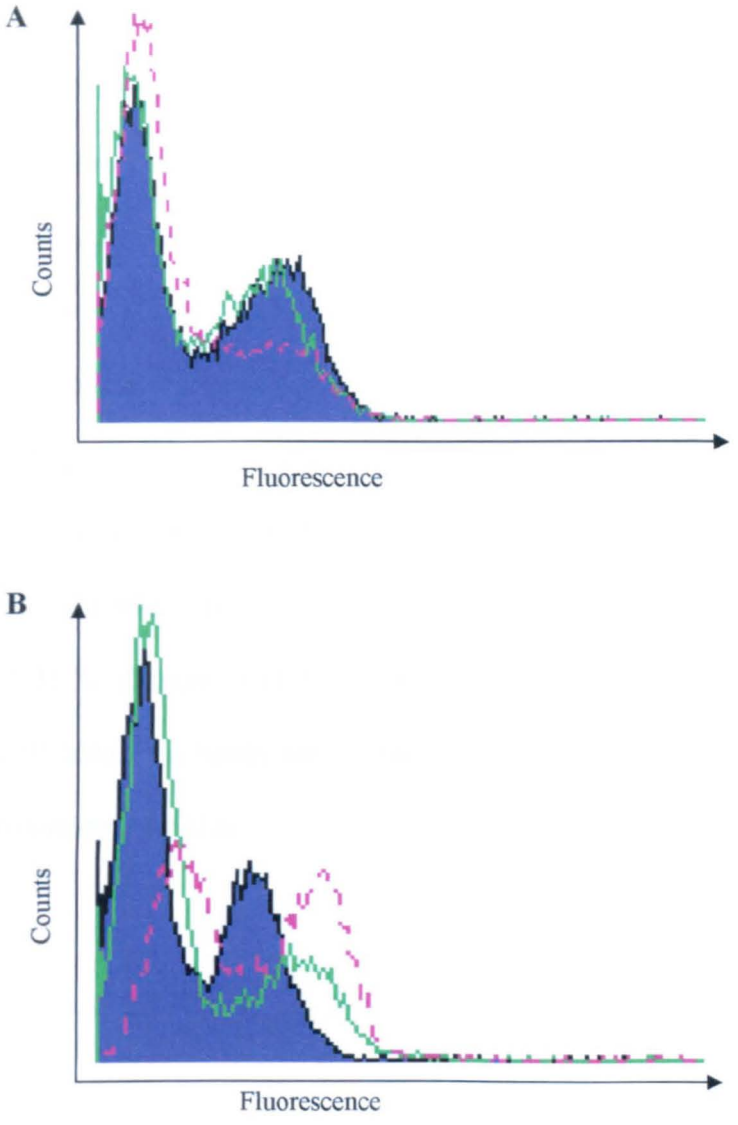


Figure 4.10. Flow cytometry analysis for rTRPV1-Pro5, rTRPV1N604Q-Pro5, rTRPV1-Lec2, rTRPV1N604Q-Lec2 and mock transfected Pro5 and Lec2 cell lines. Flow cytometry analysis for (A)TRPV1 expression on mock transfected Pro5 (■), rTRPV1-Pro5 (-----) and rTRPV1N604Q-Pro5 cells (——). (B) TRPV1 expression on mock transfected Lec2 (■), rTRPV1-Lec2 (-----) and rTRPV1N604Q-Lec2 cells (——). The results are representative of 3 separate experiments.

4.3.4 rTRPV1 is not Heavily Glycosylated

Membrane preparations and immunoprecipitations were performed for rTRPV1-Pro5, rTRPV1N604Q-Pro5, rTRPV1-Lec2 and rTRPV1N604Q-Lec2 as well as mock transfected Pro5 and Lec2 cell lines. However, not enough protein was extracted to be detected on the transfer membrane despite cells from up to ten 175 cm^2 flasks being utilised. A non reducing blot was performed for rTRPV1-Pro5, rTRPV1N604Q-Pro5, rTRPV1-Lec2 and rTRPV1N604Q-Lec2 and a band was clearly visible at approximately 100kDa for rTRPV1-Lec2 and rTRPV1N604Q-Lec2 with no band observed for the mock transfected Lec2 cells (Figure 4.11A). As a positive control, membrane preparations and immunoprecipitation was performed for rTRPV1E648A-HEK (Figure 4.11B). The blot clearly shows a major band at approximately 95 kDa. No bands were detected in the mock transfected HEK cells, except at approximately 60 kDa.

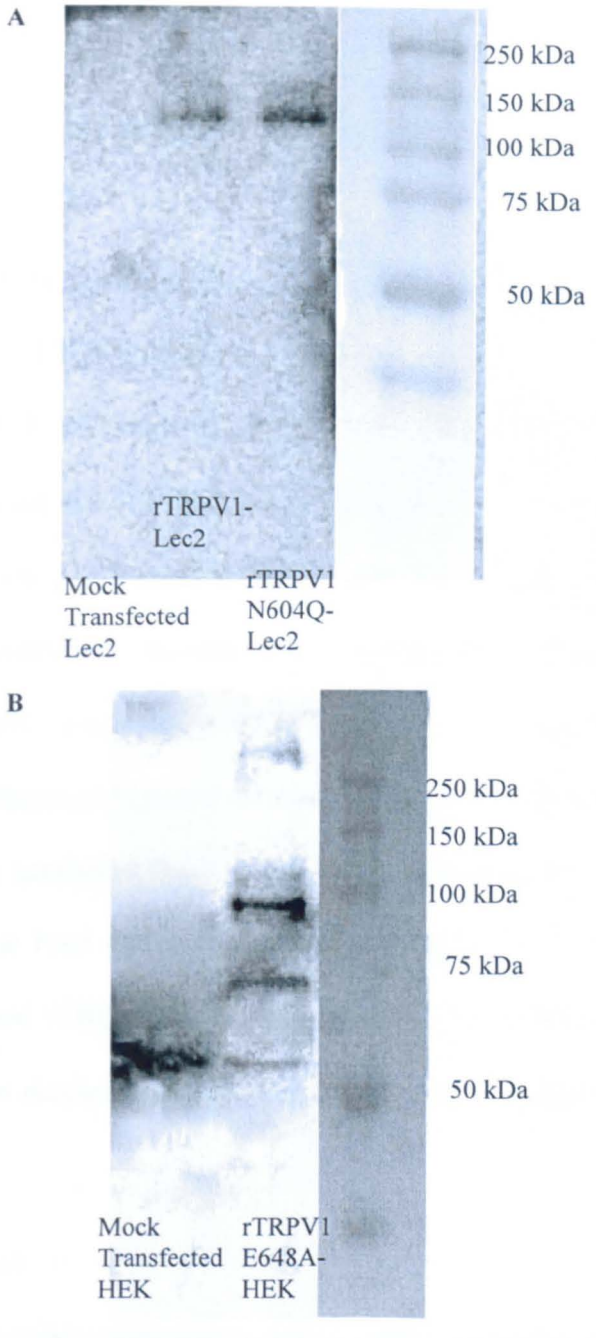


Figure 4.11. Western Blot analysis of HA11 tagged rTRPV1-Lec2, rTRPV1N604Q-Lec2 and rTRPV1E648A-HEK. (A) Western blot analysis of rTRPV1-Lec2, rTRPV1N604Q-Lec2 and mock transfected Lec2 cell lines. (B) Western blot analysis of rTRPV1E648A-HEK and mock transfected HEK293 cells. Crude membrane preps were immunoprecipitated using an HA11 immunoprecipitation kit and proteins were separated by SDS-PAGE before immunoblotting with a monoclonal HA11 antibody.

4.3.5 Tunicamycin and Neuraminidase Severely Reduce the Function of rTRPV1 expressed in HEK293 Cells

To determine whether the results obtained with rTRPV1-Pro5, rTRPV1N604Q-Pro5, rTRPV1-Lec2 and rTRPV1N604Q-Lec2 were cell specific we expressed rTRPV1E648A in HEK293 cells (see chapter 3). Capsaicin concentration effect curves were constructed for rTRPV1E648A-HEK before and following treatment with either neuraminidase or tunicamycin treatment (Figure 4.12A). Untreated cells (open diamonds) responded to capsaicin from 1 nM reaching a maximum by 300 nM with an EC_{50} of 13 nM. The neuraminidase (Figure 4.12A, cross) and tunicamycin (Figure 4.12, closed diamond) treated cells both responded to capsaicin between 10 nM again reaching a maximum by 300 nM with EC_{50} values of 17 nM and 8 nM respectively. Like the Pro5 cell lines, the maximal response of the neuraminidase and tunicamycin treated rTRPV1E648A cells resulted in a striking reduction (~68% for both treatments) in maximal response compared to the untreated rTRPV1E648A-HEK cells.

4.3.6 Tunicamycin Does Not Affect Global Expression of rTRPV1E648A-HEK

To show whether the reduction in response following tunicamycin treatment was due to a loss of TRPV1, flow cytometry was performed to compare channel densities before and after treatment (Figure 4.12B). The results show a similar shift in fluorescence for both the treated and untreated cell lines compared to mock transfected HEK cells.

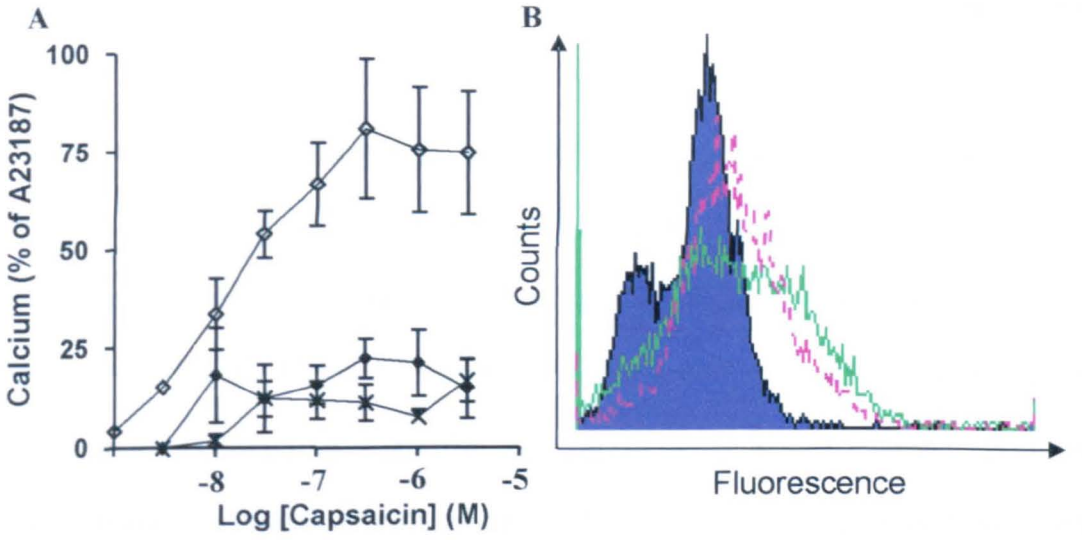


Figure 4.12. Capsaicin concentration-effect curves for rTRPV1E648A-HEK with and without tunicamycin and neuraminidase treatment and flow cytometry analysis to compare TRPV1 expression before and after tunicamycin treatment. (A) Capsaicin concentration-effect curve for untreated rTRPV1E648A-HEK cells (◊), tunicamycin treated rTRPV1E648A-HEK (♦) and neuraminidase treated rTRPV1E648A-HEK (×). Results are expressed as the mean ± SEM of 3 to 5 experiments each performed in duplicate. (B) Flow cytometry analysis for TRPV1 expression on mock transfected HEK cells (■), untreated rTRPV1E648A-HEK (-----) and tunicamycin treated rTRPV1E648A-HEK cells (——). The results are representative of 3 separate experiments.

4.3.7 Tunicamycin and Neuraminidase Treatment Reduces the Responsiveness of hTRPV1 to Capsaicin

Finally, the role of glycosylation on hTRPV1 was investigated. The response of hTRPV1 expressing HEK cells to capsaicin with and without neuraminidase or tunicamycin treatment was measured and capsaicin concentration-effect curves were constructed (Figure 4.13). Untreated cells responded to capsaicin from 300 pM reaching a maximum by 300 nM with an EC_{50} of 8 nM (Stars). Both the neuraminidase and tunicamycin treated cells responded to capsaicin from 1 nM reaching a maximum by 100 nM with EC_{50} values of 6 nM for both cell lines (cross and open triangle respectively). Both the neuraminidase and tunicamycin treated cells exhibited a ~44% reduction in maximal response compared to the untreated hTRPV1-HEK cells.

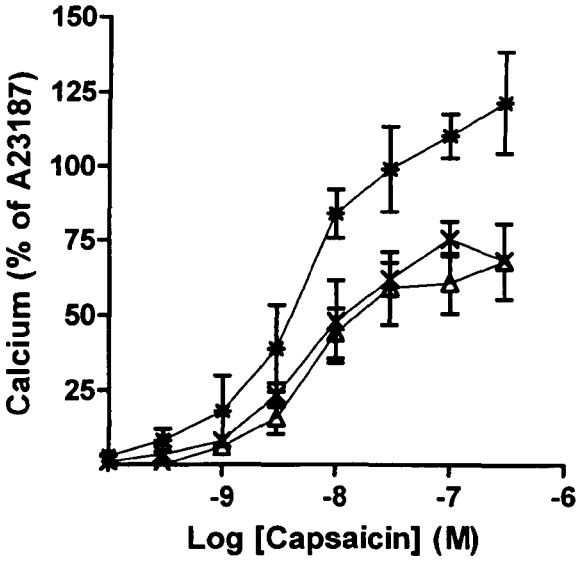


Figure 4.13. Capsaicin concentration-effect curves for hTRPV1-HEK cells with and without tunicamycin and neuraminidase treatment. Capsaicin concentration-effect curve for untreated hTRPV1 expressed in HEK cells (*), tunicamycin treated hTRPV1-HEK (Δ) and neuraminidase treated hTRPV1-HEK (×). Results are expressed as the mean ± SEM of 3 to 4 experiments each performed in duplicate.

4.4 Discussion

The results from this chapter provide novel and convincing data that glycosylation and more specifically sialylation of TRPV1 modulates receptor function in response to capsaicin. Using calcium signalling we have shown pharmacologically, molecularly and genetically that TRPV1 lacking either its glycan or sialic acid is strikingly less responsive to capsaicin. Our findings uncover a novel mechanism whereby TRPV1 activation is regulated.

Initially western blot analysis with rTRPV1-Pro5, rTRPV1-Lec2, rTRPV1N604Q-Pro5 and rTRPV1N604Q-Lec2 cells was employed to confirm that rTRPV1 was glycosylated, however purifying enough of the channel proved difficult. Confocal microscopy may provide one explanation as to why the membrane preparations consistently failed to produce enough TRPV1. The receptor isolation method utilised in this thesis discarded the cytosolic fraction of the cells. It was presumed that the receptor would be largely at the cell surface, hence why crude membrane preparations were used. However, confocal results indicated that TRPV1 was uniformally distributed throughout the cell. Thus, in hindsight it would have been better perhaps to have collected whole cell lysates for subsequent western blot analysis. Nevertheless, western blot results were obtained for the rTRPV1-Lec2 and rTRPV1N604Q-Lec2 cell lines. The blot clearly shows a band for each cell line at a similar molecular weight suggesting the *N*-glycan on TRPV1 is not large, a finding consistent with previous reports (Jahnel et al., 2001, Kedei et al., 2001, Rosenbaum et al., 2002). Western blot analysis was also successful for rTRPV1E648A-HEK due to the exceptionally high levels of receptor expression in this cell line as depicted by the flow cytometry data (see Figure 3.5), calcium signalling (see Figure 3.4) and

confocal microscopy (see Figure 4.9). Comparison to previously published studies suggests the band at approximately 95 kDa may represent unglycosylated rTRPV1 and the band just over 100 kDa may represent glycosylated rTRPV1. Finally, the band at over 250 kDa may represent trimeric or tetrameric rTRPV1 (Kedei et al., 2001, Jahnel et al., 2001, Rosenbaum et al., 2002). The single band observed in the Lec2 cell lines appears to be greater than 100 kDa. The difference in weight between rTRPV1 in the Lec2 and HEK cell line may be due to cell line differences or the fact that the protein from the Lec2 cell lines was not reduced prior to separation on SDS polyacrylamide gel. Moreover, proteins lacking sialic acid (i.e. those isolated from the Lec2 cell line) may also appear to have a greater molecular mass on SDS PAGE because sialic acid confers a negative charge to proteins and thus may cause proteins to migrate towards the positive electrode faster than non sialylated proteins. Nevertheless, our results support previous reports that TRPV1 is not a heavily glycosylated protein.

We next utilised flow cytometry and confocal microscopy to assess the expression levels of each of the wild type and glycosylation mutant receptor cell lines. Flow cytometry data indicated that the rTRPV1-Lec2 and rTRPV1N604Q-Lec2 cell lines displayed a greater global TRPV1 expression than the rTRPV1-Pro5 cell line, suggesting that differences observed in calcium signalling are unlikely to be attributable to differences in receptor expression. Further, to assess whether tunicamycin altered TRPV1 expression levels, we employed the rTRPV1E648A-HEK cell line, since receptor levels could clearly be observed using flow cytometry. The results clearly indicate that tunicamycin treated rTRPV1E648A-HEK cells

exhibit similar expression levels to non treated cells, leading us to conclude that rTRPV1 expression is not significantly affected by removal of the *N*-linked glycan.

Confocal microscopy was then utilised to determine the cellular distribution of TRPV1 in each cell line. As described above the rTRPV1E648A-HEK cell line was used as a positive control. Interestingly, staining revealed rTRPV1 was expressed throughout the cell, not just at the plasma membrane. This is consistent with findings by others (Olah et al., 2001b, Hellwig et al., 2005, Jahnel et al., 2001) and confirm findings described in chapter 2 which suggested that TRPV1 was expressed on the plasma membrane as well as the membrane of intracellular stores. Surprisingly however, the intracellular distribution appeared to be throughout the cytosolic compartment and did not appear to be confined to specific regions. The same pattern of rTRPV1 staining was observed in rTRPV1-Pro5 cells. Further investigation is needed to show that the expression of the channel exhibits the same distribution in Lec2 cells or N604Q mutant cell lines. However, due to time constraints these experiments could not be performed before the writing of this thesis.

Following challenge with capsaicin, rTRPV1-Pro5 cells exhibited a robust concentration dependent increase in intracellular calcium. However, removal of the *N*-linked glycan either pharmacologically using tunicamycin or molecularly by mutation of the potential *N*-linked glycosylation site, N604Q, caused a significant reduction in the maximal capsaicin induced calcium influx. Further investigation revealed that it was not just removal of the glycosylation site, but more specifically the removal of the sialic acid on the *N*-linked glycan which could be important for

determining TRPV1 function in response to capsaicin. Removal of sialic acid from the wild type rTRPV1-Pro5 cell line by neuraminidase again caused a significant reduction in the maximal calcium response following capsaicin challenge, an observation which was confirmed genetically by expression of wild type TRPV1 in the mutant cell line Lec2, rTRPV1-Lec2. Finally, the importance of sialic acid and glycosylation on rTRPV1 function did not appear to be cell type specific as rTRPV1E648A-HEK cells also exhibited a significantly reduced capsaicin response following tunicamycin or neuraminidase treatment.

From the results presented here, it was not possible to assess whether the reduction in rTRPV1 function in the Lec2 cell lines was a result of a reduction in channel expression at the cell surface *per se*. Nevertheless, a number of lines of evidence clearly demonstrated that TRPV1 global expression in either pharmacologically treated or glycosylation mutant expressing cell lines was either the same or higher than in their respective controls. Firstly, western blot data showed bands for rTRPV1 in both rTRPV1-Lec2 and rTRPV1N604Q-Lec2 following preparation of crude membrane samples. However despite preparing samples from the same number of cells, no bands were observed for the rTRPV1 expressing Pro5 cell lines, despite greater responses to capsaicin in rTRPV1-Pro5. Secondly, flow cytometry data indicated that the rTRPV1 Lec2 cell lines had greater global expression than the Pro5 cell lines. Thirdly, the magnitude of capsaicin evoked response in tunicamycin and neuraminidase treated rTRPV1-Pro5 cells was comparable to the magnitude of response in the rTRPV1N604 and rTRPV1 Lec2 cell lines. The data therefore suggests that the rTRPV1 reduction in response to capsaicin in cell lines lacking the *N*-glycan or sialic acid is not due to loss of channel expression. The data even

suggests cell surface expression in these cell lines may be increased, a finding seen in other TRP channels lacking *N*-glycans (Xu et al., 2006). The reduction in response may therefore be due to impared agonist binding, impared cation channel transport throught the channel or impared folding of the channel. Further experimentation is required to decipher the exact cause of this reduction in capsaicin evoked response.

The effect of removal of the rTRPV1 *N*-linked glycosylation site on channel function has been investigated in two previous studies (Rosenbaum et al., 2002, Wirkner et al., 2005). Rosenbaum and colleagues performed the mutation N604S and using xenopus oocytes as an expression system showed that although the channel appeared to be glycosylated, unlike our data their results showed no significant change in function in response to capsaicin compared to the wild type channel (Rosenbaum et al., 2002). They concluded that glycosylation of TRPV1 has no effect on TRPV1 folding or targeting to the plasma membrane (Rosenbaum et al., 2002), a result consistent with our findings. Wirkner and colleague, on the other hand, showed HEK293 cells expressing the mutant rTRPV1N604T, like us, exhibited a significant reduction in the maximal response to capsaicin (Wirkner et al., 2005). However, in contrast to our findings they observed a slight leftward shift in the capsaicin concentration-effect curve (Wirkner et al., 2005). They also showed the mutant channel was more sensitive to low pH and low capsaicin concentrations and suggested that glycosylation may be involved in the regulation of pH-sensitivity of the channel (Wirkner et al., 2005). Our data pointing clearly to a significant role for sialylation *per se* in regulating TRPV1 activation by capsaicin has uncovered a novel mechanism of TRPV1 regulation.

Finally, we sought to determine whether glycosylation is important for hTRPV1 function. Tunicamycin and neuraminidase treatment of hTRPV1-HEK cells resulted in a similar reduction in the maximal obtainable response with capsaicin. The reduction in maximal response was similar to that obtained when extracellular calcium was removed from the assay buffer (see chapter 2). Thus, one would conclude that the drop in response in neuraminidase treated cells was a result of unresponsive TRPV1 at the cell surface, as found for rTRPV1, and that the remaining response was indicative of intracellular TRPV1 signalling. Further, the fact that tunicamycin treated cells were as responsive to capsaicin as neuramindase treated cells raises the question, does TRPV1 have to be fully glycosylated at the cell membrane to signal, but not require glycosylation to signal from internal stores? Detailed experiments are required to answer this question categorically, however, this is a likely scenario because we know that hTRPV1, unlike rTRPV1 does appear to signal from internal stores. Thus, we conclude that sialylation, also plays an pivotal role in regulating hTRPV1 in addition to rTRPV1.

Chapter 5

TRPV1 Upregulation by Inflammatory Mediators

5.1 Introduction

A number of recent studies have indicated that TRPV1 may be upregulated following nerve injury or in certain painful disease states (Hudson et al., 2001, Kanai et al., 2005, Rashid et al., 2003, Yiangou et al., 2001, Zhou et al., 2002). Using a number of animal models, Hudson and colleagues demonstrated that in rats TRPV1 was upregulated in undamaged afferent nerve fibres following nerve injury (Hudson et al., 2001). Using immunostaining they showed an increase in TRPV1 protein in undamaged neurons but not in the damaged nerves (Hudson et al., 2001). Interestingly Rashid and colleagues showed again using immunostaining following nerve injury, mice also exhibited an increase in TRPV1 protein expression, however the increase was seen mainly on A fibres previously lacking the channel (Rashid et al., 2003). This increase can also be seen following non injurious painful stimuli to the skin caused by chronic constriction (Kanai et al., 2005). Again TRPV1 was upregulated on sensory neurons as demonstrated by Western Blot analysis (Kanai et al., 2005). In rats TRPV1 upregulation has also been demonstrated by immunostaining in the dorsal horn of the spinal cord following spinal cord injury (Zhou et al., 2002).

TRPV1 upregulation has also been described in some painful, inflammatory disease states. Yiangou showed using immunostaining that TRPV1 appeared to be upregulated, compared to normal tissue, in nerve fibres from patients with inflammatory bowel syndrome, a disease which can often cause abdominal pain (Yiangou et al., 2001). TRPV1 upregulation has also recently been described in chronic cough using immunostaining. The levels of TRPV1 protein in human

airways smooth muscle have been shown in some cases to be higher than in normals (Mitchell et al., 2005).

It appears that following nerve injury or inflammation TRPV1 can be upregulated. It has been proposed that during inflammation TRPV1 is sensitised by mediators such as bradykinin and nerve growth factor, and now evidence is mounting that the amount of protein can also be increased following inflammation (Chuang et al., 2001, Hwang et al., 2000, Yiangou et al., 2001). This indicates that TRPV1 has an important role in inflammation and indeed it is well documented that inflamed tissue is more sensitive to heat and low pH, the TRPV1 agonists (Caterina et al., 1997). This is probably due to sensitised TRPV1 and may also therefore be due to an increase in the protein too.

Although much work has been done characterising TRPV1 on sensory neurons, it is now emerging that TRPV1 may also be expressed in non neuronal cells. Recently TRPV1 has been identified in keratinocytes as well as rat skeletal muscles, rat urethelial cells, human airway smooth muscle cells and human dental pulp fibroblasts (Mitchell et al., 2005, Inoue et al., 2002, Denda et al., 2001, Xin et al., 2005, Birder et al., 2001, Miyamoto et al., 2005). We have cloned human TRPV1 from MRC-5 cDNA, a cell line derived from human foetal lung fibroblasts. We therefore sought to determine whether primary human lung fibroblasts also express TRPV1 and if so whether the channel can be upregulated by inflammatory mediators. We have investigated not only mRNA expression but protein expression and channel function following human bronchial fibroblast exposure to three potent inflammatory

mediators, namely, tumour necrosis factor α (TNF-α), lipopolysaccharide (LPS) and interleukin 1 α (IL-1α).

5.2 Materials and Methods

5.2.1 Materials

The fibroblast growth medium kits including fibroblast basal medium, fibroblast growth supplements and 0.05mg gentamycin and 0.1μg amphotericin B were purchased from TCS Cell Works (Botolph Claydon, Buckinghamshire, UK). The rabbit anti-capsaicin receptor polyclonal antibody was bought from Chemicon International, Hampshire, UK. For all other reagents see section 2.2.1.

5.2.2 Culture of Human Primary Bronchial Fibroblasts.

Human primary bronchial fibroblasts (HPBF) were cultured using methods based on those by Akers and colleagues (Akers et al., 2000). Ethics approval was obtained from the Hull and East Riding local research ethics committee. Explants were taken from the conducting bronchi with informed consent from patients undergoing thoracotomy. The tissue was cut into pieces of less than 1 mm^3. These sections were placed into individual wells of a 24 well culture plate with DMEM containing 20% FBS, 1% penicillin/streptomycin and 100 μM sodium pyruvate. The medium was changed every three to five days. After two to three weeks, fibroblasts were observed growing from the bronchial tissue pieces. Confluent fibroblast cultures were harvested by trypsinisation and transferred to 75 cm^2 flasks containing selective human primary bronchial fibroblast medium (10 ml, TCS Cellworks). Confluent cultures were passaged with a split ratio of 1:3 and cells were used between passages 1-10 for all experiments. Purity of fibroblast cultures was established morphologically by observing the typical spindle shape of the cells and the characteristic swirls they formed. Immunohistochemical analysis was performed by

Mr. Rithwik Ramachandran by staining with a fibroblast specific markers (Rithwik Ramachandran PhD thesis). Two fibroblast markers were typically used, these were vimentin and prolyl 4-hydroxylase (D'Andrea et al., 2001, Vernet et al., 2002).

5.2.3 Upregulation of hTRPV1 mRNA

HPBF were grown to confluence in 6 well cell culture plates and were then allowed to quiesce for a further 48 hours in serum replacement medium (DMEM, 1x serum replacement (Sigma), 1% penicillin/streptomycin and sodium pyruvate). The cells were then treated with either IL-1α (10 ng/ml, for 0, 3, 6, 24, 48 or 72 hours), TNF-α (50 ng/ml, for 0, 3, 6, 24 or 48 hours), or LPS (100 ng/ml for 0, 3, 6, 24, 48 or 72 hours). mRNA was extracted using an RNeasy minikit (Qiagen, Hilden, Germany) according to manufacturer's instructions and then quantified using a GeneQuant™ spectrophotometer (Amersham Biosciences, Buckinghamshire, UK). cDNA was synthesised by reverse transcription of 2 μg of mRNA with an omniscript RT kit (Qiagen) and random primers (Invitrogen). The reverse transcription reaction was carried out at 24°C for 10 minutes, 37°C for 50 minutes and 95°C for 10 minutes. PCR was performed to compare mRNA levels as described in section 2.2.2.12 (primers sense 5'-TTCTTGTTCGGGTTTTCCAC-3' and antisense 5'-TCACTTCTCCCCGGAAGCGG-3'). A representative RT-PCR sample was sent sent to Kings College, London to confirm that it was TRPV1.

5.2.4 Flow Cytometry

Flow cytometry was used to compare the density of TRPV1 on IL1-α, LPS or TNF-α treated and untreated HPBF as described in section 3.2.6. Cells were grown to

confluence in 6 well plates in HPBF growth media. This media was replaced with serum free media for 24 hours before the cells were treated with IL-1α (10 ng/ml, for 0, 12, 24, 48, 72 or 96 hours), TNF-α (50 ng/ml, for 0, 3, 6, 12, 24 or 48 hours), or LPS (100 ng/ml for 0, 6, 12, 24, 48 or 72 hours) in serum media. At the end of the time course, cells were washed and harvested with cell dissociation buffer before incubation on ice in PBS with 4% formaldehyde for 30 minutes. The cells were then washed twice by centrifugation at 4°C with cold PBS followed by incubation on ice with rabbit anti-capsaicin receptor polyclonal antibody (1 in 400, chemicon international) in cold basal DMEM culture medium for 1 hour. The cells were again washed twice by centrifugation at 4°C with cold PBS and incubated on ice with anti-rabbit IgG FITC conjugated monoclonal antibody (1 in 100, sigma) for 45 minutes. The cells were then washed two more times by centrifugation at 4°C with cold PBS and then analysed using a Becton Dickinson Flow Cytometer.

5.2.5 Calcium Signalling

HPBF cells were seeded on 12 mm glass coverslips and grown to confluence in 2 ml of HPBF medium in 60 mm Petri dishes. Upon confluence the cells were grown for 24 hours in serum free media before treatment with IL-1α (10 ng/ml, for 0, 24, 48,72 or 96 hours), TNF-α (50 ng/ml, for 0, 6, 12, 24 or 48 hours), or LPS (100 ng/ml for 0, 12, 24, 48 or 72 hours) in serum free media. Following treatment for the correct time length, the cells were incubated in HPBF media containing 0.25 mM sulfinpyrazone and 2.5 mg/ml Fluo-3 acetoxymethyl ester (Cambridge Bioscience or later Invitrogen) for 25 minutes at 37°C. The coverslips were briefly washed in calcium assay buffer (CAB) (150 mM sodium chloride, 3 mM potassium chloride,

1.5 mM calcium chloride, 10 mM glucose, 20 mM HEPES, 0.25 mM sulfinpyrazone, pH 7.4) before mounting in a perfusion chamber (Warner instruments, CT, USA) and covering with 500 µl of calcium assay buffer. Cells were then stimulated with capsaicin (30 µM) or resiniferatoxin (30 nM) followed by calcium ionophore (A23187, 6 µM).

Increases in intracellular calcium were measured through a 20x objective using ImageMaster system software and DeltaRAM rapid wavelength-switching illuminator (Photon Technology International, London, ON, CA) with excitation at 480 nm and emission at 530 nm.

5.3 Results

5.3.1 TRPV1 mRNA is Upregulated Following Treatment of HPBF with TNF-α, LPS or IL-1α.

TNF-α treatment of HPBF induced upregulation of hTRPV1 mRNA as early as 3 hours however, significant increases were not seen until 48 hours (Figure 5.1). LPS caused upregulation of hTRPV1 mRNA as early as 6 hours, peaking at 48 hours (Figure 5.2). mRNA was still detected at the latest time point tested 48 hours (Figure 5.2). Following treatment with IL-1α, mRNA for hTRPV1 could be seen as early as 6 hours and increased over time until it plateaued at 48 hours. Of the eleven RT-PCRs carried out, just four showed any TRPV1 mRNA expression before treatment (data not shown). However, following incubation with each of the inflammatory mediators, TNF-α, LPS or IL-1α, hTRPV1 mRNA consistently appeared to be upregulated. The PCR fragment was at the expected size (~761 bp) and was sent for sequencing to Kings College, London, to confirm that the band was indeed part of hTRPV1. The bar charts in figures 5.1, 5.2 and 5.3 represent data from three to five separate RT-PCR reactions from cultures grown from samples from different patients.

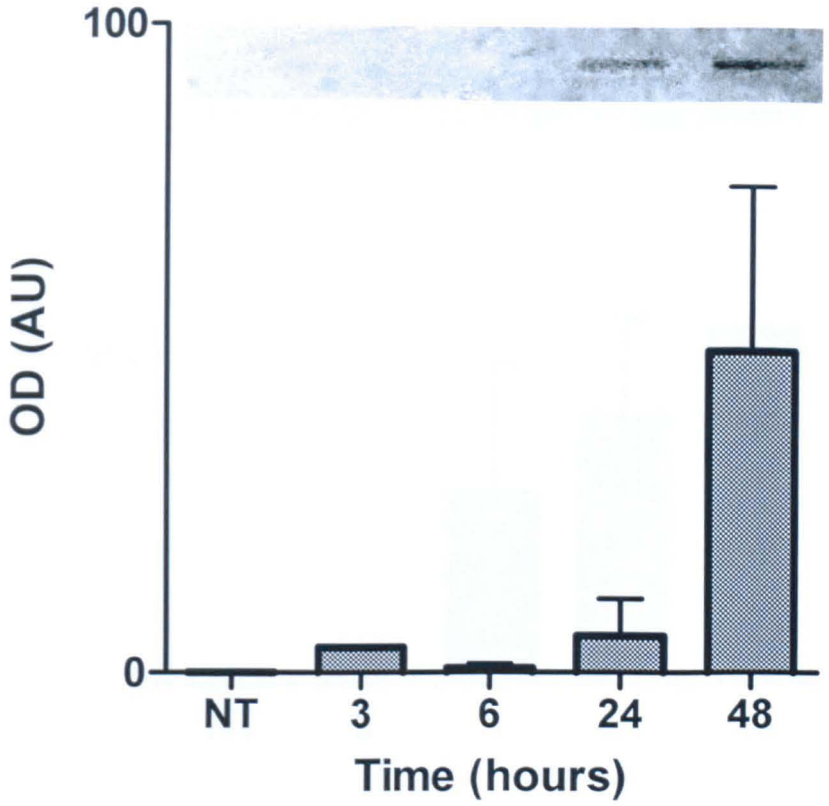


Figure 5.1. TRPV1 mRNA upregulation over 48 hours following TNF-α treatment. Top: Representative RT-PCR of hTRPV1 mRNA expression following TNF-α treatment. The product (~761 bp) was separated on a 1.3% agarose gel containing ethidium bromide and visualised under UV light. The picture is representative of three separate RT-PCR experiments from three different tissues. Bottom: The bar chart shows increases in TRPV1 mRNA above no treatment (NT) analysis was performed using Labworks imaging acquisition and analysis software. Results are expressed as the mean ± SEM of three RT-PCR reactions. OD, optical density. AU, arbitary units

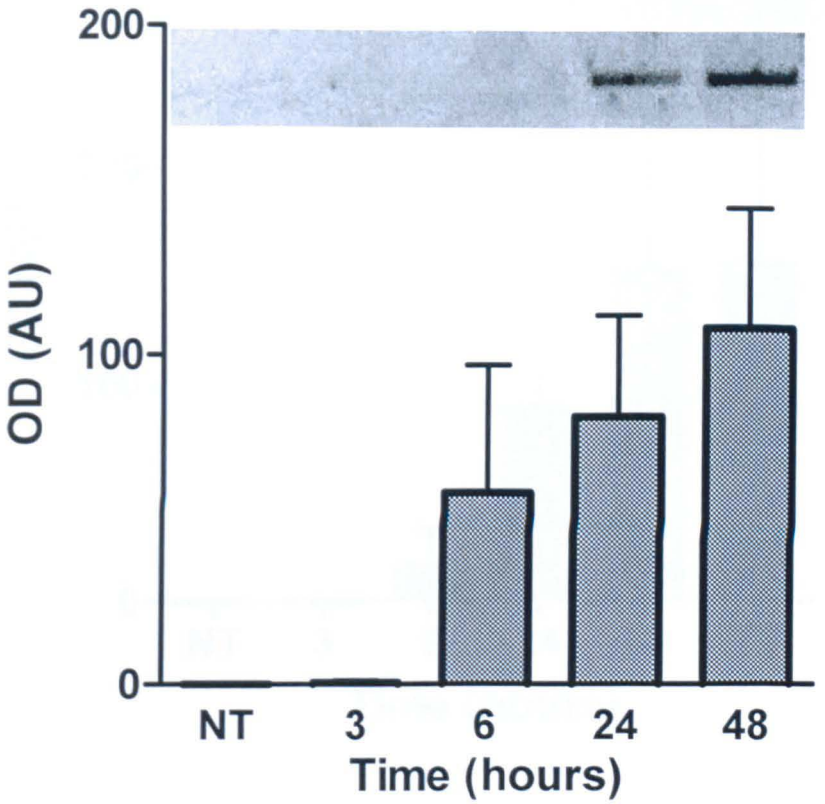


Figure 5.2. TRPV1 mRNA upregulation over 48 hours following LPS treatment. Top: Representative RT-PCR of hTRPV1mRNA expression following LPS treatment. The product (~761 bp) was separated on a 1.3% agarose gel containing ethidium bromide and visualised under UV light. The picture is representative of five separate RT-PCR experiments from five different tissues. Bottom: The bar chart shows increases in TRPV1 mRNA above no treatment (NT) analysis was performed using Labworks imaging acquisition and analysis software. Results are expressed as the mean ± SEM of five RT-PCR reactions. OD, optical density. AU, arbitary units

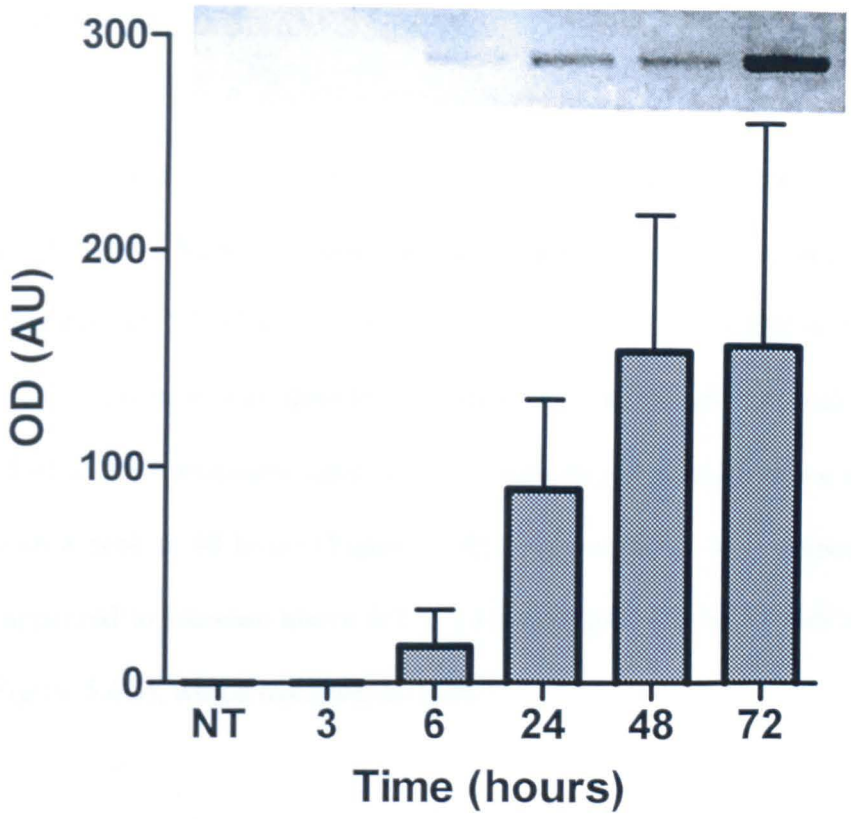


Figure 5.3. TRPV1 mRNA upregulation over 72 hours following IL1-α treatment. Top: Representative RT-PCR of hTRPV1 mRNA expression following IL-1α treatment. The product (~761 bp) was separated on a 1.3% agarose gel containing ethidium bromide and visualised under UV light. The picture is representative of three separate RT-PCR experiments from three different tissues. Bottom: The bar chart shows increases in TRPV1 mRNA above no treatment (NT) analysis was performed using Labworks imaging acquisition and analysis software. Results are expressed as the mean ± SEM of three RT-PCR reactions. OD, optical density. AU, arbitary units

5.3.2 TRPV1 Protein is Increased Following Treatment of HPBF with TNF-α, LPS or IL-1α.

Figures 5.4, 5.5 and 5.6 represent TRPV1 channel density on HPBF cells as shown by flow cytometry. Minor increases in fluorescence were detected in all the treated cells compared to NT (Figure 5.4A, 5.5A and 5.6A). For TNF-α treated cells increased fluorescence was detected as early as 3 hours with a peak at 6 hours (Figure 5.4B). LPS treatment again showed an increase in fluorescence as early as 6 hours, with a peak at 48 hours (Figure 5.5B). Following IL-1α treatment, hTRPV1 protein appeared to increase above NT at 12 hours post treatment with a peak at 72 hours (Figure 5.6B), which declined thereafter.

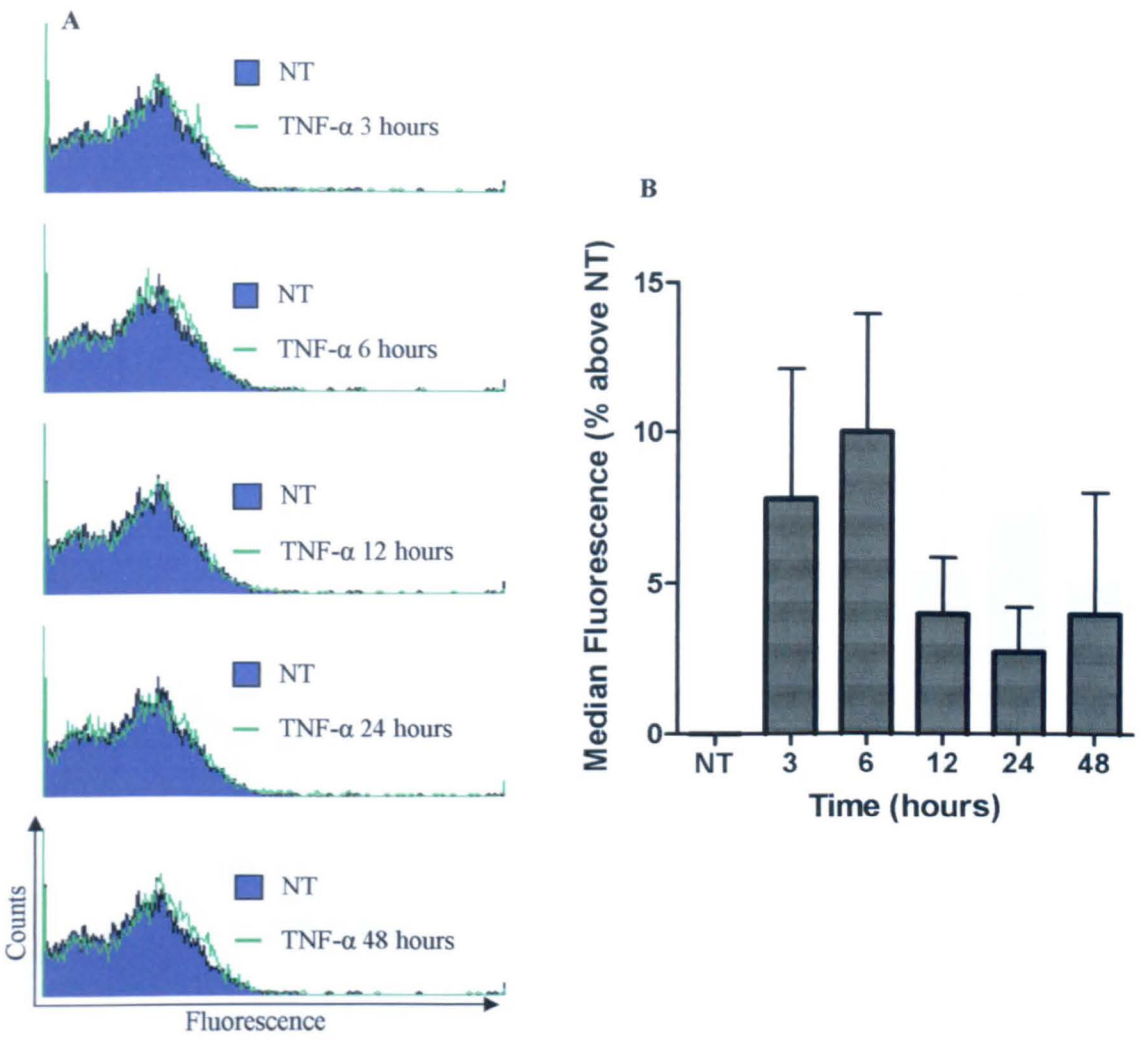


***Figure 5.4.* Flow Cytometry Data Showing TNF-α Upregulation of TRPV1 in HPBF.** (A) Representative flow cytometry traces of TRPV1 expression over 48 hours following TNF-α treatment. The trace is respresentative of four separate experiments. (B) Bar chart depicting change in HPBF TRPV1 expression, as assessed by flow cytometry over 48 hours following TNF-α treatment. Results are expressed as mean ± SEM of four separate flow cytometry experiments. NT, no treatment.

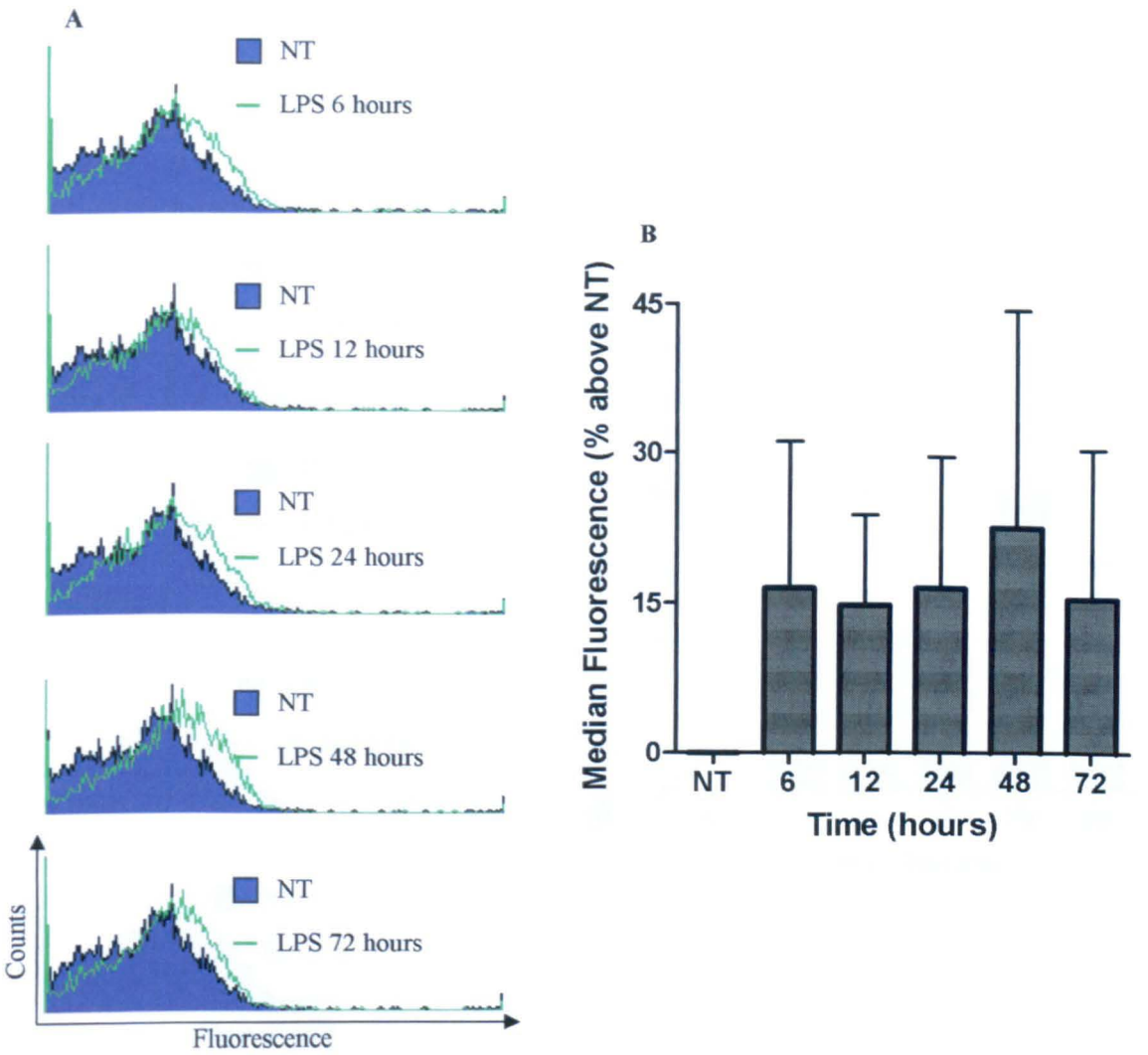


Figure 5.5. Flow Cytometry Data Showing LPS Upregulation of TRPV1 in HPBF. (A) Representative flow cytometry traces of TRPV1 expression over 72 hours following LPS treatment.. The trace is respresentative of four separate experiments. (B) Bar chart depicting change in HPBF TRPV1 expression, as assessed by flow cytometry over 72 hours following LPS treatment HPBF. Results are expressed as the mean ± SEM of four separate flow cytometry experiments. NT, no treatment.

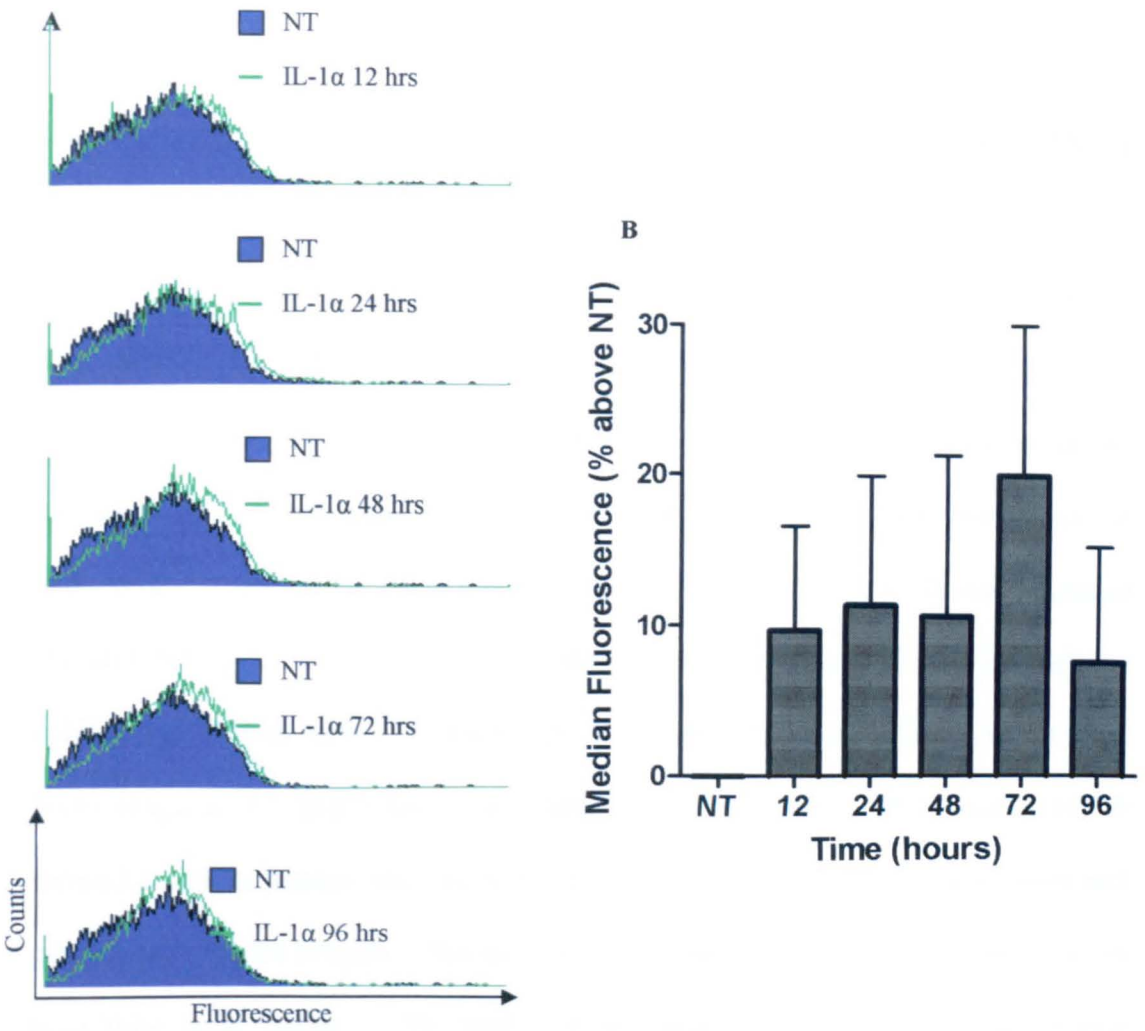


Figure 5.6. Flow Cytometry Data Showing IL-1α Upregulation of TRPV1 in HPBF. (A) Representative flow cytometry traces of TRPV1 expression over 96 hours following IL-1α treatment. The trace is respresentative of four separate experiments. (B) Bar chart depicting change in HPBF TRPV1 expression, as assessed by flow cytometry over 96 hours following IL-1α treatment. Results are expressed as the mean ± SEM of four separate experiments. NT, no treatment.

5.3.3 Functional TRPV1 is Increased Following Treatment of HPBF with TNF-α, LPS or IL-1α.

Comparisons of TRPV1 function were made for HPBF before and after treatment with either IL-1α, LPS or TNF-α. Representative traces for resiniferatoxin activation of HPBF TRPV1 over full TNF-α, LPS and IL-1α treatment time courses are shown in Figures 5.7, 5.9 and 5.11. Bar charts were also constructed for both resiniferatoxin and capsaicin activation of HPBF following TNF-α, LPS and IL-1α treatment (Figures 5.8, 5.10 and 5.12). Resiniferatoxin triggered increases in intracellular calcium in HPBF as early at 6 hours post TNF-α treatment with a peak detected at 24 hours (Figures 5.7 and 5.8A). Activation by capsaicin in TNF-α treated HPBF showed a similar pattern, with increases in intracellular calcium seen at 6 hours and peaking at 12 hours (Figure 5.8B) and remained responsive to capsaicin up to the 48 hour time point tested. LPS treated HPBF displayed increases in intracellular calcium following both resiniferatoxin (Figures 5.9 and 5.10A) and capsaicin (Figure 5.10B) challenge as early as 12 hours. The LPS treated HPBF remained responsive to capsaicin or resiniferatoxin upto the 72 hour time point tested. Treatment with IL-1α again showed increases in intracellular calcium following challenge with either resiniferatoxin or capsaicin from the earliest time point tested 24 hours (Figures 5.12A and 5.12B). The calcium signalling remained elevated above NT for the full time course tested (96 hours).

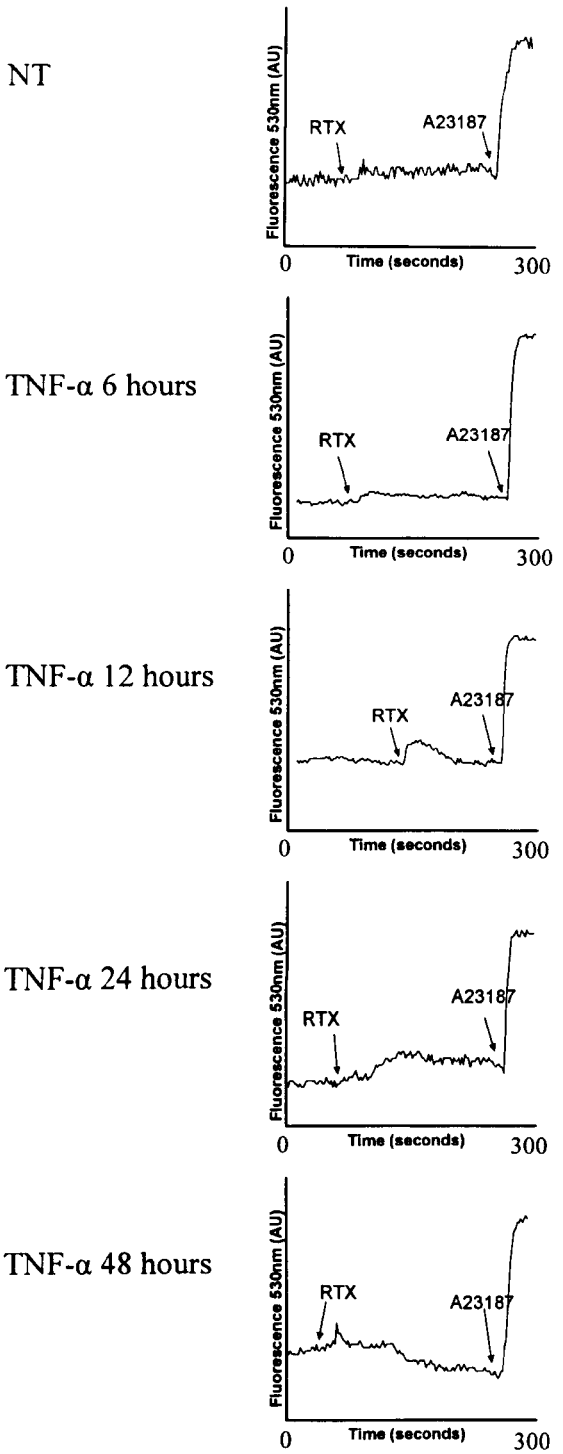


Figure 5.7. Induction of functional TRPV1 in TNF-α treated HPBF. Increases in intracellular calcium levels were detected following challenge of TNF-α (50 ng/ml) treated HPBF with resiniferatoxin (30 nM). These responses were compared to the maximum response evoked by calcium ionophore (A23187, 6 μM). Representative traces from four separate experiments.

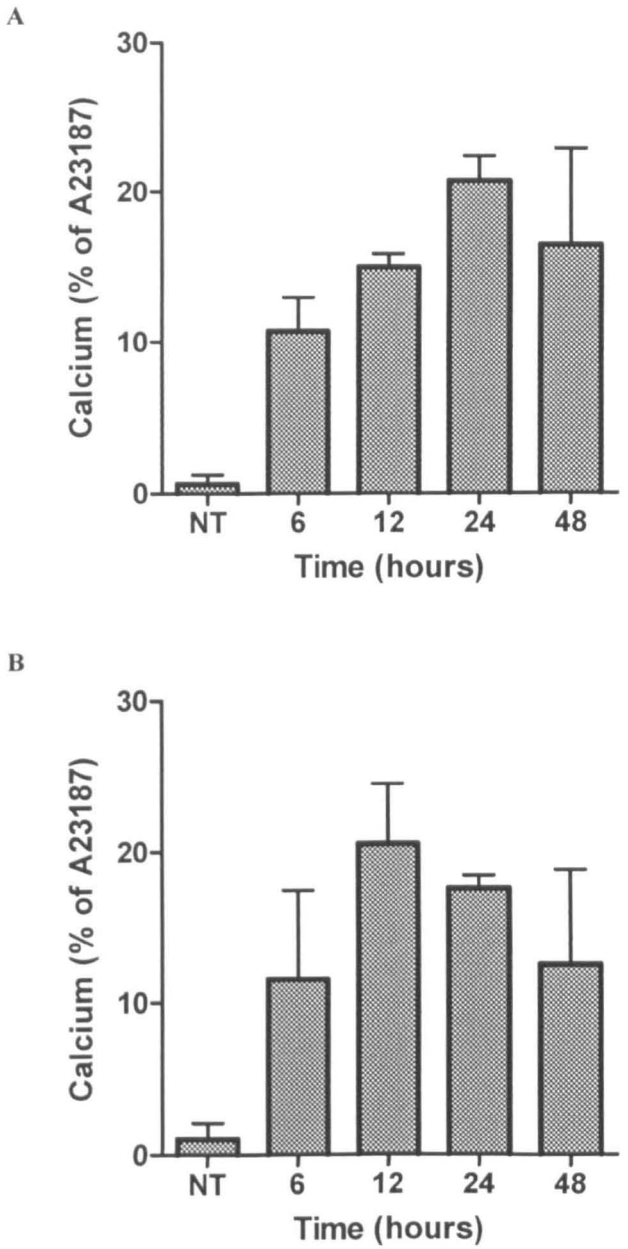


Figure 5.8. Resiniferatoxin and Capsaicin Evoked Calcium Signalling in TNF-α Treated HPBF. (A) Resiniferatoxin and (B) capsaicin evoked increases in intracellular calcium levels in TNF-α treated HPBF cells expressed as a percentage of the maximum (A23187). The results are expressed as mean ± SEM of 3 to 4 experiments. NT, no treatment.

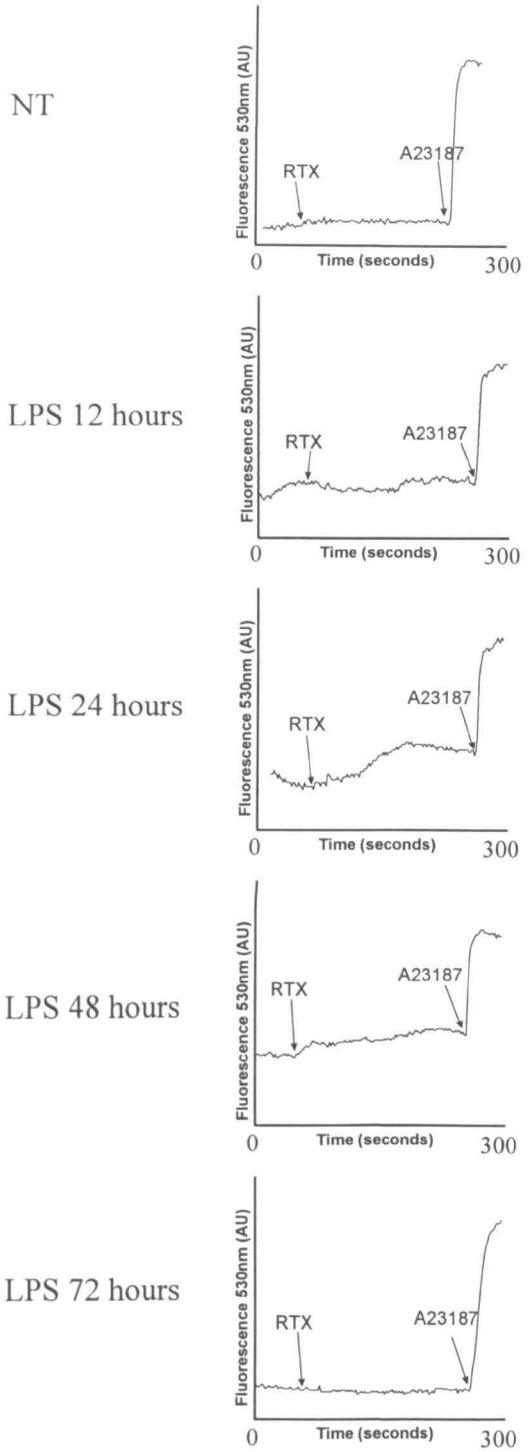


Figure 5.9. Induction of functional TRPV1 in LPS treated HPBF. Increases in intracellular calcium levels were detected following challenge of LPS (100 ng/ml) treated HPBF with resiniferatoxin (30 nM). These responses were compared to the maximum response evoked by calcium ionophore (A23187, 6 μM). Representative traces from three separate experiments.

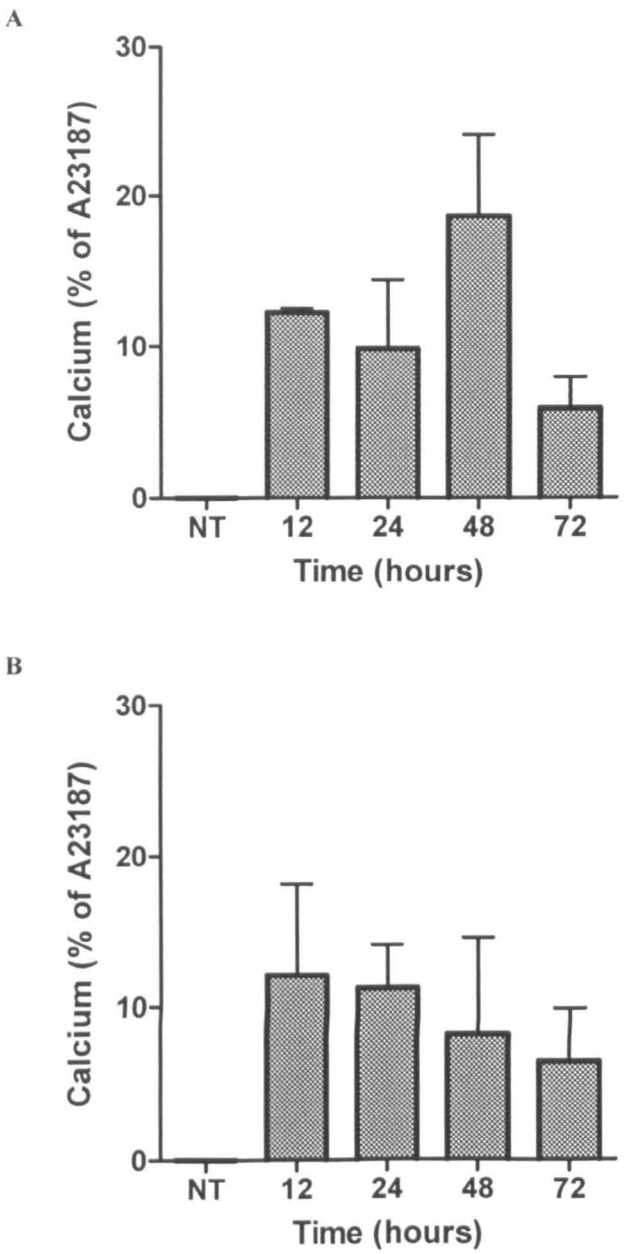


Figure 5.10. Resiniferatoxin and Capsaicin Evoked Calcium Signalling in LPS Treated HPBF. (A) Resiniferatoxin and (B) capsaicin evoked increases in intracellular calcium levels in LPS treated HPBF cells expressed as a percentage of the maximum (A23187). The results are expressed as mean ± SEM of 3 experiments. NT, no treatment.

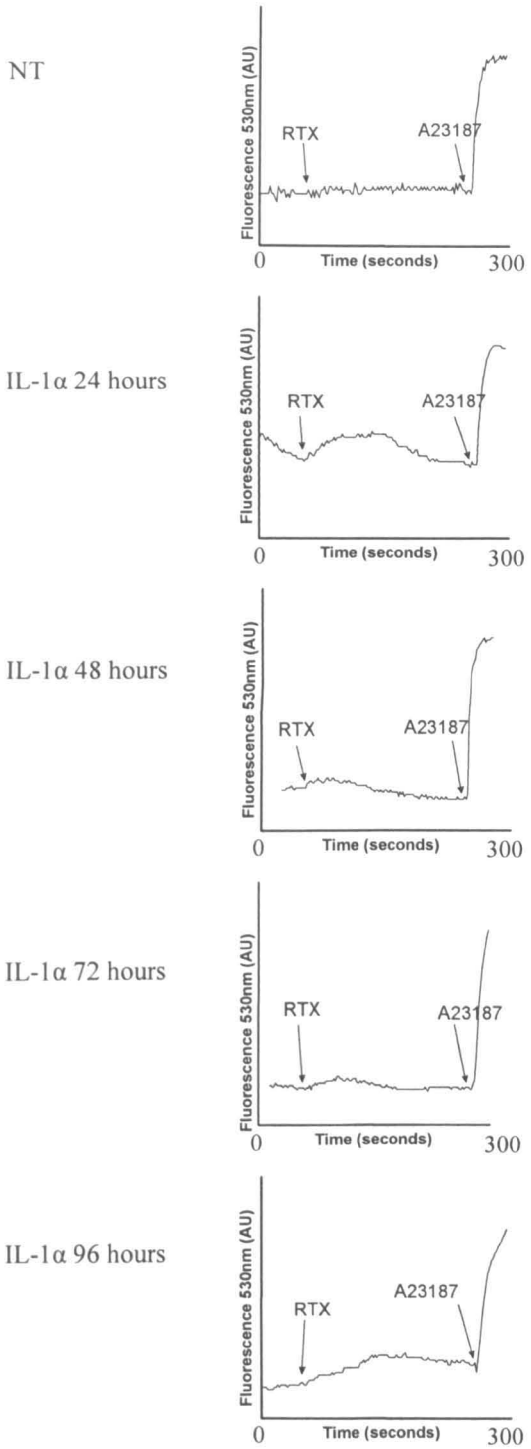


Figure 5.11. Induction of functional TRPV1 in IL-1α treated HPBF. Increases in intracellular calcium levels were detected following challenge of IL-1α (10 ng/ml) treated HPBF with resiniferatoxin (30 nM). These responses were compared to the maximum response evoked by calcium ionophore (A23187, 6 μM). Representative traces from four separate experiments.

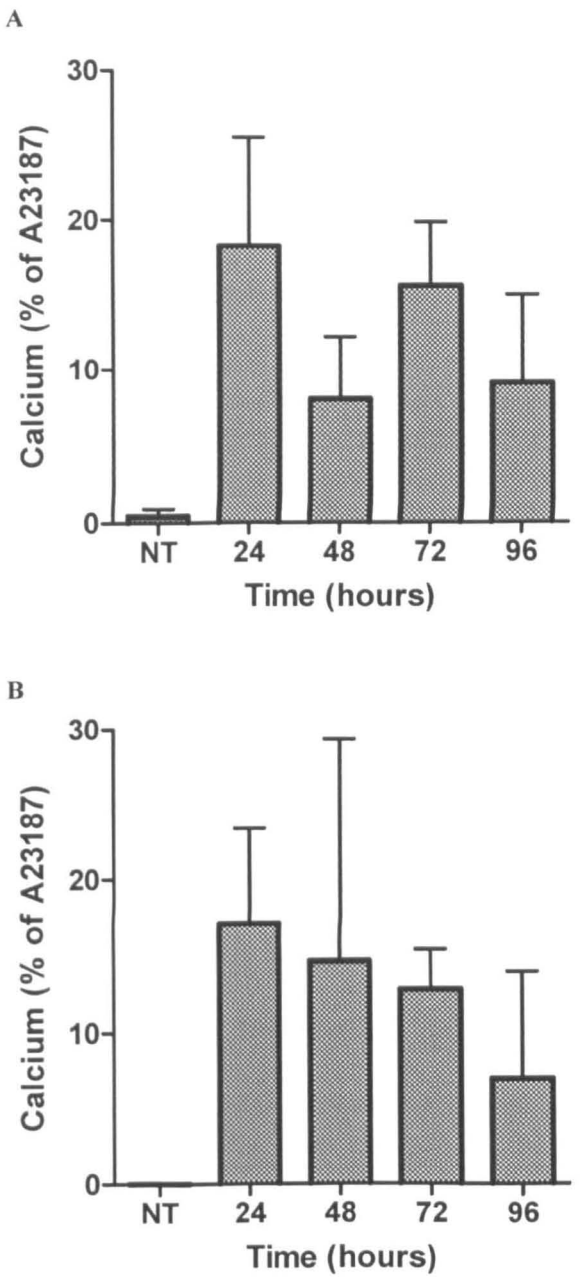


Figure 5.12. Resiniferatoxin and Capsaicin Evoked Calcium Signalling in IL-1α Treated HPBF. (A) Resiniferatoxin and (B) capsaicin evoked increases in intracellular calcium levels in IL-1α treated HPBF cells expressed as a percentage of the maximum (A23187). The results are expressed as mean ± SEM of 3 to 5 experiments.

5.4 Discussion

We have shown here for the first time that TRPV1 expression in HPBF can be induced following incubation with the inflammatory mediators TNF-α, LPS or IL-1α. Such findings may provide a novel insight into the potential role of TRPV1 in airways disease where inflammation is an important factor.

hTRPV1 in this report was initially cloned from MRC5 cDNA a cell line derived from human foetal lung fibroblast cells. Since there were no reports at the time of TRPV1 expression in lung fibroblasts, we therefore sought to determine whether primary human lung fibroblasts express TRPV1 and if so, could TRPV1 be upregulated by inflammatory mediators? Our finding that HPBF can express TRPV1, adds to the emerging data which suggests that TRPV1 is not confined to sensory neurons as was initially thought (Caterina et al., 1997). For example, TRPV1 has been detected on human keratinocytes, rat skeletal muscle cells and rat urothelial cells (Birder et al., 2001, Denda et al., 2001, Inoue et al., 2002, Xin et al., 2005). More interestingly TRPV1 has very recently been detected in human dental pulp fibroblasts (Miyamoto et al., 2005). However this is the first report that TRPV1 expression can be induced by inflammatory mediators in either non-neuronal or neuronal cells.

RT-PCR and calcium signalling experiments consistently showed increased TRPV1 mRNA and function respectively in TNF-α, LPS or IL-1α treated HPBF. However flow cytometry once again proved difficult. Although plotting increases in median fluorescence suggested treated cells exhibited an increased fluorescence, implying an increase in TRPV1 protein, increases were small and not statistically significant.

This is probably due to the low specificity of the TRPV1 polyclonal antibody which has been a reoccurring problem throughout this thesis. The antibody cannot detect low levels of TRPV1 expression, however when TRPV1 levels are high, flow cytometry has successfully been used to detect TRPV1 using this polyclonal antibody (see Chapter 3, Figure 3.5).

Although clear upregulation of TRPV1 was observed in HPBF, considerable variability was also noted. The degree of variablilty between cultures was attributed to 1) the magnitude of TRPV1 induction by inflammatory mediators and 2) endogenous expression of TRPV1 in some cultures. The tissue samples donated were from patients where the only information known was that they were undergoing a thoracotomy. No data about lung diseases including asthma or chronic cough were given nor was data indicating the patients' sex, age or smoking habits. As has been shown here, TRPV1 can be upregulated during inflammation, therefore if the tissue was taken from lungs which were already inflamed this could influence expression of proteins, possibly including TRPV1. Most of the HPBF cultures used in this study did not endogenously express TRPV1. However, the HPBF that did endogenously express TRPV1 may be indicative of inflamed airways. If this is the case it would be interesting to determine whether those endogenously expressing the channel had hightened sensitivity to capsaicin or citric acid or suffered from an inflammatory lung disease or chronic cough.

By immunostaining, it has been suggested previously by our group, that TRPV1 expression is increased in airways smooth muscle cells in patients suffering from chronic cough compared to normal airways (Mitchell et al., 2005). In this report,

only one out of twenty one of the normal patient samples showed positive staining for TRPV1 whereas eleven out of nineteen of the chronic cough samples showed staining from TRPV1. This report is therefore consistent with the results from our cultured fibroblasts. Firstly, samples endogenously expressing TRPV1 may have been taken from patients suffering from inflamed lungs or chronic cough and secondly, it shows a difference in TRPV1 during inflammation, as shown by our HPBF following inflammatory mediator treatment. Increases in TRPV1 during inflammation, have also been suggested previously where tissue samples from patients with inflammatory bowl disease displayed greater immunoreactivity compared to controls (Yiangou et al., 2001). As well as detecting increased TRPV1 in disease states upregulation of the channel has also been suggested previously in nerve fibres following injury (Hudson et al., 2001, Kanai et al., 2005, Mitchell et al., 2005, Rashid et al., 2003, Yiangou et al., 2001, Zhou et al., 2002). The recent report that TRPV1 activation induces IL-6 release from dental pulp fibroblasts further implicates this channel in the inflammatory process (Miyamoto et al., 2005). The results in this chapter highlight the ability of TRPV1 expression to be directly induced by inflammatory mediators and thus its potentially important role in the inflammatory process.

Chapter 6

General Discussion

Evidence has been provided in this thesis which adds to the current understanding of TRPV1 regulation and activation. Both rTRPV1 and hTRPV1 were found to be expressed on intracellular calcium stores, however, rTRPV1 is expressed on thapsigargin sensitive stores, where as hTRPV1 is expressed on thapsigargin insensitive intracellular stores. Using pharmacological and molecular methods it has been demonstrated that citric acid, an agent commonly used for inhalation cough challenges (Morice et al., 2001), directly activates TRPV1 by a proton dependent mechanism. Again using molecular and pharmacological techniques, TRPV1 sialylation was shown to regulate capsaicin evoked responses of both rTRPV1 and hTRPV1. Finally, TRPV1 expression was induced in HPBF following treatment of the cells with the potent inflammatory mediators TNF-α, LPS and IL-1α.

6.1 Cloning and Characterisation of rTRPV1 and hTRPV1

In order to study rTRPV1 and hTRPV1, both channels were cloned and permanently expressed in a cell line which did not endogenously express the channel, HEK293 cells. Characterisation of both channels revealed that they conformed to the known pharmacology of the channel responding in a concentration dependent manner to both capsaicin and resiniferatoxin (Caterina et al., 1997, Cortright et al., 2001, Hayes et al., 2000, Jerman et al., 2000, Smart et al., 2001, Witte et al., 2002). Resiniferatoxin was shown to be more potent than capsaicin, and displayed different activation kinetics. Resiniferatoxin, at a concentration which activated the channels to the same degree as capsaicin (determined by expression as a percentage of calcium ionophore, A23187) produced a slower onset of channel activation than the less potent agonist, capsaicin. The two TRPV1 specific antagonists, capsazepine and iodoresiniferatoxin also inhibited the capsaicin evoked responses of the cloned

channel. These characteristics conform to the pharmacology of the channel shown in previously published literature (Caterina et al., 1997, Jerman et al., 2000, Toth et al., 2005, Witte et al., 2002).

6.2 rTRPV1 and hTRPV1 Expression on Intracellular Stores

Having characterised the two channels, the location of TRPV1 within the cell was investigated, and we have reported that rTRPV1 and hTRPV1 are present on intracellular calcium stores as well as the plasma membrane (Mitchell et al., 2005). This finding is consistent with the current literature (Karai et al., 2004, Liu et al., 2003, Olah et al., 2001b, Turner et al., 2003, Wisnoskey et al., 2003). Interestingly, Xin and colleagues have recently shown that TRPV1 is expressed on rat skeletal muscle but mainly on the sarcoplasmic reticulum (Xin et al., 2005). Experiments with and without extracellular calcium revealed that both rTRPV1 and hTRPV1 still responded to capsaicin in a concentration dependent manner, a response which was antagonised by capsazepine in rTRPV1. This suggests that both rat and human TRPV1 are expressed not only on the plasma membrane but on the membrane of some intracellular stores. For the rat channel this is consistent with published literature (Karai et al., 2004, Liu et al., 2003, Olah et al., 2001b, Turner et al., 2003, Wisnoskey et al., 2003) with intracellular hTRPV1 being reported by us (Mitchell et al., 2005). The response of hTRPV1 to capsaicin in the absence of extracellular calcium was of a greater magnitude than in rTRPV1, therefore suggesting that the majority of functional rTRPV1 is expressed at the cell surface and functional hTRPV1 is found both intracellularly and at the cell surface

The use of thapsigargin, an endoplasmic reticulum calcium ATPase inhibitor, revealed rTRPV1 was expressed on thapsigargin sensitive stores, most likely the endoplasmic reticulum. In addition we found that hTRPV1 appeared to be expressed on thapsigargin insensitive stores (Mitchell et al., 2005). All of the organelles of the cell are now thought to be able to store calcium (Michelangeli et al., 2005), therefore the exact location of intracellular hTRPV1 cannot currently be deduced but the results shown here seem to eliminate the endoplasmic reticulum. The literature regarding rTRPV1 expression on the endoplasmic reticulum is currently confusing and there is debate as to whether the channel is expressed on thapsigargin sensitive (Kanai et al., 2005, Wisnoskey et al., 2003) or insensitive stores (Liu et al., 2003, Turner et al., 2003). However the results in this thesis demonstrate that intracellular rTRPV1 is expressed on thapsigargin sensitive stores (Mitchell et al., 2005). Further work is required to determine the exact localisation of hTRPV1 within the cell. Confocal microscopy could be utilised using TRPV1 antibodies and organelle specific markers.

6.3 Citric Acid Activation of rTRPV1

The data within this thesis provide the first evidence that citric acid directly activates TRPV1. Our findings that citric acid can activate rTRPV1 is consistent with a study carried out by Kollarik and colleagues in guinea pig trachea/ bronchus afferent nerves. In their study, they reported that citric acid evoked responses in C-fibres in a TRPV1 dependent manner (Kollarik and Undem, 2002). Consistent with Kollarick, inhalation cough challenges with guinea pigs have demonstrated that both citric acid and capsaicin evoked cough could be inhibited by capsazepine and iodoresiniferatoxin, again suggesting that citric acid evokes cough via TRPV1

(Trevisani et al., 2004, Lalloo et al., 1995). In our study however, iodoresiniferatoxin but not capsazepine inhibited citric acid evoked responses. This is consistent with previously published data which shows capsazepine can antagonise hTRPV1 and guinea pig TRPV1, but not rTRPV1 proton activation (McIntyre et al., 2001, Savidge et al., 2002, Gavva et al., 2005a), yet iodoresiniferatoxin is capable of inhibiting both rTRPV1 and hTRPV1 (Seabrook et al., 2002). We further confirmed the importance of protons in citric acid activation of TRPV1 when we mutated one of the potential proton binding sites (E648 to A648), which subsequently removed the channels ability to respond to citric acid. This is consistent with the findings of Jordt who showed removal of E648 inhibited proton activation of TRPV1 without affecting capsaicin responses (Jordt et al., 2000).

Activation of TRPV1 by low pH may also be of importance during inflammation (Bevan and Geppetti, 1994). Inflammation is often characterised by a reduction in the pH in affected tissues (Bevan and Geppetti, 1994) and the pH of exhaled airway vapour from asthmatic patients is thought to be over two log orders lower than in non asthmatics (~pH 5.2 for asthmatics compared to ~pH 7.7 in normals) (Hunt et al., 2000). This pH lowering may therefore sensitise or activate TRPV1 (Caterina et al., 1997, Tominaga et al., 1998) and may provide an explanation for the prevalence of cough as a major symptom of asthma and other inflammatory lung dieases (Corrao et al., 1979). Activation of TRPV1 by low pH may also provide a mechanism by which GOR causes cough, by reflux of acidic stomach contents into the airways (Everett and Morice, 2004).

6.4 Role of *N*-Glycosylation and Sialylation on rTRPV1

Previous studies have shown that rTRPV1 is expressed in both the glycosylated and unglycosylated forms, however reports regarding the effects of *N*-linked gylcans on channel function are conflicting (Jahnel et al., 2001, Kedei et al., 2001, Rosenbaum et al., 2002, Wirkner et al., 2005).

Consistent with Wirkner and colleagues, but in contrast to Rosenbaum, mutation to remove the potential glycosylation site of rTRPV1 caused a significant reduction in the maximal response of the channel to capsaicin (Rosenbaum et al., 2002, Wirkner et al., 2005). Further to this, our results demonstrate that this loss of response is due more specifically to loss of sialic acid. Despite the significant loss of capsaicin induced response following deglycosylation or sialic acid removal, the global expression of the channel remained unchanged, as determined by flow cytometry of permeabolised tunicamycin treated and untreated rTRPV1E648A-HEK cells. Although the exact localisation of the channel requires confirmation by confocal microscopy with rTRPV1N604Q-Pro5 and rTRPV1-Lec2 cells, it appears that TRPV1 production is not affected.

Removal of sialic acid from cell surface rTRPV1 by neuraminidase caused a similar response to removal of global sialic acid by expression of TRPV1 in Lec2 cells, or by removal of the *N*-linked glycan by tunicamycin or site directed mutagenesis. Thus, if neuraminidase treated cells respond similarly to capsaicin as cells lacking all sialic acid (i.e. Lec2 cells), the results suggest that intracellular rTRPV1 is not sensitive to capsaicin. Despite seemingly high expression of intracellular rTRPV1 as seen with confocal microscopy, the response of intracellular rTRPV1 to capsaicin is

fairly small as demonstrated by removal of extracellular calcium. It therefore appears that removal of sialic acid has a significant effect on rTRPV1 function in response to capsaicin and therefore suggests that sialylation is a novel mechanism of TRPV1 regulation. Sialic acid regulation of TRPV1 may have important consequences during infection by neuraminidase-producing viruses, such as influenza or in painful inflammatory condition such as rheumatoid arthritis where modifications in the expression of the glycosylation machinery have been detected (Axford, 1999).

Pretreatment of hTRPV1-HEK with either neuraminidase or tunicamycin caused an equal reduction in the channels response to capsaicin, confirming the observation made with the rTRPV1, and suggesting that again sialic acid plays a major role in TRPV1 activation by capsaicin. Interestingly, the reduction in response to capsaicin for hTRPV1 following neuraminidase or tunicamycin treatment was of a smaller magnitude than that seen in the rat channel. This is consistent with functional hTRPV1 expression on intracellular stores, as indicated by the observation that hTRPV1 was less sensitive to depletion of extracellular calcium than rTRPV1. Our data indicates that intracellular hTRPV1 is more responsive to capsaicin than intracellular rTRPV1. Thus, channel sialylation is important for regulating both human and rat TRPV1 activation at least by capsaicin.

Although it is likely that many more of the TRP channels are glycosylated (Apweiler et al., 1999), currently the literature only describes glycosylation in TRPV2, TRPV4, TRPC3 and TRPC6 (Xu et al., 2006, Dietrich et al., 2003, Jahnel et al., 2003). Jahnel and colleagues showed that the over expressed TRPV2 is present in both

glycosylated and unglycosylated forms whereas F-11 endogenously expressed TRPV2 is only present in the unglycosylated form, confined to intracellular stores (Jahnel et al., 2003). The role of sialic acid was not investigated in this study, however, the results suggested that TRPV2, required glycosylation to target the protein to the plasma membrane (Jahnel et al., 2003). In contrast, the results of our study suggest that glycosylation is not essential for targeting of TRPV1 to the plasma membrane a finding which was also reported for TRPC6 (Dietrich et al., 2003). Like TRPV1, the glycosylation site of TRPV2 is located extracellularly between transmembrane domain five and the pore loop (Jahnel et al., 2003). No comparison of TRPV2 function between *N*-glycosylated and unglycosylated forms has been performed and it would be interesting to determine whether glycosylation and sialic acid affected TRPV2 function to a similar degree as we have observed with TRPV1.

6.5 TRPV1 Upregulation by Inflammatory Mediators

It is now emerging that unlike initially thought, TRPV1 is expressed not only on neuronal tissue but also on some other cell types including human keratinocytes, dental pulp fibroblasts and rat skeletal muscle cells (Denda et al., 2001, Inoue et al., 2002, Xin et al., 2005, Miyamoto et al., 2005). Upregulation of TRPV1 has been shown previously by immunostaining or western blot analysis following nerve injury (Hudson et al., 2001, Rashid et al., 2003, Zhou et al., 2002) and an increase in TRPV1 protein was detected by immunostaining in patients with inflammatory bowl syndrome compared to normals (Yiangou et al., 2001). More recently increased cell surface expression of TRPV1 following nerve growth factor (NGF) treatment has been demonstrated (Zhang et al., 2005). In this thesis upregulation of TRPV1 in human primary bronchial fibroblasts was shown from mRNA levels through to

function following incubation with the inflammatory mediators TNF-α, LPS and IL-1α. We have therefore shown for the first time that human lung fibroblasts can be induced to express functional TRPV1, during inflammatory conditions. This finding has important implications and is consistent with the findings of Mitchell *et al* which suggest that tissue from chronic cough patients displayed increased TRPV1 expression (Mitchell et al., 2005). It is also consistent with increased expression of TRPV1 in inflammatory bowl syndrome (Yiangou et al., 2001). This data adds to the understanding of the already complicated regulation mechanisms of TRPV1. The ability of human lung fibroblasts to upregulate TRPV1 during inflammation as well as sensitise the channel under inflammatory conditions by inflammatory mediators such as bradykinin, or even activation of the channel by lipoxygenase products may explain why in inflammatory lung diseases such as asthma, coughing is a prevalent symptom (Hwang et al., 2000). Further work is required to determine the exact mechanisms of TRPV1 upregulation. Previous studies have suggested that the MAPK pathway may be involved (Bron et al., 2003, Ji et al., 2002) and it is known that all three inflammatory mediators used in the study, TNF-α, LPS and IL-1α, can cause upregulation of other inflammatory proteins via this mechanism (Hopkins and Sriskandan, 2005, Liu, 2005, Palsson-McDermott and O'Neill, 2004).

The complexities of TRPV1 activity regulation are now beginning to be uncovered and it has even been suggested that regulation of TRPV1 may be one of the most complex examples (De Petrocellis and Di Marzo, 2005). Section 1.5 describes the current known regulators of TRPV1, however in this thesis we have provided evidence adding to the known activators and regulators of the channels function.

It is well established that TRPV1 is activated by protons (Caterina et al., 1997, Jordt et al., 2000, Tominaga et al., 1998), and a number of studies have investigated the activation of the channel in nerve fibres and whole animals by citric acid, the agent used routinely in the investigation of the cough reflex (Kollarik and Undem, 2002, Tanaka and Maruyama, 2005, Trevisani et al., 2004), however the molecular mechanism whereby citric acid stimulated TRPV1 was not yet fully understood. Confirming findings in nerve fibres and whole animals the present study showed citic acid actived rTRPV1. Using site directed mutagenesis we demonstrated the responses were caused by proton activation of the channel and responses were antagonised by iodoresiniferatoxin but not capsazepine. Again this is consistent with the current literature which shows that resiniferatoxin inhibits proton activation of rTRPV1 but capsazepine does not and further suggests citric acid activation of rTRPV1 is proton dependent (Gavva et al., 2005a, McIntyre et al., 2001, Phillips et al., 2004, Savidge et al., 2002, Seabrook et al., 2002). To date TRPV1 is the only TRP channel known to be activated by protons, however, as their name suggests ASIC are activated by protons, and ASIC1a and ASIC3 are co-localised with TRPV1 in rat neurons (Ugawa et al., 2005).

Preliminary, conflicting studies have investigated the role of glycosylation on rTRPV1 function (Rosenbaum et al., 2002, Wirkner et al., 2005), however, the role of sialylation on TRPV1 was unknown. Our data suggested that glycosylation, and more specifically sialylation affected rTRPV1 function in response to capsaicin. The pharmacological, molecular and genetic data indicated that although there did not appear to be a reduction in cell surface, or global expression of the channel in the

absence of sialylation compared to the wild type channel, the function of the channel was reduced. Rosenbaum and colleague saw no change in channel function and concluded that glycosylation of TRPV1 has no effect on TRPV1 folding or targeting to the plasma membrane (Rosenbaum et al., 2002). Wirkner and colleague, on the other hand, showed HEK293 cells expressing the mutant rTRPV1N604T, like us, exhibited a significant reduction in the maximal response to capsaicin (Wirkner et al., 2005). From the results shown in this thesis it is difficult to conclude why TRPV1 function is impared in the absence of sialic acid and continued experimentation will hopefully clarify the mechanism, however the results suggest that the channel is still able to reach the cell surface and so trafficking is not impared. We have demonstrated that rTRPV1 is expressed on intracellular stores in chapter 2 and by confocal microscopy in chapter 4. However, signalling in response to capsaicin from intracellular rTRPV1 was much reduced. In the case of hTRPV1, cells treated with neuraminidase or tunicamycin also displayed a reduction in capsaicin evoked response, however the reduction in response was less than that demonstrated in rTRPV1. In chapter 2 we demonstrated that hTRPV1 was also expressed on intracellular stores and signalling from these stores in response to capsaicin was of a greater magnitude than rTRPV1. Although no conclusions can be made and further experimentation is required, the data may suggest that TRPV1 has to be fully glycosylated at the cell membrane to signal, but does not require glycosylation to signal from internal stores.

There is little information on the role of sialylation on TRP channels, however some work has been published on the role of glycosylation on TRP channels. In contrast to TRPV1, TRPV4, the osmotically sensitive channel, displayed an increase in

calcium response following hypotonic stress in cells expressing the channel lacking *N*-linked glycan N651 (Xu et al., 2006). This increase was attributed to increased cell surface expression of the channel compared to the wild type TRPV4 expressing cells. This finding suggests that as the TRPV4 glycosylation site is in a similar position to the TRPV1 glycosylation site (near the P-loop, see figure 1.3), the TRPV1 glycosylation site does indeed play an important role in channel function. The role of sialylation on TRPV4 function was not investigated by Xu and colleagues, and it would have been interesting to determine, like TRPV1, whether it was in fact sialylation that affected channel function, rather than glycosylation (Xu et al., 2006).

The role of TRPV1 regulation by sialylation and glycosylation in disease states cannot be deciphered from the evidence presented here, however, impared glycosylation and sialylation has been implicated in a number of disorders including cystic fibrosis and influenza (Kube et al., 2001, Nayak et al., 2004). TRPV1 is involved in inflammation, pain and cough, therefore removal of *N*-linked glycosylation or sialic acid may have implications for diseases or illnesses where these symptoms are prevelant.

We have shown here for the first time that hTRPV1 expression in primary bronchial fibroblasts can be induced following exposure to inflammatory mediators. There is little information regarding induction of TRP channels following inflammation. It has been demonstrated that TRPA1 DRG mRNA can be upregulated following challenge with inflammatory mediators (Obata et al., 2005) and a number of studies have shown an increase in TRPV1 protein following injury or during some

inflammatory diseases (Hudson et al., 2001, Kanai et al., 2005, Mitchell et al., 2005, Rashid et al., 2003, Yiangou et al., 2001, Zhou et al., 2002) however the current study describes induction of TRP expression for the first time and suggests another possible mechanism of TRPV1 regulation. TRPV1 may therefore play an even more important role in inflammation than previously thought. As described in section 1.5.3, BK and NGF produced during inflammation sensitise TRPV1, lowering the activation threshold of the channel, thus causing pain in the inflammed tissue. Protons produced during inflammation are also known TRPV1 agonists, thus the channel is activated by protons during inflammation (Caterina et al., 1997, Tominaga et al., 1998). Added to this, the finding of this thesis suggests TRPV1 may also be induced and upregulated under inflammatory conditions. Therefore during inflammation TRPV1 can be sensitised, directly activated and expression can be upregulated. Together these characteristics of TRPV1 explain how inflammed tissues cause pain and further support the idea that TRPV1 is an ideal target for drugs to relieve pain caused by inflammation.

In conclusion, this thesis provides novel insights into the potential activation and regulation of TRPV1 in respiratory conditions where cough and inflammation are contributing factors.

References

AKERS, I. A., PARSONS, M., HILL, M. R., HOLLENBERG, M. D., SANJAR, S., LAURENT, G. J. & MCANULTY, R. J. (2000) Mast cell tryptase stimulates human lung fibroblast proliferation via protease-activated receptor-2. *Am J Physiol Lung Cell Mol Physiol,* 278, L193-201.

ANDERSON, G. P. (2004) TRPV1 and cough. *Thorax,* 59, 730-1.

ANGATA, K. & FUKUDA, M. (2003) Polysialyltransferases: major players in polysialic acid synthesis on the neural cell adhesion molecule. *Biochimie,* 85, 195-206.

APWEILER, R., HERMJAKOB, H. & SHARON, N. (1999) On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim Biophys Acta,* 1473, 4-8.

AVELINO, A., CRUZ, C., NAGY, I. & CRUZ, F. (2002) Vanilloid receptor 1 expression in the rat urinary tract. *Neuroscience,* 109, 787-98.

AXFORD, J. S. (1999) Glycosylation and rheumatic disease. *Biochim Biophys Acta,* 1455, 219-29.

BAUSE, E. (1983) Structural requirements of N-glycosylation of proteins. Studies with proline peptides as conformational probes. *Biochem J,* 209, 331-6.

BAUSE, E. & LEGLER, G. (1981) The role of the hydroxy amino acid in the triplet sequence Asn-Xaa-Thr(Ser) for the N-glycosylation step during glycoprotein biosynthesis. *Biochem J,* 195, 639-44.

BERGH, M. L., CEPKO, C. L., WOLF, D. & ROBBINS, P. W. (1987) Expression of the Saccharomyces cerevisiae glycoprotein invertase in mouse fibroblasts: glycosylation, secretion, and enzymatic activity. *Proc Natl Acad Sci U S A,* 84, 3570-4.

BEVAN, S. & GEPPETTI, P. (1994) Protons: small stimulants of capsaicin-sensitive sensory nerves. *Trends Neurosci,* 17, 509-12.

BHAVE, G., HU, H. J., GLAUNER, K. S., ZHU, W., WANG, H., BRASIER, D. J., OXFORD, G. S. & GEREAU, R. W. T. (2003) Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). *Proc Natl Acad Sci U S A,* 100, 12480-5.

BHAVE, G., ZHU, W., WANG, H., BRASIER, D. J., OXFORD, G. S. & GEREAU, R. W. T. (2002) cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. *Neuron,* 35, 721-31.

BIRDER, L. A., KANAI, A. J., DE GROAT, W. C., KISS, S., NEALEN, M. L., BURKE, N. E., DINELEY, K. E., WATKINS, S., REYNOLDS, I. J. & CATERINA, M. J. (2001) Vanilloid receptor expression suggests a sensory role for urinary bladder epithelial cells. *Proc Natl Acad Sci U S A,* 98, 13396-401.

BIRDER, L. A., NAKAMURA, Y., KISS, S., NEALEN, M. L., BARRICK, S., KANAI, A. J., WANG, E., RUIZ, G., DE GROAT, W. C., APODACA, G., WATKINS, S. & CATERINA, M. J. (2002) Altered urinary bladder function in mice lacking the vanilloid receptor TRPV1. *Nat Neurosci,* 5, 856-60.

BIRO, T., BRODIE, C., MODARRES, S., LEWIN, N. E., ACS, P. & BLUMBERG, P. M. (1998a) Specific vanilloid responses in C6 rat glioma cells. *Brain Res Mol Brain Res,* 56, 89-98.

BIRO, T., MAURER, M., MODARRES, S., LEWIN, N. E., BRODIE, C., ACS, G., ACS, P., PAUS, R. & BLUMBERG, P. M. (1998b) Characterization of functional vanilloid receptors expressed by mast cells. *Blood,* 91, 1332-40.

BISOGNO, T., MELCK, D., BOBROV, M., GRETSKAYA, N. M., BEZUGLOV, V. V., DE PETROCELLIS, L. & DI MARZO, V. (2000) N-acyl-dopamines: novel synthetic CB(1) cannabinoid-receptor ligands and inhibitors of anandamide inactivation with cannabimimetic activity in vitro and in vivo. *Biochem J,* 351 Pt 3, 817-24.

BONINI, S., LAMBIASE, A., ANGELUCCI, F., MAGRINI, L., MANNI, L. & ALOE, L. (1996) Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. *Proc Natl Acad Sci U S A,* 93, 10955-60.

BRON, R., KLESSE, L. J., SHAH, K., PARADA, L. F. & WINTER, J. (2003) Activation of Ras is necessary and sufficient for upregulation of vanilloid receptor type 1 in sensory neurons by neurotrophic factors. *Mol Cell Neurosci,* 22, 118-32.

BURDA, P. & AEBI, M. (1998) The ALG10 locus of Saccharomyces cerevisiae encodes the alpha-1,2 glucosyltransferase of the endoplasmic reticulum: the

terminal glucose of the lipid-linked oligosaccharide is required for efficient N-linked glycosylation. *Glycobiology,* 8, 455-62.

BURDA, P., JAKOB, C. A., BEINHAUER, J., HEGEMANN, J. H. & AEBI, M. (1999) Ordered assembly of the asymmetrically branched lipid-linked oligosaccharide in the endoplasmic reticulum is ensured by the substrate specificity of the individual glycosyltransferases. *Glycobiology,* 9, 617-25.

CARR, M. J. & UNDEM, B. J. (2001) Inflammation-induced plasticity of the afferent innervation of the airways. *Environ Health Perspect,* 109 Suppl 4, 567-71.

CATERINA, M. J., ROSEN, T. A., TOMINAGA, M., BRAKE, A. J. & JULIUS, D. (1999) A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature,* 398, 436-41.

CATERINA, M. J., SCHUMACHER, M. A., TOMINAGA, M., ROSEN, T. A., LEVINE, J. D. & JULIUS, D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature,* 389, 816-24.

CHUANG, H. H., PRESCOTT, E. D., KONG, H., SHIELDS, S., JORDT, S. E., BASBAUM, A. I., CHAO, M. V. & JULIUS, D. (2001) Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature,* 411, 957-62.

COMPTON, S. J., CAIRNS, J. A., PALMER, K. J., AL-ANI, B., HOLLENBERG, M. D. & WALLS, A. F. (2000) A polymorphic protease-activated receptor 2 (PAR2) displaying reduced sensitivity to trypsin and differential responses to PAR agonists. *J Biol Chem,* 275, 39207-12.

COMPTON, S. J., RENAUX, B., WIJESURIYA, S. J. & HOLLENBERG, M. D. (2001) Glycosylation and the activation of proteinase-activated receptor 2 (PAR(2)) by human mast cell tryptase. *Br J Pharmacol,* 134, 705-18.

COMPTON, S. J., SANDHU, S., WIJESURIYA, S. J. & HOLLENBERG, M. D. (2002) Glycosylation of human proteinase-activated receptor-2 (hPAR2): role in cell surface expression and signalling. *Biochem J,* 368, 495-505.

COREY, D. P., GARCIA-ANOVEROS, J., HOLT, J. R., KWAN, K. Y., LIN, S. Y., VOLLRATH, M. A., AMALFITANO, A., CHEUNG, E. L., DERFLER, B. H., DUGGAN, A., GELEOC, G. S., GRAY, P. A., HOFFMAN, M. P., REHM, H. L., TAMASAUSKAS, D. & ZHANG, D. S. (2004) TRPA1 is a

candidate for the mechanosensitive transduction channel of vertebrate hair cells. *Nature,* 432, 723-30.

CORRAO, W. M., BRAMAN, S. S. & IRWIN, R. S. (1979) Chronic cough as the sole presenting manifestation of bronchial asthma. *N Engl J Med,* 300, 633-7.

CORTRIGHT, D. N., CRANDALL, M., SANCHEZ, J. F., ZOU, T., KRAUSE, J. E. & WHITE, G. (2001) The tissue distribution and functional characterization of human VR1. *Biochem Biophys Res Commun,* 281, 1183-9.

CRONIN, N. B., O'REILLY, A., DUCLOHIER, H. & WALLACE, B. A. (2005) Effects of deglycosylation of sodium channels on their structure and function. *Biochemistry,* 44, 441-9.

CRUZ, F., GUIMARAES, M., SILVA, C. & REIS, M. (1997) Suppression of bladder hyperreflexia by intravesical resiniferatoxin. *Lancet,* 350, 640-1.

D'ANDREA, M. R., DERIAN, C. K., SANTULLI, R. J. & ANDRADE-GORDON, P. (2001) Differential expression of protease-activated receptors-1 and -2 in stromal fibroblasts of normal, benign, and malignant human tissues. *Am J Pathol,* 158, 2031-41.

DE PETROCELLIS, L., CHU, C. J., MORIELLO, A. S., KELLNER, J. C., WALKER, J. M. & DI MARZO, V. (2004) Actions of two naturally occurring saturated N-acyldopamines on transient receptor potential vanilloid 1 (TRPV1) channels. *Br J Pharmacol,* 143, 251-6.

DE PETROCELLIS, L. & DI MARZO, V. (2005) Lipids as regulators of the activity of transient receptor potential type V1 (TRPV1) channels. *Life Sci,* 77, 1651-66.

DE PETROCELLIS, L., HARRISON, S., BISOGNO, T., TOGNETTO, M., BRANDI, I., SMITH, G. D., CREMINON, C., DAVIS, J. B., GEPPETTI, P. & DI MARZO, V. (2001) The vanilloid receptor (VR1)-mediated effects of anandamide are potently enhanced by the cAMP-dependent protein kinase. *J Neurochem,* 77, 1660-3.

DELANY, N. S., HURLE, M., FACER, P., ALNADAF, T., PLUMPTON, C., KINGHORN, I., SEE, C. G., COSTIGAN, M., ANAND, P., WOOLF, C. J., CROWTHER, D., SANSEAU, P. & TATE, S. N. (2001) Identification and characterization of a novel human vanilloid receptor-like protein, VRL-2. *Physiol Genomics,* 4, 165-74.

DELMAS, P., NAULI, S. M., LI, X., COSTE, B., OSORIO, N., CREST, M., BROWN, D. A. & ZHOU, J. (2004) Gating of the polycystin ion channel signaling complex in neurons and kidney cells. *Faseb J,* 18, 740-2.

DENDA, M., FUZIWARA, S., INOUE, K., DENDA, S., AKAMATSU, H., TOMITAKA, A. & MATSUNAGA, K. (2001) Immunoreactivity of VR1 on epidermal keratinocyte of human skin. *Biochem Biophys Res Commun,* 285, 1250-2.

DEVANE, W. A., HANUS, L., BREUER, A., PERTWEE, R. G., STEVENSON, L. A., GRIFFIN, G., GIBSON, D., MANDELBAUM, A., ETINGER, A. & MECHOULAM, R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science,* 258, 1946-9.

DI PALMA, F., BELYANTSEVA, I. A., KIM, H. J., VOGT, T. F., KACHAR, B. & NOBEN-TRAUTH, K. (2002) Mutations in Mcoln3 associated with deafness and pigmentation defects in varitint-waddler (Va) mice. *Proc Natl Acad Sci U S A,* 99, 14994-9.

DIETRICH, A., MEDEROS Y SCHNITZLER, M., EMMEL, J., KALWA, H., HOFMANN, T. & GUDERMANN, T. (2003) N-linked protein glycosylation is a major determinant for basal TRPC3 and TRPC6 channel activity. *J Biol Chem,* 278, 47842-52.

DOCHERTY, R. J., YEATS, J. C., BEVAN, S. & BODDEKE, H. W. (1996) Inhibition of calcineurin inhibits the desensitization of capsaicin-evoked currents in cultured dorsal root ganglion neurones from adult rats. *Pflugers Arch,* 431, 828-37.

EKLUND, E. A. & FREEZE, H. H. (2005) Essentials of glycosylation. *Semin Pediatr Neurol,* 12, 134-43.

EVERETT, C. F. & MORICE, A. H. (2004) Gastroesophageal reflux and chronic cough. *Minerva Gastroenterol Dietol,* 50, 205-13.

FACER, P., KNOWLES, C. H., TAM, P. K., FORD, A. P., DYER, N., BAECKER, P. A. & ANAND, P. (2001) Novel capsaicin (VR1) and purinergic (P2X3) receptors in Hirschsprung's intestine. *J Pediatr Surg,* 36, 1679-84.

GALA, F. A. & MORRISON, S. L. (2002) The role of constant region carbohydrate in the assembly and secretion of human IgD and IgA1. *J Biol Chem,* 277, 29005-11.

GARCIA-MARTINEZ, C., MORENILLA-PALAO, C., PLANELLS-CASES, R., MERINO, J. M. & FERRER-MONTIEL, A. (2000) Identification of an aspartic residue in the P-loop of the vanilloid receptor that modulates pore properties. *J Biol Chem,* 275, 32552-8.

GARCIA-SANZ, N., FERNANDEZ-CARVAJAL, A., MORENILLA-PALAO, C., PLANELLS-CASES, R., FAJARDO-SANCHEZ, E., FERNANDEZ-BALLESTER, G. & FERRER-MONTIEL, A. (2004) Identification of a tetramerization domain in the C terminus of the vanilloid receptor. *J Neurosci,* 24, 5307-14.

GAVVA, N. R., TAMIR, R., KLIONSKY, L., NORMAN, M. H., LOUIS, J. C., WILD, K. D. & TREANOR, J. J. (2005a) Proton activation does not alter antagonist interaction with the capsaicin-binding pocket of TRPV1. *Mol Pharmacol.*

GAVVA, N. R., TAMIR, R., KLIONSKY, L., NORMAN, M. H., LOUIS, J. C., WILD, K. D. & TREANOR, J. J. (2005b) Proton activation does not alter antagonist interaction with the capsaicin-binding pocket of TRPV1. *Mol Pharmacol,* 68, 1524-33.

GEIRSSON, G., FALL, M. & SULLIVAN, L. (1995) Clinical and urodynamic effects of intravesical capsaicin treatment in patients with chronic traumatic spinal detrusor hyperreflexia. *J Urol,* 154, 1825-9.

GIANNANTONI, A., DI STASI, S. M., STEPHEN, R. L., NAVARRA, P., SCIVOLETTO, G., MEARINI, E. & PORENA, M. (2002) Intravesical capsaicin versus resiniferatoxin in patients with detrusor hyperreflexia: a prospective randomized study. *J Urol,* 167, 1710-4.

GONZALEZ-PERRETT, S., KIM, K., IBARRA, C., DAMIANO, A. E., ZOTTA, E., BATELLI, M., HARRIS, P. C., REISIN, I. L., ARNAOUT, M. A. & CANTIELLO, H. F. (2001) Polycystin-2, the protein mutated in autosomal dominant polycystic kidney disease (ADPKD), is a Ca2+-permeable nonselective cation channel. *Proc Natl Acad Sci U S A,* 98, 1182-7.

GORINA, S. & PAVLETICH, N. P. (1996) Structure of the p53 tumor suppressor bound to the ankyrin and SH3 domains of 53BP2. *Science,* 274, 1001-5.

GOTTSCHALK, A. (1958) Neuraminidase; its substrate and mode of action. *Adv Enzymol Relat Subj Biochem,* 20, 135-46.

GUNTHORPE, M. J., SMITH, G. D., DAVIS, J. B. & RANDALL, A. D. (2001) Characterisation of a human acid-sensing ion channel (hASIC1a) endogenously expressed in HEK293 cells. *Pflugers Arch,* 442, 668-74.

HAMMOND, C., BRAAKMAN, I. & HELENIUS, A. (1994) Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proc Natl Acad Sci U S A,* 91, 913-7.

HARDIE, R. C. & RAGHU, P. (2001) Visual transduction in Drosophila. *Nature,* 413, 186-93.

HARDUIN-LEPERS, A., VALLEJO-RUIZ, V., KRZEWINSKI-RECCHI, M. A., SAMYN-PETIT, B., JULIEN, S. & DELANNOY, P. (2001) The human sialyltransferase family. *Biochimie,* 83, 727-37.

HAYES, P., MEADOWS, H. J., GUNTHORPE, M. J., HARRIES, M. H., DUCKWORTH, D. M., CAIRNS, W., HARRISON, D. C., CLARKE, C. E., ELLINGTON, K., PRINJHA, R. K., BARTON, A. J., MEDHURST, A. D., SMITH, G. D., TOPP, S., MURDOCK, P., SANGER, G. J., TERRETT, J., JENKINS, O., BENHAM, C. D., RANDALL, A. D., GLOGER, I. S. & DAVIS, J. B. (2000) Cloning and functional expression of a human orthologue of rat vanilloid receptor-1. *Pain,* 88, 205-15.

HELENIUS, A. & AEBI, M. (2004) Roles of N-linked glycans in the endoplasmic reticulum. *Annu Rev Biochem,* 73, 1019-49.

HELENIUS, J. & AEBI, M. (2002) Transmembrane movement of dolichol linked carbohydrates during N-glycoprotein biosynthesis in the endoplasmic reticulum. *Semin Cell Dev Biol,* 13, 171-8.

HELLWIG, N., ALBRECHT, N., HARTENECK, C., SCHULTZ, G. & SCHAEFER, M. (2005) Homo- and heteromeric assembly of TRPV channel subunits. *J Cell Sci,* 118, 917-28.

HOENDEROP, J. G., NILIUS, B. & BINDELS, R. J. (2002) ECaC: the gatekeeper of transepithelial Ca2+ transport. *Biochim Biophys Acta,* 1600, 6-11.

HOENDEROP, J. G., VAN DER KEMP, A. W., HARTOG, A., VAN DE GRAAF, S. F., VAN OS, C. H., WILLEMS, P. H. & BINDELS, R. J. (1999) Molecular identification of the apical Ca2+ channel in 1, 25-dihydroxyvitamin D3-responsive epithelia. *J Biol Chem,* 274, 8375-8.

HOLTZMAN, M. J. (1992) Arachidonic acid metabolism in airway epithelial cells. *Annu Rev Physiol,* 54, 303-29.

HOLZER, P. (2002) Sensory neurone responses to mucosal noxae in the upper gut: relevance to mucosal integrity and gastrointestinal pain. *Neurogastroenterol Motil,* 14, 459-75.

HOPKINS, P. A. & SRISKANDAN, S. (2005) Mammalian Toll-like receptors: to immunity and beyond. *Clin Exp Immunol,* 140, 395-407.

HOTTA, Y. & BENZER, S. (1969) Abnormal electroretinograms in visual mutants of Drosophila. *Nature,* 222, 354-6.

HUANG, S. M., BISOGNO, T., TREVISANI, M., AL-HAYANI, A., DE PETROCELLIS, L., FEZZA, F., TOGNETTO, M., PETROS, T. J., KREY, J. F., CHU, C. J., MILLER, J. D., DAVIES, S. N., GEPPETTI, P., WALKER, J. M. & DI MARZO, V. (2002) An endogenous capsaicin-like substance with high potency at recombinant and native vanilloid VR1 receptors. *Proc Natl Acad Sci U S A,* 99, 8400-5.

HUDSON, L. J., BEVAN, S., WOTHERSPOON, G., GENTRY, C., FOX, A. & WINTER, J. (2001) VR1 protein expression increases in undamaged DRG neurons after partial nerve injury. *Eur J Neurosci,* 13, 2105-14.

HUNT, J. F., FANG, K., MALIK, R., SNYDER, A., MALHOTRA, N., PLATTS-MILLS, T. A. & GASTON, B. (2000) Endogenous airway acidification. Implications for asthma pathophysiology. *Am J Respir Crit Care Med,* 161, 694-9.

HWANG, S. W., CHO, H., KWAK, J., LEE, S. Y., KANG, C. J., JUNG, J., CHO, S., MIN, K. H., SUH, Y. G., KIM, D. & OH, U. (2000) Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances. *Proc Natl Acad Sci U S A,* 97, 6155-60.

HWANG, S. W. & OH, U. (2002) Hot channels in airways: pharmacology of the vanilloid receptor. *Curr Opin Pharmacol,* 2, 235-42.

INOUE, K., KOIZUMI, S., FUZIWARA, S., DENDA, S. & DENDA, M. (2002) Functional vanilloid receptors in cultured normal human epidermal keratinocytes. *Biochem Biophys Res Commun,* 291, 124-9.

IRWIN, R. S., BOULET, L. P., CLOUTIER, M. M., FULLER, R., GOLD, P. M., HOFFSTEIN, V., ING, A. J., MCCOOL, F. D., O'BYRNE, P., POE, R. H., PRAKASH, U. B., PRATTER, M. R. & RUBIN, B. K. (1998) Managing cough as a defense mechanism and as a symptom. A consensus panel report of the American College of Chest Physicians. *Chest,* 114, 133S-181S.

IRWIN, R. S. & MADISON, J. M. (2000) The diagnosis and treatment of cough. *N Engl J Med,* 343, 1715-21.

IRWIN, R. S., MADISON, J. M. & FRAIRE, A. E. (2000) The cough reflex and its relation to gastroesophageal reflux. *Am J Med,* 108 Suppl 4a, 73S-78S.

JAHNEL, R., BENDER, O., MUNTER, L. M., DREGER, M., GILLEN, C. & HUCHO, F. (2003) Dual expression of mouse and rat VRL-1 in the dorsal root ganglion derived cell line F-11 and biochemical analysis of VRL-1 after heterologous expression. *Eur J Biochem,* 270, 4264-71.

JAHNEL, R., DREGER, M., GILLEN, C., BENDER, O., KURRECK, J. & HUCHO, F. (2001) Biochemical characterization of the vanilloid receptor 1 expressed in a dorsal root ganglia derived cell line. *Eur J Biochem,* 268, 5489-96.

JERMAN, J. C., BROUGH, S. J., PRINJHA, R., HARRIES, M. H., DAVIS, J. B. & SMART, D. (2000) Characterization using FLIPR of rat vanilloid receptor (rVR1) pharmacology. *Br J Pharmacol,* 130, 916-22.

JI, R. R., SAMAD, T. A., JIN, S. X., SCHMOLL, R. & WOOLF, C. J. (2002) p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron,* 36, 57-68.

JOHNSON, D., MONTPETIT, M. L., STOCKER, P. J. & BENNETT, E. S. (2004) The sialic acid component of the beta1 subunit modulates voltage-gated sodium channel function. *J Biol Chem,* 279, 44303-10.

JORDT, S. E., BAUTISTA, D. M., CHUANG, H. H., MCKEMY, D. D., ZYGMUNT, P. M., HOGESTATT, E. D., MENG, I. D. & JULIUS, D. (2004) Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature,* 427, 260-5.

JORDT, S. E. & JULIUS, D. (2002) Molecular basis for species-specific sensitivity to "hot" chili peppers. *Cell,* 108, 421-30.

JORDT, S. E., TOMINAGA, M. & JULIUS, D. (2000) Acid potentiation of the capsaicin receptor determined by a key extracellular site. *Proc Natl Acad Sci U S A,* 97, 8134-9.

JUNG, J., HWANG, S. W., KWAK, J., LEE, S. Y., KANG, C. J., KIM, W. B., KIM, D. & OH, U. (1999) Capsaicin binds to the intracellular domain of the capsaicin-activated ion channel. *J Neurosci,* 19, 529-38.

JUNG, J., LEE, S. Y., HWANG, S. W., CHO, H., SHIN, J., KANG, Y. S., KIM, S. & OH, U. (2002) Agonist recognition sites in the cytosolic tails of vanilloid receptor 1. *J Biol Chem,* 277, 44448-54.

JUNG, J., SHIN, J. S., LEE, S. Y., HWANG, S. W., KOO, J., CHO, H. & OH, U. (2004) Phosphorylation of vanilloid receptor 1 by Ca2+/calmodulin-dependent kinase II regulates its vanilloid binding. *J Biol Chem,* 279, 7048-54.

KANAI, Y., NAKAZATO, E., FUJIUCHI, A., HARA, T. & IMAI, A. (2005) Involvement of an increased spinal TRPV1 sensitization through its up-regulation in mechanical allodynia of CCI rats. *Neuropharmacology.*

KANZAKI, M., ZHANG, Y. Q., MASHIMA, H., LI, L., SHIBATA, H. & KOJIMA, I. (1999) Translocation of a calcium-permeable cation channel induced by insulin-like growth factor-I. *Nat Cell Biol,* 1, 165-70.

KARAI, L., RUSSELL, J. T., IADAROLA, M. J. & OLAH, Z. (2004) Vanilloid receptor 1 regulates multiple calcium compartments and contributes to Ca2+-induced Ca2+-release in sensory neuronsn. *J Biol Chem.*

KARLSSON, J. A. & FULLER, R. W. (1999) Pharmacological regulation of the cough reflex--from experimental models to antitussive effects in Man. *Pulm Pharmacol Ther,* 12, 215-28.

KEDEI, N., SZABO, T., LILE, J. D., TREANOR, J. J., OLAH, Z., IADAROLA, M. J. & BLUMBERG, P. M. (2001) Analysis of the native quaternary structure of vanilloid receptor 1. *J Biol Chem,* 276, 28613-9.

KOLLARIK, M. & UNDEM, B. J. (2002) Mechanisms of acid-induced activation of airway afferent nerve fibres in guinea-pig. *J Physiol,* 543, 591-600.

KORNFELD, R. & KORNFELD, S. (1985) Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem,* 54, 631-64.

KOZAK, M. (1984) Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res,* 12, 857-72.

KUBE, D., ADAMS, L., PEREZ, A. & DAVIS, P. B. (2001) Terminal sialylation is altered in airway cells with impaired CFTR-mediated chloride transport. *Am J Physiol Lung Cell Mol Physiol,* 280, L482-92.

KUZHIKANDATHIL, E. V., WANG, H., SZABO, T., MOROZOVA, N., BLUMBERG, P. M. & OXFORD, G. S. (2001) Functional analysis of capsaicin receptor (vanilloid receptor subtype 1) multimerization and agonist

responsiveness using a dominant negative mutation. *J Neurosci,* 21, 8697-706.

KWAK, J., WANG, M. H., HWANG, S. W., KIM, T. Y., LEE, S. Y. & OH, U. (2000) Intracellular ATP increases capsaicin-activated channel activity by interacting with nucleotide-binding domains. *J Neurosci,* 20, 8298-304.

LALLOO, U. G., FOX, A. J., BELVISI, M. G., CHUNG, K. F. & BARNES, P. J. (1995) Capsazepine inhibits cough induced by capsaicin and citric acid but not by hypertonic saline in guinea pigs. *J Appl Physiol,* 79, 1082-7.

LAPLANTE, J. M., FALARDEAU, J., SUN, M., KANAZIRSKA, M., BROWN, E. M., SLAUGENHAUPT, S. A. & VASSILEV, P. M. (2002) Identification and characterization of the single channel function of human mucolipin-1 implicated in mucolipidosis type IV, a disorder affecting the lysosomal pathway. *FEBS Lett,* 532, 183-7.

LAUDE, E. A., HIGGINS, K. S. & MORICE, A. H. (1993) A comparative study of the effects of citric acid, capsaicin and resiniferatoxin on the cough challenge in guinea-pig and man. *Pulm Pharmacol,* 6, 171-5.

LAZZERI, M., BENEFORTI, P. & TURINI, D. (1997) Urodynamic effects of intravesical resiniferatoxin in humans: preliminary results in stable and unstable detrusor. *J Urol,* 158, 2093-6.

LEAVITT, R., SCHLESINGER, S. & KORNFELD, S. (1977) Impaired intracellular migration and altered solubility of nonglycosylated glycoproteins of vesicular stomatitis virus and Sindbis virus. *J Biol Chem,* 252, 9018-23.

LIEDTKE, W., CHOE, Y., MARTI-RENOM, M. A., BELL, A. M., DENIS, C. S., SALI, A., HUDSPETH, A. J., FRIEDMAN, J. M. & HELLER, S. (2000) Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell,* 103, 525-35.

LIU, M., LIU, M. C., MAGOULAS, C., PRIESTLEY, J. V. & WILLMOTT, N. J. (2003) Versatile regulation of cytosolic Ca2+ by vanilloid receptor I in rat dorsal root ganglion neurons. *J Biol Chem,* 278, 5462-72.

LIU, Z. G. (2005) Molecular mechanism of TNF signaling and beyond. *Cell Res,* 15, 24-7.

LODISH, H. F. & KONG, N. (1984) Glucose removal from N-linked oligosaccharides is required for efficient maturation of certain secretory

glycoproteins from the rough endoplasmic reticulum to the Golgi complex. *J Cell Biol,* 98, 1720-9.

LUX, S. E., JOHN, K. M. & BENNETT, V. (1990) Analysis of cDNA for human erythrocyte ankyrin indicates a repeated structure with homology to tissue-differentiation and cell-cycle control proteins. *Nature,* 344, 36-42.

MACPHERSON, L. J., GEIERSTANGER, B. H., VISWANATH, V., BANDELL, M., EID, S. R., HWANG, S. & PATAPOUTIAN, A. (2005) The pungency of garlic: activation of TRPA1 and TRPV1 in response to allicin. *Curr Biol,* 15, 929-34.

MALYKH, Y. N., SCHAUER, R. & SHAW, L. (2001) N-Glycolylneuraminic acid in human tumours. *Biochimie,* 83, 623-34.

MASON, L., MOORE, R. A., DERRY, S., EDWARDS, J. E. & MCQUAY, H. J. (2004) Systematic review of topical capsaicin for the treatment of chronic pain. *Bmj,* 328, 991.

MCINTYRE, P., MCLATCHIE, L. M., CHAMBERS, A., PHILLIPS, E., CLARKE, M., SAVIDGE, J., TOMS, C., PEACOCK, M., SHAH, K., WINTER, J., WEERASAKERA, N., WEBB, M., RANG, H. P., BEVAN, S. & JAMES, I. F. (2001) Pharmacological differences between the human and rat vanilloid receptor 1 (VR1). *Br J Pharmacol,* 132, 1084-94.

MCKEMY, D. D., NEUHAUSSER, W. M. & JULIUS, D. (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature,* 416, 52-8.

MCNAMARA, F. N., RANDALL, A. & GUNTHORPE, M. J. (2005) Effects of piperine, the pungent component of black pepper, at the human vanilloid receptor (TRPV1). *Br J Pharmacol,* 144, 781-90.

MEZEY, E., TOTH, Z. E., CORTRIGHT, D. N., ARZUBI, M. K., KRAUSE, J. E., ELDE, R., GUO, A., BLUMBERG, P. M. & SZALLASI, A. (2000) Distribution of mRNA for vanilloid receptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human. *Proc Natl Acad Sci U S A,* 97, 3655-60.

MICHELANGELI, F., OGUNBAYO, O. A. & WOOTTON, L. L. (2005) A plethora of interacting organellar Ca2+ stores. *Curr Opin Cell Biol,* 17, 135-40.

MITCHELL, J. E., CAMPBELL, A. P., NEW, N. E., SADOFSKY, L. R., KASTELIK, J. A., MULRENNAN, S. A., COMPTON, S. J. & MORICE, A.

H. (2005) Expression and characterization of the intracellular vanilloid receptor (TRPV1) in bronchi from patients with chronic cough. *Exp Lung Res,* 31, 295-306.

MIYAMOTO, R., TOKUDA, M., SAKUTA, T., NAGAOKA, S. & TORII, M. (2005) Expression and characterization of vanilloid receptor subtype 1 in human dental pulp cell cultures. *J Endod,* 31, 652-8.

MOCHIZUKI, T., WU, G., HAYASHI, T., XENOPHONTOS, S. L., VELDHUISEN, B., SARIS, J. J., REYNOLDS, D. M., CAI, Y., GABOW, P. A., PIERIDES, A., KIMBERLING, W. J., BREUNING, M. H., DELTAS, C. C., PETERS, D. J. & SOMLO, S. (1996) PKD2, a gene for polycystic kidney disease that encodes an integral membrane protein. *Science,* 272, 1339-42.

MOHAPATRA, D. P. & NAU, C. (2003) Desensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 is decreased by the cyclic AMP-dependent protein kinase pathway. *J Biol Chem,* 278, 50080-90.

MOQRICH, A., HWANG, S. W., EARLEY, T. J., PETRUS, M. J., MURRAY, A. N., SPENCER, K. S., ANDAHAZY, M., STORY, G. M. & PATAPOUTIAN, A. (2005) Impaired thermosensation in mice lacking TRPV3, a heat and camphor sensor in the skin. *Science,* 307, 1468-72.

MORICE, A. H. (2004) Post-nasal drip syndrome--a symptom to be sniffed at? *Pulm Pharmacol Ther,* 17, 343-5.

MORICE, A. H., FONTANA, G. A., SOVIJARVI, A. R., PISTOLESI, M., CHUNG, K. F., WIDDICOMBE, J., O'CONNELL, F., GEPPETTI, P., GRONKE, L., DE JONGSTE, J., BELVISI, M., DICPINIGAITIS, P., FISCHER, A., MCGARVEY, L., FOKKENS, W. J. & KASTELIK, J. (2004) The diagnosis and management of chronic cough. *Eur Respir J,* 24, 481-92.

MORICE, A. H. & GEPPETTI, P. (2004) Cough. 5: The type 1 vanilloid receptor: a sensory receptor for cough. *Thorax,* 59, 257-8.

MORICE, A. H., KASTELIK, J. A. & THOMPSON, R. (2001) Cough challenge in the assessment of cough reflex. *Br J Clin Pharmacol,* 52, 365-75.

MOSAVI, L. K., CAMMETT, T. J., DESROSIERS, D. C. & PENG, Z. Y. (2004) The ankyrin repeat as molecular architecture for protein recognition. *Protein Sci,* 13, 1435-48.

MULLER, D., HOENDEROP, J. G., MEIJ, I. C., VAN DEN HEUVEL, L. P., KNOERS, N. V., DEN HOLLANDER, A. I., EGGERT, P., GARCIA-

NIETO, V., CLAVERIE-MARTIN, F. & BINDELS, R. J. (2000) Molecular cloning, tissue distribution, and chromosomal mapping of the human epithelial Ca2+ channel (ECAC1). *Genomics,* 67, 48-53.

MUNSTER-KUHNEL, A. K., TIRALONGO, J., KRAPP, S., WEINHOLD, B., RITZ-SEDLACEK, V., JACOB, U. & GERARDY-SCHAHN, R. (2004) Structure and function of vertebrate CMP-sialic acid synthetases. *Glycobiology,* 14, 43R-51R.

MURAKAMI, M., XU, F., MIYOSHI, I., SATO, E., ONO, K. & IIJIMA, T. (2003) Identification and characterization of the murine TRPM4 channel. *Biochem Biophys Res Commun,* 307, 522-8.

NAUSEEF, W. M., MCCORMICK, S. J. & CLARK, R. A. (1995) Calreticulin functions as a molecular chaperone in the biosynthesis of myeloperoxidase. *J Biol Chem,* 270, 4741-7.

NAYAK, D. P., HUI, E. K. & BARMAN, S. (2004) Assembly and budding of influenza virus. *Virus Res,* 106, 147-65.

NILIUS, B., PRENEN, J., VENNEKENS, R., HOENDEROP, J. G., BINDELS, R. J. & DROOGMANS, G. (2001) Pharmacological modulation of monovalent cation currents through the epithelial Ca2+ channel ECaC1. *Br J Pharmacol,* 134, 453-62.

NILIUS, B., VENNEKENS, R., PRENEN, J., HOENDEROP, J. G., BINDELS, R. J. & DROOGMANS, G. (2000) Whole-cell and single channel monovalent cation currents through the novel rabbit epithelial Ca2+ channel ECaC. *J Physiol,* 527 Pt 2, 239-48.

NOLANO, M., SIMONE, D. A., WENDELSCHAFER-CRABB, G., JOHNSON, T., HAZEN, E. & KENNEDY, W. R. (1999) Topical capsaicin in humans: parallel loss of epidermal nerve fibers and pain sensation. *Pain,* 81, 135-45.

NUMAZAKI, M., TOMINAGA, T., TAKEUCHI, K., MURAYAMA, N., TOYOOKA, H. & TOMINAGA, M. (2003) Structural determinant of TRPV1 desensitization interacts with calmodulin. *Proc Natl Acad Sci U S A,* 100, 8002-6.

NUMAZAKI, M., TOMINAGA, T., TOYOOKA, H. & TOMINAGA, M. (2002) Direct phosphorylation of capsaicin receptor VR1 by protein kinase Cepsilon and identification of two target serine residues. *J Biol Chem,* 277, 13375-8.

OBATA, K., KATSURA, H., MIZUSHIMA, T., YAMANAKA, H., KOBAYASHI, K., DAI, Y., FUKUOKA, T., TOKUNAGA, A., TOMINAGA, M. & NOGUCHI, K. (2005) TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. *J Clin Invest,* 115, 2393-401.

OLAH, Z., KARAI, L. & IADAROLA, M. J. (2001a) Anandamide activates vanilloid receptor 1 (VR1) at acidic pH in dorsal root ganglia neurons and cells ectopically expressing VR1. *J Biol Chem,* 276, 31163-70.

OLAH, Z., KARAI, L. & IADAROLA, M. J. (2002) Protein kinase C(alpha) is required for vanilloid receptor 1 activation. Evidence for multiple signaling pathways. *J Biol Chem,* 277, 35752-9.

OLAH, Z., SZABO, T., KARAI, L., HOUGH, C., FIELDS, R. D., CAUDLE, R. M., BLUMBERG, P. M. & IADAROLA, M. J. (2001b) Ligand-induced dynamic membrane changes and cell deletion conferred by vanilloid receptor 1. *J Biol Chem,* 276, 11021-30.

PALMER, K. J., WATSON, P. & STEPHENS, D. J. (2005) The role of microtubules in transport between the endoplasmic reticulum and Golgi apparatus in mammalian cells. *Biochem Soc Symp,* 1-13.

PALOMBINI, B. C., VILLANOVA, C. A., ARAUJO, E., GASTAL, O. L., ALT, D. C., STOLZ, D. P. & PALOMBINI, C. O. (1999) A pathogenic triad in chronic cough: asthma, postnasal drip syndrome, and gastroesophageal reflux disease. *Chest,* 116, 279-84.

PALSSON-MCDERMOTT, E. M. & O'NEILL, L. A. (2004) Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology,* 113, 153-62.

PEDERSEN, S. F., OWSIANIK, G. & NILIUS, B. (2005) TRP channels: an overview. *Cell Calcium,* 38, 233-52.

PENG, J. B., CHEN, X. Z., BERGER, U. V., VASSILEV, P. M., BROWN, E. M. & HEDIGER, M. A. (2000) A rat kidney-specific calcium transporter in the distal nephron. *J Biol Chem,* 275, 28186-94.

PENG, J. B., CHEN, X. Z., BERGER, U. V., VASSILEV, P. M., TSUKAGUCHI, H., BROWN, E. M. & HEDIGER, M. A. (1999) Molecular cloning and characterization of a channel-like transporter mediating intestinal calcium absorption. *J Biol Chem,* 274, 22739-46.

PETERSON, J. R., ORA, A., VAN, P. N. & HELENIUS, A. (1995) Transient, lectin-like association of calreticulin with folding intermediates of cellular and viral glycoproteins. *Mol Biol Cell,* 6, 1173-84.

PETRESCU, A. J., MILAC, A. L., PETRESCU, S. M., DWEK, R. A. & WORMALD, M. R. (2004) Statistical analysis of the protein environment of N-glycosylation sites: implications for occupancy, structure, and folding. *Glycobiology,* 14, 103-14.

PHILLIPS, E., REEVE, A., BEVAN, S. & MCINTYRE, P. (2004) Identification of species-specific determinants of the action of the antagonist capsazepine and the agonist PPAHV on TRPV1. *J Biol Chem,* 279, 17165-72.

PIPER, A. S., YEATS, J. C., BEVAN, S. & DOCHERTY, R. J. (1999) A study of the voltage dependence of capsaicin-activated membrane currents in rat sensory neurones before and after acute desensitization. *J Physiol,* 518 (Pt 3), 721-33.

PREMKUMAR, L. S. & AHERN, G. P. (2000) Induction of vanilloid receptor channel activity by protein kinase C. *Nature,* 408, 985-90.

PRESCOTT, E. D. & JULIUS, D. (2003) A modular PIP2 binding site as a determinant of capsaicin receptor sensitivity. *Science,* 300, 1284-8.

RASHID, M. H., INOUE, M., KONDO, S., KAWASHIMA, T., BAKOSHI, S. & UEDA, H. (2003) Novel expression of vanilloid receptor 1 on capsaicin-insensitive fibers accounts for the analgesic effect of capsaicin cream in neuropathic pain. *J Pharmacol Exp Ther,* 304, 940-8.

ROSENBAUM, T., AWAYA, M. & GORDON, S. E. (2002) Subunit modification and association in VR1 ion channels. *BMC Neurosci,* 3, 4.

ROSENBAUM, T., GORDON-SHAAG, A., MUNARI, M. & GORDON, S. E. (2004) Ca2+/calmodulin modulates TRPV1 activation by capsaicin. *J Gen Physiol,* 123, 53-62.

SAMUELSSON, B. (1983) Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science,* 220, 568-75.

SANDFORD, R., SGOTTO, B., APARICIO, S., BRENNER, S., VAUDIN, M., WILSON, R. K., CHISSOE, S., PEPIN, K., BATEMAN, A., CHOTHIA, C., HUGHES, J. & HARRIS, P. (1997) Comparative analysis of the polycystic kidney disease 1 (PKD1) gene reveals an integral membrane glycoprotein with multiple evolutionary conserved domains. *Hum Mol Genet,* 6, 1483-9.

SAVIDGE, J., DAVIS, C., SHAH, K., COLLEY, S., PHILLIPS, E., RANASINGHE, S., WINTER, J., KOTSONIS, P., RANG, H. & MCINTYRE, P. (2002) Cloning and functional characterization of the guinea pig vanilloid receptor 1. *Neuropharmacology,* 43, 450-6.

SEABROOK, G. R., SUTTON, K. G., JAROLIMEK, W., HOLLINGWORTH, G. J., TEAGUE, S., WEBB, J., CLARK, N., BOYCE, S., KERBY, J., ALI, Z., CHOU, M., MIDDLETON, R., KACZOROWSKI, G. & JONES, A. B. (2002) Functional properties of the high-affinity TRPV1 (VR1) vanilloid receptor antagonist (4-hydroxy-5-iodo-3-methoxyphenylacetate ester) iodo-resiniferatoxin. *J Pharmacol Exp Ther,* 303, 1052-60.

SEDGWICK, S. G. & SMERDON, S. J. (1999) The ankyrin repeat: a diversity of interactions on a common structural framework. *Trends Biochem Sci,* 24, 311-6.

SHIN, J., CHO, H., HWANG, S. W., JUNG, J., SHIN, C. Y., LEE, S. Y., KIM, S. H., LEE, M. G., CHOI, Y. H., KIM, J., HABER, N. A., REICHLING, D. B., KHASAR, S., LEVINE, J. D. & OH, U. (2002) Bradykinin-12-lipoxygenase-VR1 signaling pathway for inflammatory hyperalgesia. *Proc Natl Acad Sci U S A,* 99, 10150-5.

SIDI, S., FRIEDRICH, R. W. & NICOLSON, T. (2003) NompC TRP channel required for vertebrate sensory hair cell mechanotransduction. *Science,* 301, 96-9.

SILBERSTEIN, S. & GILMORE, R. (1996) Biochemistry, molecular biology, and genetics of the oligosaccharyltransferase. *Faseb J,* 10, 849-58.

SIMONE, D. A., NOLANO, M., JOHNSON, T., WENDELSCHAFER-CRABB, G. & KENNEDY, W. R. (1998) Intradermal injection of capsaicin in humans produces degeneration and subsequent reinnervation of epidermal nerve fibers: correlation with sensory function. *J Neurosci,* 18, 8947-59.

SMART, D., GUNTHORPE, M. J., JERMAN, J. C., NASIR, S., GRAY, J., MUIR, A. I., CHAMBERS, J. K., RANDALL, A. D. & DAVIS, J. B. (2000) The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br J Pharmacol,* 129, 227-30.

SMART, D., JERMAN, J. C., GUNTHORPE, M. J., BROUGH, S. J., RANSON, J., CAIRNS, W., HAYES, P. D., RANDALL, A. D. & DAVIS, J. B. (2001)

Characterisation using FLIPR of human vanilloid VR1 receptor pharmacology. *Eur J Pharmacol,* 417, 51-8.

SMITH, G. D., GUNTHORPE, M. J., KELSELL, R. E., HAYES, P. D., REILLY, P., FACER, P., WRIGHT, J. E., JERMAN, J. C., WALHIN, J. P., OOI, L., EGERTON, J., CHARLES, K. J., SMART, D., RANDALL, A. D., ANAND, P. & DAVIS, J. B. (2002) TRPV3 is a temperature-sensitive vanilloid receptor-like protein. *Nature,* 418, 186-90.

STEIN, R. J., SANTOS, S., NAGATOMI, J., HAYASHI, Y., MINNERY, B. S., XAVIER, M., PATEL, A. S., NELSON, J. B., FUTRELL, W. J., YOSHIMURA, N., CHANCELLOR, M. B. & DE MIGUEL, F. (2004) Cool (TRPM8) and hot (TRPV1) receptors in the bladder and male genital tract. *J Urol,* 172, 1175-8.

STORY, G. M., PEIER, A. M., REEVE, A. J., EID, S. R., MOSBACHER, J., HRICIK, T. R., EARLEY, T. J., HERGARDEN, A. C., ANDERSSON, D. A., HWANG, S. W., MCINTYRE, P., JEGLA, T., BEVAN, S. & PATAPOUTIAN, A. (2003) ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell,* 112, 819-29.

STROTMANN, R., HARTENECK, C., NUNNENMACHER, K., SCHULTZ, G. & PLANT, T. D. (2000) OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. *Nat Cell Biol,* 2, 695-702.

STRUBING, C., KRAPIVINSKY, G., KRAPIVINSKY, L. & CLAPHAM, D. E. (2003) Formation of novel TRPC channels by complex subunit interactions in embryonic brain. *J Biol Chem,* 278, 39014-9.

SUN, M., GOLDIN, E., STAHL, S., FALARDEAU, J. L., KENNEDY, J. C., ACIERNO, J. S., JR., BOVE, C., KANESKI, C. R., NAGLE, J., BROMLEY, M. C., COLMAN, M., SCHIFFMANN, R. & SLAUGENHAUPT, S. A. (2000) Mucolipidosis type IV is caused by mutations in a gene encoding a novel transient receptor potential channel. *Hum Mol Genet,* 9, 2471-8.

TANAKA, M. & MARUYAMA, K. (2005) Mechanisms of capsaicin- and citric-acid-induced cough reflexes in guinea pigs. *J Pharmacol Sci,* 99, 77-82.

THAYER, S. A., PERNEY, T. M. & MILLER, R. J. (1988) Regulation of calcium homeostasis in sensory neurons by bradykinin. *J Neurosci,* 8, 4089-97.

THORNHILL, W. B., WU, M. B., JIANG, X., WU, X., MORGAN, P. T. & MARGIOTTA, J. F. (1996) Expression of Kv1.1 delayed rectifier potassium

channels in Lec mutant Chinese hamster ovary cell lines reveals a role for sialidation in channel function. *J Biol Chem,* 271, 19093-8.

TKACZ, J. S. & LAMPEN, O. (1975) Tunicamycin inhibition of polyisoprenyl N-acetylglucosaminyl pyrophosphate formation in calf-liver microsomes. *Biochem Biophys Res Commun,* 65, 248-57.

TOGNETTO, M., AMADESI, S., HARRISON, S., CREMINON, C., TREVISANI, M., CARRERAS, M., MATERA, M., GEPPETTI, P. & BIANCHI, A. (2001) Anandamide excites central terminals of dorsal root ganglion neurons via vanilloid receptor-1 activation. *J Neurosci,* 21, 1104-9.

TOMINAGA, M., CATERINA, M. J., MALMBERG, A. B., ROSEN, T. A., GILBERT, H., SKINNER, K., RAUMANN, B. E., BASBAUM, A. I. & JULIUS, D. (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron,* 21, 531-43.

TOTH, A., WANG, Y., KEDEI, N., TRAN, R., PEARCE, L. V., KANG, S. U., JIN, M. K., CHOI, H. K., LEE, J. & BLUMBERG, P. M. (2005) Different vanilloid agonists cause different patterns of calcium response in CHO cells heterologously expressing rat TRPV1. *Life Sci,* 76, 2921-32.

TREVISANI, M., MILAN, A., GATTI, R., ZANASI, A., HARRISON, S., FONTANA, G., MORICE, A. H. & GEPPETTI, P. (2004) Antitussive activity of iodo-resiniferatoxin in guinea pigs. *Thorax,* 59, 769-72.

TREVISANI, M., SMART, D., GUNTHORPE, M. J., TOGNETTO, M., BARBIERI, M., CAMPI, B., AMADESI, S., GRAY, J., JERMAN, J. C., BROUGH, S. J., OWEN, D., SMITH, G. D., RANDALL, A. D., HARRISON, S., BIANCHI, A., DAVIS, J. B. & GEPPETTI, P. (2002) Ethanol elicits and potentiates nociceptor responses via the vanilloid receptor-1. *Nat Neurosci,* 5, 546-51.

TURNER, H., FLEIG, A., STOKES, A., KINET, J. P. & PENNER, R. (2003) Discrimination of intracellular calcium store subcompartments using TRPV1 (transient receptor potential channel, vanilloid subfamily member 1) release channel activity. *Biochem J,* 371, 341-50.

UGAWA, S., UEDA, T., YAMAMURA, H. & SHIMADA, S. (2005) In situ hybridization evidence for the coexistence of ASIC and TRPV1 within rat single sensory neurons. *Brain Res Mol Brain Res,* 136, 125-33.

VASSILEV, P. M., PENG, J. B., JOHNSON, J., HEDIGER, M. A. & BROWN, E. M. (2001) Inhibition of CaT1 channel activity by a noncompetitive IP3 antagonist. *Biochem Biophys Res Commun,* 280, 145-50.

VAZQUEZ, G., WEDEL, B. J., AZIZ, O., TREBAK, M. & PUTNEY, J. W., JR. (2004) The mammalian TRPC cation channels. *Biochim Biophys Acta,* 1742, 21-36.

VELLANI, V., MAPPLEBECK, S., MORIONDO, A., DAVIS, J. B. & MCNAUGHTON, P. A. (2001) Protein kinase C activation potentiates gating of the vanilloid receptor VR1 by capsaicin, protons, heat and anandamide. *J Physiol,* 534, 813-25.

VENNEKENS, R., HOENDEROP, J. G., PRENEN, J., STUIVER, M., WILLEMS, P. H., DROOGMANS, G., NILIUS, B. & BINDELS, R. J. (2000) Permeation and gating properties of the novel epithelial Ca(2+) channel. *J Biol Chem,* 275, 3963-9.

VENNEKENS, R., PRENEN, J., HOENDEROP, J. G., BINDELS, R. J., DROOGMANS, G. & NILIUS, B. (2001) Pore properties and ionic block of the rabbit epithelial calcium channel expressed in HEK 293 cells. *J Physiol,* 530, 183-91.

VERNET, D., FERRINI, M. G., VALENTE, E. G., MAGEE, T. R., BOU-GHARIOS, G., RAJFER, J. & GONZALEZ-CADAVID, N. F. (2002) Effect of nitric oxide on the differentiation of fibroblasts into myofibroblasts in the Peyronie's fibrotic plaque and in its rat model. *Nitric Oxide,* 7, 262-76.

VLACHOVA, V., TEISINGER, J., SUSANKOVA, K., LYFENKO, A., ETTRICH, R. & VYKLICKY, L. (2003) Functional role of C-terminal cytoplasmic tail of rat vanilloid receptor 1. *J Neurosci,* 23, 1340-50.

WAHL, P., FOGED, C., TULLIN, S. & THOMSEN, C. (2001) Iodo-resiniferatoxin, a new potent vanilloid receptor antagonist. *Mol Pharmacol,* 59, 9-15.

WALKER, R. G., WILLINGHAM, A. T. & ZUKER, C. S. (2000) A Drosophila mechanosensory transduction channel. *Science,* 287, 2229-34.

WARD, S. M., BAYGUINOV, J., WON, K. J., GRUNDY, D. & BERTHOUD, H. R. (2003) Distribution of the vanilloid receptor (VR1) in the gastrointestinal tract. *J Comp Neurol,* 465, 121-35.

WATANABE, N., HORIE, S., MICHAEL, G. J., SPINA, D., PAGE, C. P. & PRIESTLEY, J. V. (2005) Immunohistochemical localization of vanilloid

receptor subtype 1 (TRPV1) in the guinea pig respiratory system. *Pulm Pharmacol Ther,* 18, 187-97.

WES, P. D., CHEVESICH, J., JEROMIN, A., ROSENBERG, C., STETTEN, G. & MONTELL, C. (1995) TRPC1, a human homolog of a Drosophila store-operated channel. *Proc Natl Acad Sci U S A,* 92, 9652-6.

WICTOME, M., HENDERSON, I., LEE, A. G. & EAST, J. M. (1992) Mechanism of inhibition of the calcium pump of sarcoplasmic reticulum by thapsigargin. *Biochem J,* 283 (Pt 2), 525-9.

WIRKNER, K., HOGNESTAD, H., JAHNEL, R., HUCHO, F. & ILLES, P. (2005) Characterization of rat transient receptor potential vanilloid 1 receptors lacking the N-glycosylation site N604. *Neuroreport,* 16, 997-1001.

WISNOSKEY, B. J., SINKINS, W. G. & SCHILLING, W. P. (2003) Activation of vanilloid receptor type I in the endoplasmic reticulum fails to activate store-operated Ca2+ entry. *Biochem J,* 372, 517-28.

WISSENBACH, U., BODDING, M., FREICHEL, M. & FLOCKERZI, V. (2000) Trp12, a novel Trp related protein from kidney. *FEBS Lett,* 485, 127-34.

WISSENBACH, U., NIEMEYER, B. A., FIXEMER, T., SCHNEIDEWIND, A., TROST, C., CAVALIE, A., REUS, K., MEESE, E., BONKHOFF, H. & FLOCKERZI, V. (2001) Expression of CaT-like, a novel calcium-selective channel, correlates with the malignancy of prostate cancer. *J Biol Chem,* 276, 19461-8.

WITTE, D. G., CASSAR, S. C., MASTERS, J. N., ESBENSHADE, T. & HANCOCK, A. A. (2002) Use of a fluorescent imaging plate reader--based calcium assay to assess pharmacological differences between the human and rat vanilloid receptor. *J Biomol Screen,* 7, 466-75.

WONG, C. H., MATAI, R. & MORICE, A. H. (1999) Cough induced by low pH. *Respir Med,* 93, 58-61.

XIN, H., TANAKA, H., YAMAGUCHI, M., TAKEMORI, S., NAKAMURA, A. & KOHAMA, K. (2005) Vanilloid receptor expressed in the sarcoplasmic reticulum of rat skeletal muscle. *Biochem Biophys Res Commun,* 332, 756-62.

XU, H., FU, Y., TIAN, W. & COHEN, D. M. (2006) Glycosylation of the osmoresponsive transient receptor potential channel TRPV4 on Asn-651 influences membrane trafficking. *Am J Physiol Renal Physiol,* 290, F1103-9.

XU, H., RAMSEY, I. S., KOTECHA, S. A., MORAN, M. M., CHONG, J. A., LAWSON, D., GE, P., LILLY, J., SILOS-SANTIAGO, I., XIE, Y., DISTEFANO, P. S., CURTIS, R. & CLAPHAM, D. E. (2002) TRPV3 is a calcium-permeable temperature-sensitive cation channel. *Nature,* 418, 181-6.

XU, S. Z. & BEECH, D. J. (2001) TrpC1 is a membrane-spanning subunit of store-operated Ca(2+) channels in native vascular smooth muscle cells. *Circ Res,* 88, 84-7.

YIANGOU, Y., FACER, P., DYER, N. H., CHAN, C. L., KNOWLES, C., WILLIAMS, N. S. & ANAND, P. (2001) Vanilloid receptor 1 immunoreactivity in inflamed human bowel. *Lancet,* 357, 1338-9.

YUE, L., PENG, J. B., HEDIGER, M. A. & CLAPHAM, D. E. (2001) CaT1 manifests the pore properties of the calcium-release-activated calcium channel. *Nature,* 410, 705-9.

ZHANG, X., HUANG, J. & MCNAUGHTON, P. A. (2005) NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *Embo J.*

ZHOU, Y., WANG, Y., ABDELHADY, M., MOURAD, M. S. & HASSOUNA, M. M. (2002) Change of vanilloid receptor 1 following neuromodulation in rats with spinal cord injury. *J Surg Res,* 107, 140-4.

ZHU, X., CHU, P. B., PEYTON, M. & BIRNBAUMER, L. (1995) Molecular cloning of a widely expressed human homologue for the Drosophila trp gene. *FEBS Lett,* 373, 193-8.

ZYGMUNT, P. M., PETERSSON, J., ANDERSSON, D. A., CHUANG, H., SORGARD, M., DI MARZO, V., JULIUS, D. & HOGESTATT, E. D. (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature,* 400, 452-7.