INVESTIGATING THE ROLE OF TRPA1 AND TRPV1 ION CHANNELS IN THE COUGH REFLEX

Megan Stacey Grace

2011

A Thesis Submitted for the Degree of Doctor of Philosophy in the Faculty of Medicine of Imperial College London

Respiratory Pharmacology Group
National Heart and Lung Institute
Faculty of Medicine
Imperial College London
Sir Alexander Fleming Building
Exhibition Road
London
SW7 2AZ

Abstract

Cough is under the control of sensory afferents which innervate the airways via the vagus nerve. Cough is an important protective reflex that clears the airway, but can become exacerbated and deleterious when associated with airways diseases, in which there is enhanced release of inflammatory mediators and a decrease in lung pH. These mediators sensitise airway afferents and could be driving enhanced cough associated with inflammation.

Transient Receptor Potential (TRP) ion channels are associated with several disease pathologies. TRPV1 has an established role in cough, and is implicated in the aetiology of chronic cough; and TRPA1 is a promising new target. Involvement of these ion channels in the tussive reflex is awaiting comprehensive investigation. I have therefore explored the role of TRPA1 and TRPV1 in tussive responses to the endogenous irritants prostaglandin E_2 (PGE_2), bradykinin (BK) and low pH. To do this I have used selective antagonists and genetically modified mice in models of human, guinea pig and mouse vagal sensory nerve depolarisation; conscious guinea pig cough; and guinea pig primary ganglia cell imaging.

TRPA1 and TRPV1 were shown to mediate PGE_2 and BK-induced nerve depolarisation, cough, and activation of ganglia cells. In contrast, low pH-induced nerve depolarisation and ganglia cell activation was mediated via TRPV1 or Acid Sensing Ion Channels (ASICs); whereas, cough was partially attenuated with TRPA1 or TRPV1 antagonists.

In summary, I have identified that TRPA1 and TRPV1 mediate PGE_2 and BK-induced cough; and provided evidence that low pH-induced sensory nerve activation is mediated via TRPV1 and ASICs, but a role for TRPA1 is still unclear. These are exciting findings which add to our understanding of the mechanisms that drive the cough reflex in the healthy state; builds a base for investigating cough hypersensitivity in disease; and could help to guide the development of novel efficacious anti-tussive therapies.

Acknowledgements

Foremost, I wish to thank my supervisors Professor Maria Belvisi and Dr. Mark Birrell for their support, advice and scientific expertise, and especially for allowing me the opportunity to stay in the UK and complete my PhD. It has been a challenging experience but extremely rewarding and I will benefit greatly from having the pleasure of working with you.

It has also been a pleasure to work with both past and present members of the Respiratory Pharmacology group. The support and advice that each person has given me has been invaluable. Particular thanks goes to Dr. Sarah Maher, Dr. Deborah Clarke, Dr. Kristof Raemdonck, Dr. Sissie Wong, Dr. Suffwan Eltom, and Dr. Jorge De Alba, who have all played a big part in helping me through these past few years. An extra-special thank you to Dr. Eric Dubuis, who has provided frequent support with the calcium imaging system, and helped to compose some fantastic illustrations for me to utilise!

Thank you to Professor Fugger and Dr. Welsh for kindly providing us with the ASIC knockout mice; and GSK for providing the TRPV1 antagonist for my *in vivo* studies. I am thankful to Dr. Saleem Haj-Yahia, Dr. Daniel Harris and the Transplant Coordinators at the Royal Brompton and Harefield NHS Trust, as well as Professor Alistair Proudfoot at Imperial College who have enabled me to utilise human tissue to support my research. I am also very grateful to the MRC and Wellcome Trust for providing the funding to support my studies.

My love, respect and thanks goes to Mum, Dad, Kelly, Joan and John for supporting me in this venture from half-way across the world. To my friends who have shown support and understanding, especially James and Ryan who can always make me laugh, thank you. Special mention must go to Kelly and Wai for providing some much needed stress-relief on several occasions. And finally, to my husband Richard for his endless encouragement, patience and love throughout my PhD, Kei te aroha au ki a koe.

Statement

Concentration-responses for capsaicin and PGE_2 in the isolated vagus nerve preparation were carried out by Dr. Julie Nasra and Dr. Sarah Maher, respectively. The initial concentration-responses for acrolein, capsaicin and citric acid-induced guinea pig cough were performed by Dr. David Hele; and the concentration-response for PGE_2-induced cough was performed by Dr. Sarah Maher. Dr. Eric Dubuis also helped to establish concentration-responses for the TRPA1 and TRPV1-selective agonists and antagonists in the isolated vagal ganglia cell preparation.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

4HNE	4-hydroxy-nonenal
4ONE	4-oxononenal
15-d-PGJ_2	15-deoxy-Delta$^{12, 14}$-Prostaglandin J_2
ACE	Angiotensin converting enzyme
AITC	Allyl-isothiocyanate
Amil	Amiloride
ANOVA	Analysis of variance
ASIC	Acid sensing ion channel
A.U.C	Area under curve
B_1	Bradykinin 1 receptor
B_2	Bradykinin 2 receptor
BK	Bradykinin
BK_{Ca}	Large conductance calcium-activated potassium cannel
bp	Base pairs
BSA	Bovine serum albumin
$[Ca^{2+}]_i$	Intracellular free calcium
Caps	Capsaicin
CAPZ	Capsazepine
CB	Cannabinoid receptor
CGRP	Calcitonin gene related peptide
Cinn	Cinnamaldehyde
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
COX	Cyclo-oxygenase
CR	Concentration-response
D12-PGJ_2	Delta^{12}-prostaglandin J_2
DEG/ENaC	Degenerin/epithelium sodium channel
dH_2O	Distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate

Di-8-ANEPPS	4-[2-[6-(dioctylamino)-2-naphthalenyl]ethenyl]-1-(3-sulfopropyl)-pyridinium
DiI	DiIC18(3), 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DP	D-prostanoid receptor
DRG	Dorsal root ganglion
ECS	Extracellular solution
EDTA	Ethylenediaminetetraacetic acid
EP	E-prostanoid receptor
FBS	Foetal bovine serum
FP	F-prostanoid receptor
GABA	γ-aminobutyric acid
GPCR	G protein-coupled receptor
HBSS	Hank's balanced salt solution
HC	HC-030031
HEK	Human embryonic kidney
Indo	Indomethacin
i.p.	Intraperitoneal
IP	I-prostanoid receptor
JNJ	JNJ17203212
K50	50 mM potassium chloride
KO	Knockout
mV	millivolts
NaV	Voltage-gated sodium channel
NK	Neurokinin
NTS	Nucelus tractus solitarius
OTC	Over-the-counter
PBS	Phsophate buffered saline
PCR	Polymerase chain reaction
PGA	Prostaglandin A
PGD	Prostaglandin D
PGE	Prostaglandin E
PGF	Prostaglandin F
PGI	Prostaglandin I

RAR	Rapidly adapting receptor
RLN	Recurrent laryngeal nerve
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Real-time polymerase chain reaction
RTX	Resiniferatoxin
SAR	Slowly adapting receptor
s.e.m.	Standard error of the mean
SLN	Superior laryngeal nerve
SP	Substance P
TBE	Tris Borate EDTA
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin 1
TRPM8	Transient receptor potential melastatin 8
TRPV1	Transient receptor potential vanilloid 1
TRPV2	Transient receptor potential vanilloid 2
TRPV3	Transient receptor potential vanilloid 3
TRPV4	Transient receptor potential vanilloid 4
TP	Tx-prostanoid receptor
Tween 80	Polyoxyethylene sorbitan monooleate
TxA	Thromboxane A
Veh	Vehicle
WT	Wild type

CHAPTER 1

Introduction

1.1 Cough

Cough is one of the most common complaints for which patients seek medical advice, and is reported as a troublesome symptom by a significant proportion of the population (Ford et al., 2006; McCormick et al., 1995). Under normal circumstances, cough is an important protective mechanism that helps to clear foreign material from the airway and aids in immune defence (Fontana et al., 1999; Irwin et al., 1998). In contrast, chronic cough of various aetiologies often serves no useful purpose and can lead to a dramatic decrease in quality of life (Irwin et al., 1998; Morice et al., 2007). Chronic cough, which can be defined as a cough persisting for longer than 8 weeks (Harding, 2006; Irwin et al., 1998), is associated with a number of inflammatory airway diseases such as chronic obstructive pulmonary disease (COPD), asthma, reflux, postnasal drip, cancer and viral infections; it is also a side-effect of some medical treatments such as angiotensin-converting enzyme inhibitors used in the prevention of cardiovascular disorders; and can be idiopathic in nature (Fuller & Choudry, 1987; Irwin et al., 1998; Morice et al., 2007). During development of chronic cough the cell structure and protective function of human bronchial mucosa are altered, providing strong evidence to suggest that repetitive physical damage of airway mucosa resulting from persistent cough may induce additional injury and inflammation of the airways. This mucosal injury could in turn enhance cough sensitivity and initiate a vicious cycle, which completely offsets the normal physiological functions of cough (reviewed in Niimi & Chung, 2004).

Although we know a great deal about the physiology of the cough response, we still know very little about which particular channels on the sensory nerves are central to the tussive reflex. Low pH and capsaicin are well-established tussive agents known to activate the Transient Receptor Potential Vanilloid 1 (TRPV1) ion channel. These ligands are frequently used in clinical trials to assess potential anti-tussives and hypersensitvity associated with disease states, largely because they produce a dose-dependent response which is highly reproducible and well tolerated (Karlsson & Fuller, 1999; Morice et al., 2007; Morice et al., 2001; Pounsford et al., 1985). However, these models may not be physiologically relevant since we do not currently know which stimuli or receptors are driving the cough response, either in healthy or diseased patients. Although some data exists to suggest that TRPV1 is involved in responses to certain tussive stimuli, no one has yet characterised the involvement of this or other ion

channels in the response to many of the other irritant substances which are known to produce cough. Furthermore, very little research has been carried out to discover if the same channels are driving the enhanced/excessive cough observed in disease.

1.2 Current therapies

Currently available anti-tussive therapies are largely offered over-the-counter (OTC) as self-medication programmes, and are among the most widely used OTC drugs. However, many OTC remedies are believed only to provide relief of the symptoms of cough (e.g. lubrication of a dry throat), or to act via a placebo effect (Karlsson & Fuller, 1999; Vassilev et al., 2009). Indeed, there are no data to support that OTC products are effective in children under 2 years of age, and may in fact cause adverse events in children fewer than 11 years of age (American Academy of Pediatrics, 1997; Centre for Disease Control, 2007; Gunn et al., 2001; Vassilev et al., 2009). In January 2008, the U.S. Food and Drug Administration released an advisory notice recommending that OTC cough and cold medicines not be used to treat children under 2 years due to potentially life-threatening side-effects (US Food and Drug Administration, 2008).

Anti-tussive therapies can be grouped in to two different types: (i) peripherally acting anti-tussives which directly target the afferent neuronal pathways to inhibit cough, and (ii) centrally acting treatments, which are generally considered to be the most effective anti-tussives currently available, and are thought to have their effect by suppressing a putative cough centre within the central nervous system (CNS). The major issue with these treatments are the associated side-effects, including drowsiness, constipation, nausea, and in the case of opiates physical addiction (Belvisi & Geppetti, 2004; Karlsson & Fuller, 1999; Reynolds et al., 2004). Hence, cough presents as a significant unmet medical need, and novel pharmacological approaches to treat cough (with fewer and/or less severe side effects) are required. It is important to note that coughing is a vital defence reflex that clears unwanted and potentially dangerous matter from the respiratory system. The ideal anti-tussive therapy would therefore inhibit an enhanced problematic cough without affecting the normal protective cough associated with health benefits. Interference with the afferent signal is thought to provide the best opportunity for pharmacological intervention, as the putative cough centre in the brain has yet to be clearly defined, and may be diffuse. Development of these therapies requires more in

depth understanding of the underlying mechanisms that exacerbate cough, and appropriate pharmacological tools with which to probe the pathways involved.

1.3 Physiology of cough

The physiology of the cough reflex has been well described, and can be explained as follows: rapid inspiration followed by an expiratory effort against a closed glottis and rapid generation of high intrapulmonary pressure, explosive expiration results from sudden opening of the glottis causing a high linear velocity of gas flow which sweeps irritant material up towards the pharynx (Widdicombe, 2002). The cough reflex is often involuntary, but can be accurately mimicked on a voluntary basis. We do not yet understand how the afferent nervous system is stimulated to generate the need to cough, or where in the CNS this information is integrated and organised to generate motor output (Fong et al., 2004). The cough centre of the CNS is believed to be in close proximity to the nucleus tractus solitarius (NTS), where the afferent nerves which mediate cough are known to synapse (Figure 1.1); and closely linked to the respiratory centre because a profound change in breathing pattern is an integral part of the cough reflex (Karlsson & Fuller, 1999).

1.4 Sensory nerves and the cough reflex

Airway afferent nerves express a number of different receptors and ion channels that modulate nerve activity when acted upon by pharmacological agents. Cough can be initiated by a wide variety of stimuli that trigger specialised peripheral cough receptors (Figure 1.1). Opening of ion channels on the airway sensory nerve terminals leads to membrane depolarisation, and if this depolarisation is of sufficient magnitude the peripheral nervous system will send signals to the CNS in the form of action potentials. Action potentials are carried by subsets of airway sensory fibres through the vagus nerves to the medulla where they terminate in the NTS. Second order neurons then relay the message to a respiratory pattern generator within the CNS, resulting in activation of motor neurons and initiation of the cough reflex. In addition, some mediators may interact with the nerve terminals or ion channels to inhibit depolarisation, alter the response to activating stimuli, or lead to changes in gene expression (Taylor-Clark & Undem, 2006). Cell bodies of the vagal sensory fibres originate in the nodose and

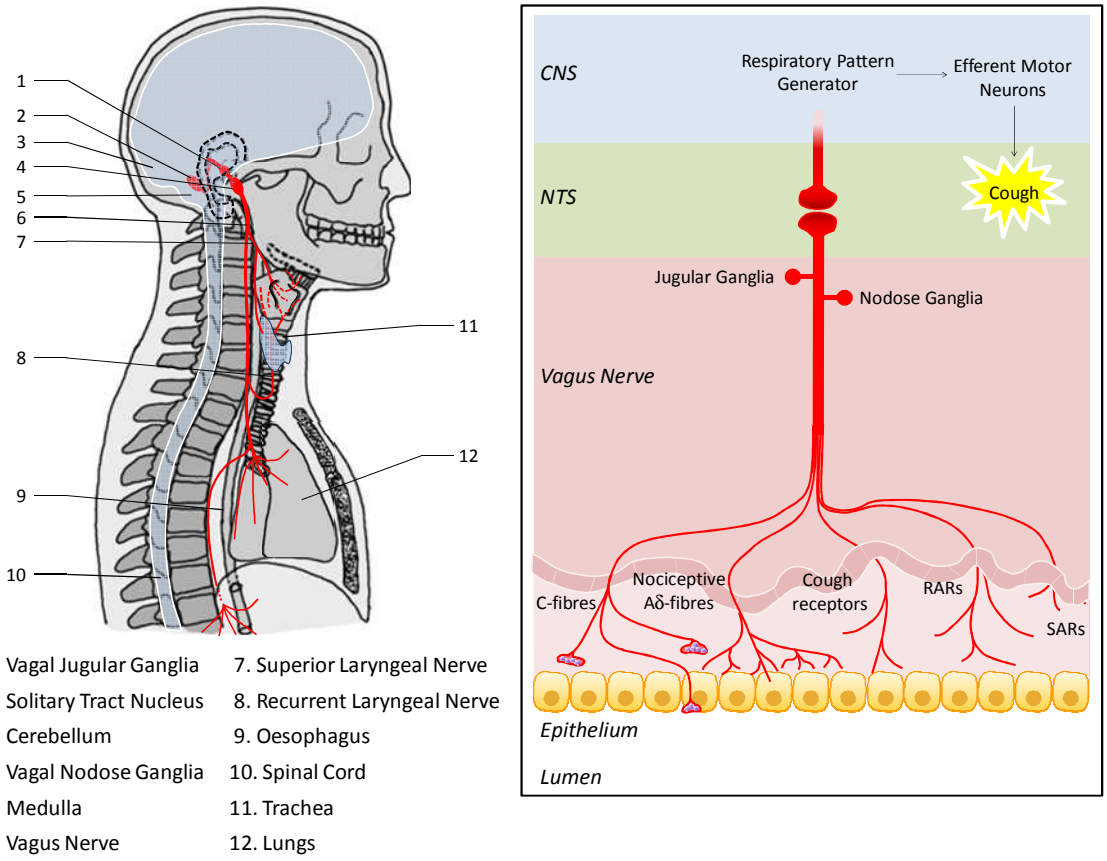
jugular ganglia, and branch in to the superior laryngeal nerves (SLN) and recurrent laryngeal nerves (RLN), which carry the airway fibres to the bronchi and trachea (Belvisi, 2003; Canning et al., 2004). A summary of the characteristics of each fibre type is presented in Table 1.1. This data should be interpreted with some caution, as all of this information has been extrapolated from experiments on guinea pigs; and there is yet to be any translational studies investigating airway fibre types in humans.

1.4.1 Rapidly Adapting Receptors (RARs)

RARs are irritant sensory receptors that respond with rapid adaptation to mechanical stimulation (e.g. bronchoconstriction, lung inflation, mucus production). They are myelinated, Aδ fast-velocity fibres that originate in the nodose ganglia, terminate predominantly in the airway epithelium, and are insensitive to direct chemical stimulation (though may be indirectly mechanically stimulated e.g. with associated mucus secretion or bronchoconstriction; Widdicombe, 2002). The evidence supporting a role for RAR fibres in cough comes mainly from work with anaesthetised animals (Tatar et al., 1994; Widdicombe, 1998). However, substances such as substance P and histamine, that have been shown to stimulate RARs under anaesthesia, are ineffective at causing cough (Canning et al., 2004; Chou et al., 2008). Although likely to be involved in the cough reflex, these fibres have few targets for pharmacological anti-tussive therapy.

1.4.2 Slowly Adapting Receptors (SARs)

Like RARs, SARs conduct action potentials in the ‘A’ range. But the activity of these fibres is not altered by stimuli that evoke cough, and they are thus not believed to be directly involved in the cough reflex. However, this does not preclude the possibility that SARs could facilitate the cough reflex by either permitting or preventing RAR activity (Reviewed in Canning & Mori, 2011; Canning et al., 2006; Reynolds et al., 2004).



1. Vagal Jugular Ganglia
2. Solitary Tract Nucleus
3. Cerebellum
4. Vagal Nodose Ganglia
5. Medulla
6. Vagus Nerve
7. Superior Laryngeal Nerve
8. Recurrent Laryngeal Nerve
9. Oesophagus
10. Spinal Cord
11. Trachea
12. Lungs

Figure 1.1. Airway sensory nerves and cough.

Cell bodies for airway nerve fibres originate in two ganglia, the jugular and nodose, which are located under the ear bone within the head. The airway fibres are carried via the vagus nerve, where they terminate both in and under the airway epithelium (illustrated in the enlarged panel). These fibres consist of the C-fibres, Aδ-nociceptors, polymodal Aδ-fibres (also called 'cough receptors'), rapidly adapting receptors (RARs), and slowly adapting receptors (SARs), which sense both chemical and mechanical stimuli. Of these fibres, the C-fibres and 'cough receptors' are thought to mediate cough. Once stimulated, information is carried to the nucleus of the solitary tract (NTS), located in the medulla, where the sensory fibres synapse. Second-order neurons then relay the message to a respiratory pattern generator, which activates efferent motor neurons, and leads to cough. The C-fibres also contain neuropeptides, which are released upon nerve activation and cause neurogenic inflammation (Adapted from Grace et al., 2011; Reynolds et al., 2004).

1.4.3 C-fibres

C-fibres are non-myelinated, chemosensitive, slow-velocity sensory nerves that originate in both the nodose and jugular vagal ganglia, and are found predominantly within the airway epithelium (Figure 1.1). The chemosensitivity of C-fibres makes them attractive pharmacological targets for anti-tussive treatment, and they have therefore been a major focus of research in the cough field. Two types of airway C-fibres have been identified, termed 'bronchial' and 'pulmonary' depending on their distribution along the airways. Bronchial C-fibres are present in the airway wall and are stimulated by chemicals exposed to the bronchial/systemic circulation; whereas, pulmonary C-fibres are located in the alveolar wall and activated by chemicals exposed to the pulmonary circulation (Coleridge & Coleridge, 1984). Based on their presence at the site from which the cough reflex can be triggered, it is thought that bronchial C-fibres mediate cough. Conversely, pulmonary C-fibres have been proposed to inhibit cough (Widdicombe, 1995; Widdicombe, 2002), likely through general inhibition of the respiratory centre in the CNS rather than a specific inhibition of the cough reflex (Karlsson & Fuller, 1999). Stimulation of pulmonary C-fibres triggers a chemoreflex causing apnoea, rapid shallow breathing, bradycardia and hypotension. During apnoea the respiratory rhythm generator is suppressed and inspiratory and expiratory efforts are inhibited, during which time it is not possible to trigger cough. In fact, suppression of cough appears to be directly proportional to the extent to which respiration is inhibited (Tatar et al., 1994).

C-fibres are activated by a wide range of stimuli, including food extracts (capsaicin, wasabi, ginger, allicin, mustard oil), environmental irritants (vehicle exhaust, air pollution, cigarette smoke, burning vegetation), and endogenous inflammatory mediators (prostaglandins, bradykinin) (Bautista et al., 2006; Caterina et al., 1997; Kaufman et al., 1980). In the guinea pig, exposure to aerosols of these compounds causes cough in conscious animals (Birrell et al., 2009; Canning et al., 2006; Costello et al., 1985; Kaufman et al., 1980; Lalloo et al., 1995; Laude et al., 1993), but fail to induce cough under anaesthesia (Belvisi & Hele, 2006; Karlsson & Fuller, 1999). It is possible that stimulation of C-fibres causes an "urge to cough" sensation, leading to the conscious generation of cough, which could explain the lack of effect under anaesthesia. Indeed, C-fibre stimulants such as capsaicin and bradykinin are associated with a dry

itchy sensation which is often associated with airway inflammation. Alternatively, anaesthesia may disrupt a C-fibre specific cough signalling pathway, or differentially upregulate inhibitory effects of pulmonary C-fibre activation (Canning, 2002). C-fibres also contain neuropeptides (tachykinins), such as substance P (SP), neurokinin A and calcitonin gene-related peptide (CGRP); and express Transient Receptor Potential (TRP) ion channels. In the guinea pig, stimulation of C-fibre nerve endings, for example by the TRPV1 agonist capsaicin, can result in the release of these neuropeptides which subsequently cause local neurogenic inflammation and CNS reflexes such as apnoea, mucus secretion and smooth muscle contraction (Widdicombe, 1995; Widdicombe, 2002). This process appears to play an important role in the pathogenesis of airway diseases in guinea pig models. Although, data provided from guinea pigs may not be clinically valid, as the existence of neurogenic inflammation in humans has not been comprehensively investigated (reviewed in Nassini et al., 2010).

1.4.4 Aδ nociceptors

Like RARs, Aδ nociceptors are myelinated fast-conducting neurons. These nerve fibres differ from RARs in that their cell bodies originate in the jugular ganglia, they are only modestly sensitive to mechanical stimulation, and respond to chemical nociceptive stimuli such as capsaicin and bradykinin. The pharmacology of Aδ nociceptors is thought to be similar to that of C-fibres, but they are yet to be systematically investigated, and their contribution to the cough reflex is unclear. In guinea pigs it has been found that Aδ nociceptors become ‘tachykinin positive’ during inflammatory disease states, and they may therefore play a role in the hypersensitive state seen in airway disease (Carr et al., 2002; Myers et al., 2002).

1.4.5 Cough receptors (polymodal Aδ nociceptors)

The so-called ‘cough receptors’ can be defined as extrapulmonary, myelinated, low threshold mechanosensors that originate in the nodose ganglia and are activated by punctuate mechanical stimulation, low chloride solutions and citric acid. These nerves do not express TRPV1 or SP, and are insensitive to classical C-fibre stimulants such as capsaicin and bradykinin. The cough receptor nerve fibres are distinct from RARs in that they conduct action potentials in a much slower range (approximately 5 m/s); and

are insensitive to airway stretch, alterations in airway pressure, and smooth muscle contraction evoked by histamine, which are stimuli that readily activate RAR fibres (Canning et al., 2004; Mazzone, 2004). However, ligands such as capsaicin and bradykinin which robustly cause cough in conscious animals do not activate cough receptors. It is possible that multiple cough pathways exist which contribute distinctly to different types of cough (e.g. defensive cough vs. urge to cough); or that secondary airway afferent pathways may evoke or modify cough responses via interactions with the primary cough pathway (Mazzone, 2004).

1.5 Transient Receptor Potential (TRP) channels and cough

The original TRP ion channel was isolated from the *Drosophila melanogaster* species of fly, and was named for its unique property of displaying a transient instead of a sustained response to bright light (Montell & Rubin, 1989). The TRP channel superfamily are a group of cation-selective, putative six-transmembrane-spanning proteins with a pore region localised between transmembrane segments 5 and 6 (Caterina et al., 1997). These receptors can be either directly or indirectly activated by intracellular and extracellular messengers, chemical compounds, mechanical stimuli, temperature changes and osmotic stress (Clapham, 2003). Thus far, six TRP channels – vanilloid 1-4 (TRPV1-4), melastatin 8 (TRPM8) and ankyrin 1 (TRPA1) – have been found to be expressed in the peripheral nervous system, and are activated by distinct sets of irritants and a range of temperatures (Figure 1.2) (Caspani & Heppenstall, 2009). This list may not be comprehensive, as the area of TRP channel research is fast expanding. Furthermore, since their discovery, the TRP channels have been linked to various roles in sensory perception, and associated with a wide range of diseases (Caterina et al., 1997; Nilius, 2007). As such, a great deal of research has focused on the TRPs as pharmacological targets, and it is postulated that their expression profile could be altered in disease states. An example of this has been discovered in patients suffering from chronic cough, who show an increase in TRPV1 expression in the lungs (Groneberg et al., 2004). TRPV1 has a well-established role in cough, and two known TRPV1 ligands (capsaicin and citric acid) are routinely used to assess the cough reflex in humans and animals.

Table 1.1. Characteristics of vagal afferent fibre types thought to be involved in the cough reflex.

	C-fibres	Aδ nociceptors	RAR fibres ('Aδ-like')	Cough Receptors ('Polymodal Aδ fibres')
Speed	Small diameter, unmyelinated, velocity <1 m/s	Small diameter, myelinated, velocity ~6 m/s	Myelinated, velocity 14-23 m/s	Myelinated, velocity 4-6 m/s
Stimulus sensitivity	High-threshold mechanosensitive (punctuate) Chemosensitive (BK, PGE_2, capsaicin, citric acid, acrolein etc.)	High-threshold mechanosensitive (punctuate) Chemosensitive (capsaicin, BK, acid)	Low-threshold mechanosensitive (punctuate, distension, stretch)	Mechanosensitive (punctuate)
Origin	Jugular (neural crest) Nodose (placodal)	Jugular (neural crest)	Nodose (placodal)	Nodose (placodal)
Termination	Extrapulmonary and intrapulmonary	Extrapulmonary and intrapulmonary	Intrapulmonary	Extrapulmonary, few intrapulmonary
TRP expression	Yes	Yes	No	No
Neuropeptide expression	Yes jugular fibres No nodose fibres	No (but can become positive during airways inflammation)	No	No

Abbreviations: BK – bradykinin; PGE_2 – prostaglandin E_2; TRP – transient receptor potential.

1.5.1 Transient Receptor Potential Vanilloid 1 (TRPV1)

The mammalian TRPV1 receptor, also called Vanilloid Receptor 1 (VR1) or the 'capsaicin receptor', was cloned and characterised by Caterina and colleagues in 1997. In this early study, TRPV1 was implicated in the sensation of noxious heat and pain (Caterina et al., 1997). Since then, TRPV1 has been identified as a polymodal channel that is activated by irritant chemicals, changes in physiological conditions, and endogenous mediators, including low pH (pH $\leq$ 6) (Jordt et al., 2000), anandamide (Jia et al., 2002; Kagaya et al., 2002; Zygmunt et al., 1999), heat (Caterina et al., 1997), bradykinin (Carr et al., 2003; Kollarik & Undem, 2004), neurotrophins (Ji et al., 2002), prostaglandins (Moriyama et al., 2005), ATP (Tominaga et al., 2001), and leukotrienes (Hwang et al., 2000). It is presumed that TRPV1 can form hetero- and homomultimers, which could help explain its polymodal sensitivity. However, experimental evidence along these lines is lacking (Tominaga & Tominaga, 2005). Interestingly, activation of TRPV1 by vanilloid compounds, heat and protons can be dissociated by mutagenesis of amino acid residues. This indicates that stimuli act through either distinct or incompletely overlapping channel regions, rather than a single agonist sensor domain (Kuzhikandathil et al., 2001; Tominaga & Tominaga, 2005).

Though TRPV1 is a non-selective cation channel, it is highly permeable to calcium. Calcium influx evoked by TRPV1 activation causes neurogenic inflammation in the guinea pig via the release of tachykinins, which act on a number of effector cells in the respiratory tract (Joos et al., 2000). However, an equivalent neurogenic response in humans has not yet been proven (Nassini et al., 2010). TRPV1 expression was initially believed to be largely confined to nociceptive neurons, and was originally cloned from rodent dorsal root ganglia (Caterina et al., 1997). Indeed, TRPV1 is highly expressed in rodent afferent neurons whose cell bodies reside in dorsal root, trigeminal or vagal ganglia (Ahluwalia et al., 2000; Caterina et al., 1997; Ichikawa & Sugimoto, 2003, 2004). But the TRPV1 receptor is now known to be more widely distributed, and is expressed by various neuronal and non-neuronal cells, including the skin (Denda et al., 2001), urinary tract (Avelino et al., 2002; Ward et al., 2003), bladder (Avelino et al., 2002; Birder et al., 2001), and gastrointestinal tract (Anavi-Goffer & Coutts, 2003; Poonyachoti et al., 2002; Ward et al., 2003). TRPV1 expression is also widespread in

the rat CNS, including the hippocampus, olfactory nuclei, amygdala, hypothalamus and cerebellum (Mezey et al., 2000).

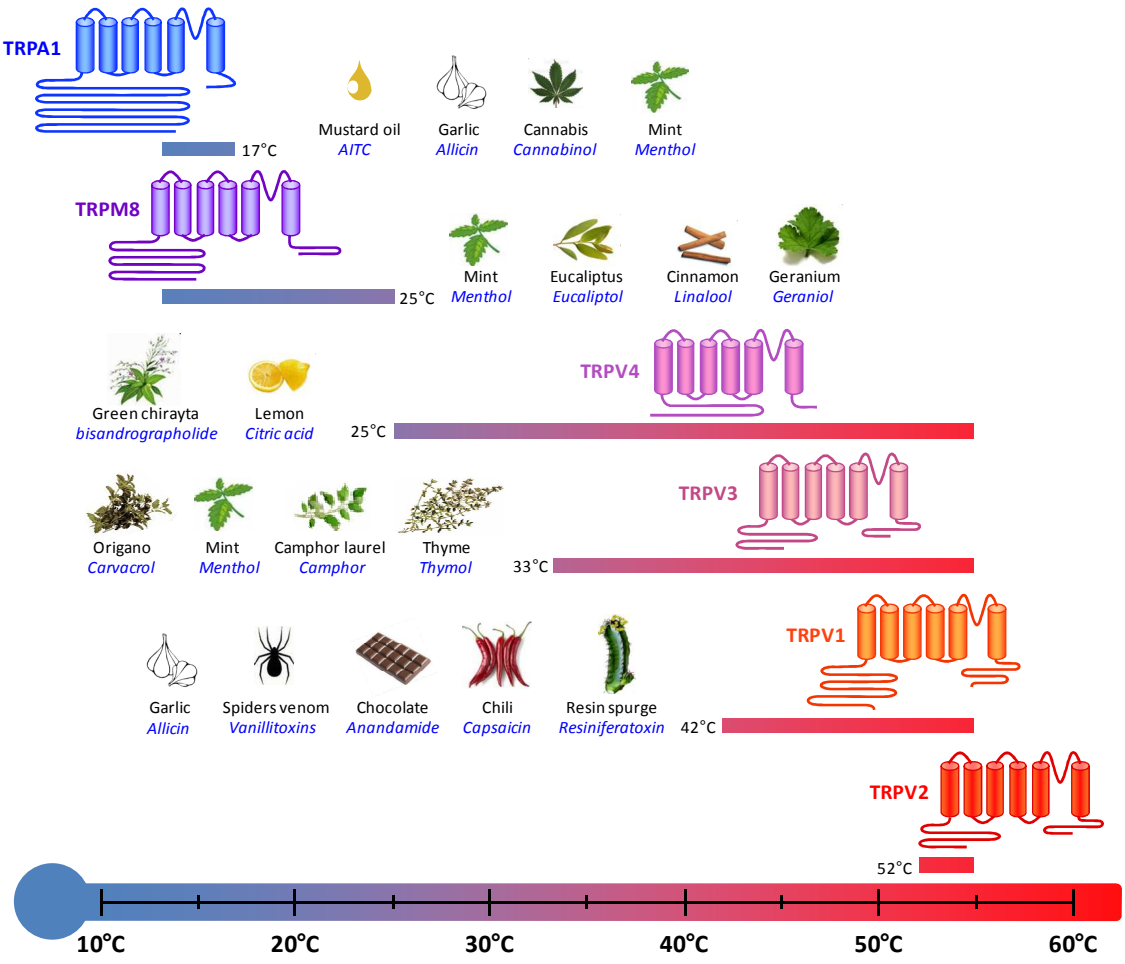


Figure 1.2. Temperature and chemical sensitivity of Transient Receptor Potential (TRP) channels expressed in sensory neurons.

The ion channels TRPA1, TRPM8 and TRPV1-4 are expressed in the peripheral sensory nervous system and mediate responses to noxious stimuli. These ion channels are activated by a range of temperatures, from noxious cold to noxious heat, as well as a number of natural exogenous irritants. A growing number of endogenous mediators are also being discovered to activate these ion channels (not illustrated).

A great deal of research has focussed on the role of TRPV1 in pain, where acute application of capsaicin induces painful sensations such as burning, itching, piercing, pricking and stinging. In animal studies, either the inhibition of TRPV1 with a selective antagonist or deletion of the TRPV1 gene (*Trpv1*$^{-/-}$) in mice blunts pain responses (Caterina et al., 2000; Davis et al., 2000; Walker et al., 2003). Therefore, the pharmaceutical industry is extremely interested in developing TRPV1 antagonists as new and effective analgesics, and there are many TRPV1 antagonists currently in clinical development. Clinical trials have had some limited success in alleviating pain with TRPV1 antagonists (Patapoutian et al., 2009). However, as discussed above, the expression profile of TRPV1 is diffuse and as such general antagonism of this receptor could be associated with unwanted side-effects. Currently, TRPV1 antagonists are known to induce hyperthermia in a range of animal models, and a recent clinical trial examining inhibition of dental pain with TRPV1 antagonists demonstrated increases in core body temperature up to as much as 40°C (Gavva et al., 2008). In response to this, there has recently been some progress toward developing TRPV1 inhibitors that do not exhibit hyperthermia (Lehto et al., 2008; Patapoutian et al., 2009; reviewed in Gunthorpe & Chizh, 2009). Nevertheless, there are other potential side-effects that may have yet to be observed. For example, TRPV1-expressing sensory neurons project to both cardiovascular and renal tissues (Singh & Deshpande, 2009; Wang & Li, 1999; Wang, 2005). Activation of these neurons can induce the release of neuropeptides, such as CGRP and SP, which are potent vasodilators and diuretics, and could be associated with cardioprotective effects (Wang, 2005). Inhibiting these neurons therefore has the potential to produce unwanted cardiovascular side-effects, though as yet there is no clinical data to support this (Patapoutian et al., 2009).

An association between hypersensitivity to pain (hyperalgesia) and hypersensitivity in the lungs (e.g. to tussive stimuli) has been postulated. Indeed, the classical TRPV1-selective agonist, capsaicin, causes airway smooth muscle contraction and sensory nerve activation *in vitro* (Belvisi et al., 1992; Patel et al., 2003) and cough *in vivo* (Lalloo et al., 1995; Trevisani et al., 2004) in animal models, which can be blocked with selective antagonists (Lalloo et al., 1995; McLeod et al., 2006; Trevisani et al., 2004). Capsaicin is also known to induce airway irritation and cough in humans (Doherty et al., 2000; Fuller & Choudry, 1987; Laude et al., 1993). Furthermore, there is evidence for an increase in TRPV1 expression in the lungs in patients exhibiting chronic cough

(Groneberg et al., 2004). As such, TRPV1 has been identified as a potential pharamacological target for the development of new anti-tussive therapies. Although TRPV1 is activated by capsaicin and low pH, it is not activated by many of the irritants known to initiate cough, thus implicating one or more other receptors in the cough reflex.

1.5.2 Transient Receptor Potential Ankyrin 1 (TRPA1)

TRPA1 (formerly ANKTM1), named for its multiple ankyrin domains in the N-terminus, is the only member of the TRPA subfamily to have been discovered thus far in mammals. Human TRPA1 was first isolated in 1999 from cultured fibroblasts (Jaquemar et al., 1999). It was later discovered that TRPA1 ion channels are expressed in a subset of TRPV1-expressing small diameter nociceptive neurons (Bautista et al., 2005; Story et al., 2003), making this channel a promising target for detection of noxious stimuli, and suggesting an involvement in processes such as inflammatory hyperalgesia and neurogenic inflammation (Andrè et al., 2008; Bautista et al., 2006; McNamara et al., 2007). Like TRPV1, TRPA1 is a polymodal non-selective cation channel that is highly permeable to calcium. Since its initial discovery, a great deal of research has focused on the role of TRPA1 in nociception. This has come about because of the diversity of compounds that have been discovered to activate TRPA1, including environmental irritants present in air pollution, tobacco smoke, and burning vegetation (e.g. acrolein); compounds present in foods and perfumes (e.g. allicin, mustard oil, wasabi and cinnamon); endogenous substances released during inflammation (bradykinin, prostaglandins) and oxidation (4-hydroxy-nonenal [4HNE] and 4-oxononenal [4ONE]); and purportedly noxious cold temperatures (Andersson et al., 2008; Bandell et al., 2004; Bautista et al., 2006, 2005; Karashima et al., 2009; Macpherson et al., 2007; Story et al., 2003; Taylor-Clark et al., 2008a, 2008b). However, a role for TRPA1 in cold sensation still remains controversial (Bautista et al., 2006; Jordt et al., 2004; Nagata et al., 2005; Zurborg et al., 2007; reviewed in Caspani & Heppenstall, 2009).

The mechanism(s) behind how many of this diverse set of compounds activate TRPA1 channels remains elusive. However, Macpherson and colleagues (2007) observed that several compounds which activate TRPA1 are electrophiles that react with cysteines,

and subsequently demonstrated that these ligands activate TRPA1 by covalently binding to cysteine residues within the cytosolic N-terminus. The molecular mechanism linking covalent bonding to channel gating has not been fully elucidated, but it has been shown that a single amino acid change in rat TRPA1 in the S6 region determines whether the channel is activated or inhibited by a compound. TRPA1 is also known to be activated by many non-electrophilic phytochemicals and some synthetic compounds, possibly in the traditional receptor-ligand binding fashion (Chen et al., 2008; Hinman et al., 2006; Macpherson et al., 2007; reviewed in Bang & Hwang, 2009). In addition, it is thought that intracellular calcium release can directly activate cation influx through TRPA1 channels (Zurborg et al., 2007), raising the possibility that TRPA1 could act as an amplifier of other signals that increase intracellular calcium. Also, G-protein coupled receptors may activate TRPA1 via intracellular secondary messengers (Bandell et al., 2004; Bautista et al., 2006).

Until very recently, it was thought that TRPA1 expression was restricted to the peripheral sensory neurons (Patapoutian et al., 2009). But there is a growing body of literature now suggesting that TRPA1 may be more widespread throughout the body, including cells such as fibroblasts, epithelial cells of the bladder and prostate gland, and on central terminals of sensory nerves in the spinal cord (reviewed in Rech et al., 2010). It is interesting to note that these cells play an important role in chronic inflammatory diseases, further implicating TRPA1 as a detector of noxious stimuli and tissue damage (Rech et al., 2010). As with TRPV1, activation of the TRPA1 ion channel has been associated with sensations of pain. A large number of chemical irritants that induce pain have been found to activate the TRPA1 receptor, including allicin, acrolein, and formalin. This has generated great interest, and a role for TRPA1 in various pain states has now been established, including acute, inflammatory and neuropathic pain. Due to the scarcity of pharmacological tools, a large part of the evidence has come from models using genetically modified mice that have had the TRPA1 gene selectively deleted (*Trpa1*$^{-/-}$). The recent development of TRPA1-selective antagonists has enabled scientists to pharmacologically verify the contribution of TRPA1 to pain sensation. In acute pain models, *Trpa1*$^{-/-}$ mice show fewer nocifensive behaviours than wild type controls with intraplantar injection and topical application of the TRPA1 agonist mustard oil, but cells from *Trpa1*$^{-/-}$ mice displayed normal responses to the TRPV1 agonist capsaicin (Bautista et al., 2006; Kwan et al., 2006). Similarly, pretreatment with

a TRPA1-selective inhibitor significantly attenuated acute nocifensive responses in rats evoked by injection of mustard oil into the hindpaw (Eid et al., 2008); and reduced nocifensive behaviours in mice treated with the TRPA1 agonist cinnamaldehyde, but not the TRPV1 agonist capsaicin (Petrus et al., 2007).

A growing number of pro-algesic endogenous compounds which are released in association with tissue injury, inflammation and oxidative stress have been identified as activators of TRPA1. Reactive electrophilic alkenals such as 4HNE and 4ONE, metabolites of arachadonic acid (e.g.15-d-PGJ_2, PGA_2), hydrogen peroxide (H_2O_2), and lipids (e.g. 4-oxo-2-nonenal and 4-hydroxyhexenal) induce pain-associated behaviours when injected in to the hindpaw of wild type mice. In contrast, these pain behaviours are blunted when the same compounds are injected into the paw of *Trpa1*$^{-/-}$ mice (Andersson et al., 2008; Materazzi et al., 2008; Trevisani et al., 2007). Moreover, activation of isolated trigeminal neurons by the metabolite 15-d-PGJ_2 is inhibited by the TRPA1 antagonist HC-030031 (Taylor-clark et al., 2008b); and activation of trigeminal neurons by bradykinin, an endogenous mediator released during inflammation, is attenuated in neurons cultured from *Trpa1*$^{-/-}$ mice in comparison to wild types (Bautista et al., 2006). Following injection of bradykinin in to the hindpaw, *Trpa1*$^{-/-}$ mice also show deficits in withdrawal latency to a heat source compared to wild types (Bautista et al., 2006). This data suggests that bradykinin-induced reductions in threshold to noxious heat are at least partially mediated via the TRPA1 ion channel. In addition, there is evidence that TRPA1 is involved in neuropathic pain, involving sensitisation of nociceptors resulting from damage to the peripheral or central nervous system (Eid et al., 2008; Obata et al., 2005), implicating a possible role for TRPA1 in chronic disease states.

Since the discovery of the TRPA1 channel, it has been postulated to be associated with respiratory irritation. A number of studies have demonstrated that stimulation of TRPA1 activates the vagal bronchopulmonary C-fibres in guinea pig and rodent lungs (Bessac et al., 2008; Nassenstein et al., 2008; Taylor-Clark et al., 2008a, 2009), and there is evidence that TRPA1 may be involved in hypersensitivity to noxious stimuli in disease states (Petrus et al., 2007). The above evidence led to the proposal that TRPA1 may be central to cough induced by a number of environmental and endogenous irritants, and could play a role in excessive cough seen in disease states. Indeed, since beginning this

project, both our lab and others have demonstrated that TRPA1 agonists can induce coughing in humans and guinea pigs, and that this effect is blocked by TRPA1-selective antagonists (Andrè et al., 2009; Birrell et al., 2009).

1.5.3 Multimerisation

Because TRPA1 and TRPV1 are co-expressed on sensory neurons, there is a possibility that they may co-operate or form heteromultimeric channels to activate nociceptors and elicit functional responses. Indeed, it has been suggested that TRPA1 channels could be activated by an overflow of calcium in the locale of other activated channels, without ever being modified by a reactive ligand. Furthermore, TRPA1 channels could act to amplify other calcium-mobilising pathways, including activation of TRPV1 (Cavanaugh et al., 2008; Zurborg et al., 2007). There is evidence for this type of coupling with bradykinin signalling in trigeminal neurons (Bautista et al., 2006). Whether this sort of cooperation exists in generating cough has yet to be determined.

1.6 Endogenous mediators involved in cough

Many compounds are released in the body in response to tissue injury and inflammation. This could be important, particularly in disease states where there is an increase in the release of endogenous inflammatory mediators within the airways. For example, PGE_2 and bradykinin are thought to be involved in hypersensitisation to tussive stimuli leading to chronic cough (Choudry et al., 1989; Fox et al., 1996). It has also been observed that patients who suffer from chronic cough exhibit a decrease in lung pH in comparison to healthy individuals (Hunt et al., 2000; Kostikas et al., 2002). As well as sensitising the cough reflex, PGE_2, BK and low pH are also capable of inducing cough when inhaled in aerosolised form (Choudry et al., 1989; Costello et al., 1985; Katsumata et al., 1991; Lalloo et al., 1995; Maher et al., 2009). It is therefore possible that enhanced release of these mediators in inflammatory airways disease could sensitise patients to environmental irritants that induce cough; or if the endogenous concentration reaches high-enough levels to activate airway sensory nerve endings, they could in fact cause cough. However, we do not know how these endogenous ligands mediate their tussive effects, either in healthy individuals or when associated with respiratory disease.

1.6.1 Prostaglandin E_2

Series 2 prostaglandins (PGE_2, PGD_2, PGI_2, PGF_2, and thromboxane [Tx]A_2) are endogenous products of arachadonic acid metabolism, and are involved in a large number of pathophysiological processes, including inflammation. Upon release, prostaglandins may interact with a number of G protein-coupled receptors (GPCRs) which are loosely divided in to EP, DP, IP, FP and TP subtypes, named for the type of prostanoid to which the series 2 prostaglandins preferentially bind – PGE_2, PGD_2, PGI_2, PGF_2, and TxA_2, respectively. In addition, the EP receptor has four known isoforms (termed EP_1 through EP_4). These GPCRs couple to one of a number of G-protein complexes that can have a variety of effects. Furthermore, there is a relative amount of cross-reactivity between the prostanoid ligands and the family of GPCRs. Thus, the area of prostanoid research can be particularly complicated (Bos et al., 2004).

In contrast to its effects in other parts of the body (McCoy et al., 2002; Wilgus et al., 2002) PGE_2 appears to have protective properties in the lungs (Kay et al., 2006; Martin et al., 2002; Walters et al., 1982), and has been identified as a potential therapy for asthmatics due to its anti-inflammatory and bronchodilator effects (Kawakami et al., 1973; Melillo et al., 1994; Walters et al., 1982). However, the development of PGE_2 as a treatment for airways disease has been hindered due to the reflex cough observed when it is inhaled (Costello et al., 1985; Gauvreau et al., 1999; Kawakami et al., 1973; Melillo et al., 1994; Pavord et al., 1993). In accordance with this, PGE_2 has been shown to excite airway afferent nerves which initiate the cough reflex (Coleridge et al., 1976; Maher et al., 2009).

The GPCR through which PGE_2 provokes cough was recently identified as the EP_3 isoform (Maher et al., 2009). Signalling downstream of the initial G-protein activation must ultimately lead to gating of ion channels in order to lead to a net change in membrane potential, but our understanding of the mechanisms behind PGE_2-induced cough is limited. There is some evidence for a role of TRPV1 in PGE_2-associated nociception in the pain field, whereby PGE_2-induced paw oedema in mice is inhibited with TRPV1-selective antagonists (Claudino et al., 2006). It is possible that the acute tussive effects of PGE_2 are also at least partially mediated via activation of the TRPV1 ion channel, which is already well-known to induce coughing. TRPA1 is another

promising candidate for mediating acute cough to PGE_2 stimulation. As discussed in section 1.5.2, TRPA1 has been implicated in the detection of noxious stimuli, and mediates the response to numerous irritant gases, dusts, vapours and chemicals linked to cough; furthermore, TRPA1 is co-expressed with TRPV1 on sensory neurons that innervate the airways (Bandell et al., 2004; Bautista et al., 2006, 2005; Jordt et al., 2004; Simon & Liedtke, 2008; Story et al., 2003; Taylor-Clark et al., 2008a, 2008b).

PGE_2 has also been implicated in the aetiology of chronic cough, as PGE_2 production is known to be upregulated in inflammatory airways disease (Choudry et al., 1989; Ho et al., 2000; Kwong & Lee, 2002; Lee et al., 2002) and could therefore contribute to the pathophysiology of cough associated with such diseases. Indeed, it has been shown that inhalation of PGE_2 can subsequently enhance the cough response to capsaicin stimulation in healthy human volunteers (Choudry et al., 1989). This effect is proposed to occur via sensitisation of the pulmonary afferent nerves, a theory supported by the finding that a low dose of exogenous PGE_2 markedly enhanced the excitability of vagal pulmonary C-fibres to capsaicin stimulation in anaesthetised rats (Ho et al., 2000). Sensitisation of TRPV1 probably occurs due to phosphorylation of the TRPV1 receptor downstream of GPCR activation, subsequently enhancing the excitability of the ion channel (Kwong & Lee, 2002; Lee et al., 2002). Alternately, PGE_2 release associated with inflammatory airways disease could directly induce coughing via opening of ion channels downstream of the EP_3 GPCR, if the endogenous concentration reaches high enough levels to induce sensory nerve activation (Maher et al., 2009).

1.6.2 Bradykinin

Bradykinin (BK) is an amino acid peptide which is derived from the precursor kininogen during inflammation and tissue injury. BK binds to the B_1 and B_2 GPCRs, through which it mediates a plethora of effects in the airways including bronchoconstriction, bronchodilation, mucus secretion, stimulation of sensory nerve afferents, and cough. A number of the effects associated with BK are indirect, caused by the subsequent release of other endogenous mediators such as prostanoids and nitrous oxide (Ellis & Fozard, 2002).

BK activates C-fibres (Kaufman et al., 1980; Reynolds et al., 2004), and has been shown to induce cough in both conscious animals (Canning, 2007; Canning et al., 2006; Kaufman et al., 1980) and humans (Choudry et al., 1989; Herxheimer & Stresemann, 1961; Katsumata et al., 1991). In cultured trigeminal neurons, application of BK elicits a robust calcium influx, which is partially attenuated in cells cultured from *Trpa1*$^{-/-}$ or *Trpv1*$^{-/-}$ mice (Bautista et al., 2006). Bautista and colleagues propose that a functional coupling may exist between TRPA1 and TRPV1 in BK signalling, whereby TRPV1 is activated upstream via production of phospholipase C and phosphokinase A. Low-level calcium influx through TRPV1, combined with release of intracellular calcium stores could then enhance the activation of TRPA1, which has been proposed to be directly gated by intracellular calcium release (Jordt et al., 2004; Zurborg et al., 2007). It has also been postulated that BK may be acting indirectly via production of other endogenous mediators, such as prostaglandins and/or 12-lipoxygenase products of arachadonic acid (Newton et al., 2002; Rodgers et al., 2002; Shin et al., 2002; Zhang et al., 2008).

The GPCR through which BK elicits its tussive effects is not known. The B_1 receptor is inducible, and not normally constitutively expressed (Calixto et al., 2000), and as such does not usually mediate the effects of BK under normal conditions. In contrast, B_2 receptors have been identified in most tissues (Ellis & Fozard, 2002), and the majority of pharmacological effects of kinins can be attributed to B_2 activation. Thus it is likely that the effects of BK in the airways are mediated via the B_2 receptor. However, as the B_1 receptor is inducible by proinflammatory agents, this isoform may be involved in the hypersensitivity to BK observed in disease states e.g. asthma (Ellis & Fozard, 2002). As mentioned above, BK is released as an endogenous by-product of inflammation and thus high levels of BK are found in patients with inflammatory airways disease. High endogenous concentrations of BK are proposed to be associated with the development of enhanced cough, as BK sensitises sensory nerve responses to capsaicin stimulation *in vitro* (Fox et al., 1996; Hwang & Oh, 2002). Inhibition of the breakdown of BK in the airways has also been linked to cough associated with patients taking angiotensin-converting-enzyme (ACE) inhibitors as a therapy for heart disease (Fox et al., 1996; Katsumata et al., 1991). ACE normally degrades endogenous BK, and thus it is thought that ACE inhibitors cause BK to accumulate, which subsequently sensitises airway sensory nerves and augments the cough reflex. An enhanced cough response with ACE

inhibitor treatment has been demonstrated in a guinea pig model, which was successfully inhibited by a B_2 receptor antagonist (Fox et al., 1996). Therefore, BK could be associated with enhanced cough in disease states either by increasing the excitability of afferent sensory neurons to other tussive stimuli; via the production of other endogenous mediators that stimulate cough; or if the BK concentration reaches high enough levels, coughing could be directly stimulated by opening of ion channels downstream of the associated GPCR, thereby activating afferent sensory nerve endings.

1.6.3 Low pH

The balance of pH within the body is maintained within a narrow range in healthy individuals. Accordingly, cells possess mechanisms for which to sense deviations from normal pH. It is therefore not surprising that acids activate nociceptive neurons within the lungs, triggering a powerful reflex cough in both animals and humans. Indeed, citric acid is a well-established tussive agent that is frequently used to assess potential anti-tussives, and the hypersensitvity associated with disease states (Karlsson & Fuller, 1999; Morice et al., 2007, 2001). Low pH-induced cough is partially mediated via the TRPV1 ion channel, but it is not currently known how protons cause ion channel opening. Acidic solutions evoke ionic currents when applied to outside-out but not inside-out membrane patches excised from HEK293 cells expressing TRPV1, suggesting that protons act on TRPV1 in the extracellular domain, in contrast to capsaicin which interacts at an intracellular/intramembrane site (Tominaga et al., 1998). It has been shown that activation of TRPV1 by vanilloid compounds, heat and protons can be dissociated by mutagenesis of amino acid residues, indicating that these stimuli act through either distinct or incompletely overlapping channel regions (Kuzhikandathil et al., 2001; Tominaga & Tominaga, 2005). This may have implications for development of future TRPV1 antagonists, as it suggests that it may be possible to block the capsaicin and/or low pH binding sites without affecting temperature regulation, as hyperthermia is a confounding factor with the currently available TRPV1 tools. This is briefly discussed in section 1.8.4.

Though low pH is mediated partially via TRPV1, it is not known what other ion channel(s) are involved. Again, TRPA1 is a likely candidate, as it is co-expressed with TRPV1 on sensory nerves, and mediates the effects of a multitude of noxious

compounds. Moreover, TRPA1 has been implicated in detecting high pH in cellular assays, and *in vivo* models of pain (Dhaka et al., 2009; Fujita et al., 2008). Recently, it has also been suggested that TRPA1 may sense acidification of the intracellular environment (Garrity, 2011; Wang et al., 2011). This is in contrast to TRPV1, which senses extracellular changes in pH (Tominaga et al., 1998).

In addition to C-fibres, low pH activates the cough receptors, and induces coughing under anaesthesia (Canning et al., 2004; Chou et al., 2008). Therefore, the mechanisms behind the tussive effects of low pH may be more complex than that of the other mediators examined in this thesis. In 2002, Kollarik & Undem observed two distinct responses to low pH stimulation in vagal afferents projecting to the airways. Of the fibres tested, both RAR-like afferents and C-fibres responded to rapid acidification, with rapidly adapting properties. In addition, C-fibres showed sustained activation in response to slow reduction in pH, whereas the RAR fibres displayed no sensitivity to this type of stimulation. The slowly inactivating response in C-fibres was attenuated with TRPV1 antagonists, but the rapidly adapting response was not affected in either fibre sub-type. This led the authors to propose two separate mechanisms for the acid-induced response in airway C-fibres: the slowly-adapting response which allows continuous monitoring of pH and is mediated by TRPV1; and a transient rapidly-adapting response displaying characteristics similar to that of the Acid Sensing Ion Channels (ASICs), which are also known to be expressed on sensory neurons. In this instance, it is also possible that the Aδ fibres could contribute to cough induced by rapid acidification, such as aspiration or inhaled irritants. It is not yet known if the transient response in C-fibres and Aδ-fibres are mediated via ASICs, or even via the same ion channels; or indeed how they contribute to low pH-induced coughing (Kollarik & Undem, 2002; Kollarik et al., 2007). The role of ASICs and other receptors in acid-sensitive sensory pathways await more definitive investigation.

The five isoforms of ASIC ion channels (ASIC1a, ASIC1b, ASIC2a, ASIC2b and ASIC3) are members of the degenerin/epithelium sodium channel (DEG/ENaC) family of sodium channels that are gated by protons. These ion channels are widely expressed in the nervous system, and have been implicated in a range of functions including peripheral sensory transduction (Gu & Lee, 2006; Kollarik & Undem, 2002). ASIC channels form both homo- and heteromultimeric channels *in vivo*, the composition of

which determines the kinetics of pH threshold and time-course of activation (Hesselager et al., 2004). Hence, these channels have the capacity to sense a wide array of pH throughout the body, from pH 7 to pH 4; and their response pattern can range from large and transient currents in response to rapid acidification, to sustained currents in the continuous presence of acid. This would then theoretically correlate to either a rapid burst of action potentials, or sustained action potential discharge in sensory nerves (Kollarik et al., 2007).

ASIC-like currents have been observed in airway vagal sensory neurons in both rats and guinea pigs (Gu & Lee, 2006; Kollarik & Undem, 2002). The transient, rapidly-inactivating profile of the responses suggests that these currents could be mediated by ASIC1 or ASIC3 ion channels. Interestingly, rat DRG neurons express both of these ASIC subunits. Specifically, medium-to-large diameter neurons (corresponding to myelinated Aδ-fibres) express both ASIC1 and ASIC3 subtypes, whereas small diameter neurons (corresponding to C-fibres) express predominantly ASIC1 (Alvarez de la Rosa et al., 2002). Unfortunately there are few selective pharmacological tools with which to probe these pathways, and this area is further complicated by the propensity of ASIC subunits to form heteromultimeric ion channels. However, it could be possible to determine a role for specific channels in the vagally-mediated response to low pH by using genetically modified mice with selected ASIC genes deleted (e.g. *Asic1*$^{-/-}$ and *Asic3*$^{-/-}$).

Patients with airway inflammatory disease exhibit lower airways pH in comparison to healthy individuals. Indeed, expired breath condensate in patients suffering from asthma, COPD and bronchiectasis can be up to 2 log orders lower than control subjects (Hunt et al., 2000; Kostikas et al., 2002). Interestingly, the threshold for TRPV1 channel opening is reduced in the presence of low pH levels (Carr, 2004; Caterina et al., 1997). As inflammation is associated with a lowering of physiological pH in the lungs, it could be postulated that airways acidity might sensitise sensory nerves, and contribute to the development of cough hypersensitivity in disease states. Due to methodological difficulties, the degree of acidification at the airway nerve terminals has not been assessed, and as such we do not know the environmental conditions at the point where the associated acid-sensitive ion channels are located. It is possible that pH within the lungs could physiologically reach a level at which ion channels are not only sensitised

but activated, thus leading to cough with no requirement for an external stimulus. Low pH could, therefore, be involved in the development of cough hypersensitivity by either sensitising sensory afferents to stimulation by other irritants; or may cause cough if lung pH becomes acidic enough to activate the airway sensory afferents.

1.7 Cough in inflammatory airways disease

Inflammation is a complex physiological process. Briefly, tissue injury caused by physical or chemical stimuli leads to vasodilatation, an increase in blood flow, vascular permeability and subsequent cellular recruitment to the site of injury. The cells that are recruited depend on the type and severity of injury, which is a biochemical process regulated by inflammatory mediators (Claudino et al., 2006). Thus far, the mechanisms driving excessive cough associated with inflammatory diseases have been elusive. Both the central and afferent nervous systems are capable of adapting to their environment, and external influences such as disease, injury and inflammation are able to induce changes in expression of various genes involved in the production of neuropeptides, neurotransmitters, and ion channels (Taylor-Clark & Undem, 2006). We do not yet understand this neuroplasticity with regard to cough-associated pathologies, but it is possible that exposure to tussigenic agents could lead to long or short-term changes in the peripheral sensory nerves; or in the central nervous system, for example the NTS where airway sensory nerves synapse (Lee & Undem, 2008).

Studies on subgroups of patients have shown that the cough reflex associated with respiratory viral infections, gastro-oesophageal reflux, COPD and 'cough-variant' asthma are hypersensitive to capsaicin challenge when compared to normal controls (Doherty et al., 2000; Higenbottam, 2002; Nakajima et al., 2006; O'Connell et al., 1996; Pecova et al., 2008; Plevkova et al., 2006). This suggests that there may be a common mechanism behind the augmented cough reflex in these diseases. As discussed above, the threshold for TRPV1 channel opening is reduced in the presence of low pH levels and inflammatory mediators. Since inflammation is associated with a lowering of physiological pH in the lungs (Hunt et al., 2000; Kostikas et al., 2002), and enhanced release of endogenous inflammatory mediators such as PGE_2 and bradykinin (Choudry et al., 1989; Ellis & Fozard, 2002; Ho et al., 2000; Lee et al., 2002), it could be postulated that this is at least partially involved in the hypersensitivity seen to capsaicin

stimulation in inflammatory disease. Furthermore, a correlation between TRPV1 expression and chronic cough has been established in humans, whereby there is increased TRPV1 channel density in the airways of patients suffering from chronic cough in comparison with healthy controls (Groneberg et al., 2004). Thus, there is growing evidence that the TRPV1 ion channel could be involved in chronic cough of various aetiologies. As yet there are no equivalent studies investigating changes in expression of other ion channels during disease states associated with excessive cough. However, an increase in TRPA1 expression in sensory nerves has been observed in models of pain (Diogenes et al., 2007; Obata et al., 2005).

Studies investigating pain states have found that neuronal inflammatory signalling pathways converge on both the TRPA1 and TRPV1 ion channels to enhance C-fibre excitability via phospholipase C and protein kinase A-dependent mechanisms. It is this process which could enable these receptors to be major integrators of diverse inflammatory signals. Moreover, inflammatory oxidants, lipid products and protons can promote the activity of these ion channels, both through direct interaction and covalent modification of the receptor. Activation of the TRPA1 receptor via covalent modification is likely to be significant in pathological situations, given that oxidant stress induced by either an inflammatory response, or by exogenous irritants such as tobacco smoke, can generate reactive electrophilic molecules including acrolein, 4-HNE and 4-ONE (Bautista et al., 2006; Taylor-Clark et al., 2008a; Trevisani et al., 2007). Production of these compounds could then lead to coughing via activation of TRPA1 ion channels. This may be of particular importance in highly polluted areas such as large cities, or in occupations where workers are chronically exposed to high levels of environmental irritants. Moreover, lipid metabolism can lead to the formation of endogenous electrophilic compounds e.g. cyclopentone ring-containing A- and J-series prostaglandins, which are formed as non-enzymatic dehydration products of PGE_2 and PGD_2, respectively. Prostanoids that contain one or two electrophilic carbons (e.g. $15PGJ_2$, $D12\text{-}PGJ_2$, 8-*iso*-PGA_2, and PGA_2) are therefore able to activate nociceptive neurons via direct interaction with TRPA1 (Taylor-Clark et al., 2008b). Prostaglandins such as PGE_2 and the inducible form of cyclooxygenase (COX-2) are elevated in respiratory disease states at the site of inflammation (Montuschi et al., 2003; Nemoto et al., 1976; Profita et al., 2003). Taken together, this information would suggest that reactive prostanoids and other endogenous TRPA1 ligands, which are produced in

greater amounts during inflammation or oxidant stress, could evoke the excessive cough seen in conditions such as asthma and COPD (Birrell et al., 2009).

1.8 Novel Therapeutic Targets for Cough

Medications currently available for cough show little efficacy, and are associated with a number of side-effects (Karlsson & Fuller, 1999; Reynolds et al., 2004; Schroeder & Fahey, 2002). In fact, certain therapeutic strategies for excessive cough associated with respiratory disease do not utilise anti-tussives *per se*, but rather treat the underlying condition. However, in many cases this type of therapy does not alleviate the associated cough. The ideal anti-tussive therapy would suppress only enhanced cough associated with disease, while leaving the protective part of the reflex functional. It is not yet known if enhanced cough is due to peripheral or central sensitisation, or a combination of both, which makes selective targeting of excessive cough difficult. In general, centrally-acting suppressants are associated with neurological side-effects such as sedation, nausea and physical dependence, which limits their effective use. In contrast, peripherally acting anti-tussives exert their effects by targeting peripheral sensory nerve afferents, and could potentially provide a better approach than centrally acting drugs.

1.8.1 Opiates

Currently, the most effective cough therapies are of the opioid class, for example codeine, morphine and dihydrocodone. These agonists are thought to act on opioid receptors both centrally and peripherally, and are associated with a number of side-effects. Codeine is associated with fewer side-effects than other opioids, and is consequently considered the gold-standard in anti-tussive therapy (McLeod et al., 2010; Reynolds et al., 2004). Dextromethorphan was developed as an isomer of the opioid levomethorphan, but acts on sigma receptors rather than classical opioid receptors. Because dextromethorphan has no analgesic or sedative properties, it is used as a constituent in many OTC preparations (Belvisi & Geppetti, 2004). New opioid peptides are also being investigated which do not bind to the classical opioid receptors. For example, nociceptin/orphanin FQ which binds to the NOP1 receptor has been shown to display anti-tussive activity in guinea pigs and cats (Lee et al., 2006; McLeod et al., 2002). This anti-tussive activity was blocked by a NOP-selective antagonist (McLeod et

al., 2002). Thus, selective NOP receptor agonists are now in clinical development, and could provide a novel therapeutic approach to the treatment of cough (McLeod et al., 2010).

1.8.2 GABA receptor agonists

Baclofen is an agonist of the γ-aminobutyric acid (GABA)-B receptor, which has been shown to inhibit capsaicin-induced cough in animal models (Bolser et al., 1993) and healthy human volunteers (Dicpinigaitis & Dobkin, 1997). Baclofen is thought to be centrally active, but there is evidence that a peripherally-restricted analogue also has anti-tussive activity (Bolser et al., 1994). Therefore, both centrally and peripherally acting GABA agonists might be useful in the treatment of cough.

1.8.3 Cannabinoids

Associated side-effects have hampered the use of non-selective cannabinoid compounds in therapeutic treatment (Belvisi et al., 2008; Patel et al., 2003). Cannabinoids mediate their effects via two known GPCRs, the CB_1 and CB_2 receptors. In rodents the CB_1 receptor is predominantly expressed throughout the CNS, but is also present at low levels in the periphery (Buckley et al., 2000; Herkenham et al., 1991). In contrast, the CB_2 receptor is found for the most part in the periphery, primarily in immune-associated tissues such as the spleen, tonsils and lymphocytes; whereas expression is limited within the CNS (Buckley et al., 2000; Galiègue et al., 1995; Griffin et al., 1997; Munro et al., 1993). Therefore, there is renewed interest in developing CB_2-selective agonists as these are likely to be devoid of the CNS mediated side-effects of the older cannabinoid class therapeutics. Indeed, a CB_2-selective agonist has been shown to inhibit sensory nerve depolarisation to a number of tussive stimuli *in vitro*, and coughing induced by citric acid *in vivo*. Moreover, this effect was blocked by a CB_2-selective antagonist, thereby confirming the selectivity of the CB_2 agonist compound (Belvisi et al., 2008).

1.8.4 TRPV1 antagonists

TRPV1 is one of the most promising targets identified for cough therapy in the last decade. The efficacy of TRPV1 antagonists have been established in preventing both citric acid and capsaicin-induced cough (Lalloo et al., 1995; Trevisani et al., 2004). Moreover, chronic coughers of various aetiologies exhibit higher expression of TRPV1 in the airways when compared to 'healthy' counterparts (Groneberg et al., 2004), indicating TRPV1 inhibitors as a potential therapy in disease. Though, it is of concern that drugs of this class may lead to unexpected side-effects if they become generally available to the public, as TRPV1 is widely expressed throughout the body. One issue which has already been identified is that currently available TRPV1 antagonists consistently cause hyperthermia, leading to the suggestion that TRPV1 is tonically active in thermoregulatory pathways (Gavva et al., 2008; Lehto et al., 2008). This side-effect is a potentially confounding factor in the clinical development of TRPV1 drugs, as current research investigating inhibitors that do not affect body temperature have had only limited success (Lehto et al., 2008). Despite this, a number of TRPV1 antagonists are currently being developed as anti-tussives and analgesics (Gunthorpe & Chizh, 2009).

1.8.5 Bradykinin receptor antagonists

Bradykinin elicits coughing in man (Choudry et al., 1989; Katsumata et al., 1991). ACE inhibitors have also been extensively reported to cause coughing in humans, an effect which has been suggested to be caused by accumulation of substances which are normally metabolised by ACE, including bradykinin (Carruthers, 1986; Fuller & Choudry, 1987; McNally, 1987). In agreement with this hypothesis, a B_2 receptor antagonist was shown to inhibit ACE inhibitor-induced cough in guinea pigs (Fox et al., 1996). More recently, it has been discovered that genetic polymorphisms exist for the BDKRB2 and PTGER3 genes (encoding the bradykinin B_2 and prostanoid EP_3 GPCRs, respectively), and that these polymorphisms are associated with ACE inhibitor cough (Grilo et al., 2011). This is a very interesting finding, as it is known that bradykinin causes cough via the B_2 GPCR (Fox et al., 1996), and PGE_2 causes cough via activation of the EP_3 GPCR in guinea pigs (Maher et al., 2009). In addition to causing cough, bradykinin can also sensitise C-fibres to activation by other tussive mediators (Fox et

al., 1996). According to this data, the cough reflex could theoretically be inhibited with bradykinin B_2 receptor antagonists, and may be effective in disease states where bradykinin is abundant in the lungs.

1.8.6 EP_3 receptor antagonists

Prostaglandin E_2 has beneficial properties in the lungs (Kay et al., 2006; Martin et al., 2002; Walters et al., 1982), and has been identified as a potential therapy for asthmatics due to its anti-inflammatory and bronchodilator effects (Kawakami et al., 1973; Melillo et al., 1994; Walters et al., 1982). Unfortunately, PGE_2 therapy induces coughing as a side-effect. Current theory suggests that the beneficial effects of PGE_2 may be able to be separated from its detrimental effects by selectively targeting the associated GPCRs (Maher et al., 2009). Indeed, it has been established that PGE_2 induces cough via the EP_3 receptor (Maher et al., 2009), whereas recent evidence suggests that the bronchodilator effects may be mediated by the EP_4 receptor (Buckley et al., 2011). Thus, cough associated with PGE_2 could be inhibited by EP_3 selective antagonists without affecting the beneficial properties of PGE_2 within the airways. Furthermore, this therapy could be useful in disease states associated with an increase PGE_2 production in the airways where cough is an aggravating symptom; or as a therapy for coughing elicited by medications, for example ACE inhibitors (see section 1.8.5).

1.8.7 β_2-adrenoceptor agonists

β_2-adrenoceptor agonists are currently one of the most effective bronchodilator treatments available, and are extensively used to alleviate bronchoconstriction associated with respiratory diseases such as COPD and asthma (Barnes, 2010a, 2010b; Campbell et al., 2005). The potential of β_2-adrenoceptor agonists as a therapy for cough has been controversial. A number of clinical trials have revealed β_2 agonists to possess anti-tussive properties in both healthy volunteers (Lowry et al., 1987) and chronic cough pathologies associated with allergic (Ellul-Micallef, 1983) or obstructive conditions (Campbell et al., 2005; Chong et al., 2005; Mulrennan et al., 2004; Pounsford et al., 1985). But other studies have not shown any anti-tussive effect (Chang et al., 1998; Smith et al., 1991). These conflicting results may be due to the data collection protocols used in these studies. For example, the use of subjective symptom

scoring measures; that few of these studies have been performed under double-blind, randomised and placebo-controlled conditions; and that cough is rarely a primary end-point of the research.

More recently, the β_2 agonist terbutaline was shown to be effective at inhibiting tussive responses to both capsaicin and citric acid in a pre-clinical guinea pig cough model (Freund-Michel et al., 2010). Furthermore, both guinea pig and human airway sensory nerve responses to a number of tussive irritants were blocked with two different β_2 agonists in an *in vitro* isolated vagus nerve preparation. This provides evidence that these compounds directly inhibit the tussive reflex rather than having an effect secondary to their bronchodilator properties, possibly via opening of the large conductance calcium-activated potassium (BK_{Ca}) channels which would inhibit sensory nerve activation by inducing hyperpolarisation (Freund-Michel et al., 2010). Further studies are required to corroborate the above findings, coupled with well-controlled and blinded clinical investigation where cough is the primary end-point. If these studies prove to be efficacious, then β_2 agonists could be introduced as an effective general (non-selective) anti-tussive therapy with an already proven acceptable safety profile in man.

1.8.8 Methylxanthines

Methylxanthines are another class of bronchodilator that are widely used in obstructive airway disease (Barnes, 2010a, 2010b), and have been proposed to inhibit the cough reflex. In particular, theophylline has been shown to exhibit anti-tussive properties in patients with poorly controlled asthma and ACE-inhibitor related cough (Bose et al., 1987; Cazzola et al., 1993; Fairfax et al., 1990). Furthermore, theophylline is effective in the treatment of pain, which indicates an inhibitory action on sensory nerves, rather than anti-tussive effects secondary to bronchodilation (Pechlivanova & Georgiev, 2005; Rao et al., 2007). There has also been interest in investigating the anti-tussive effects of theobromine, another methylxanthine compound. It was recently shown that theobromine attenuates citric acid-induced cough in guinea pigs and capsaicin-induced cough in healthy human volunteers (Usmani et al., 2005). Again, this compound was demonstrated to inhibit sensory nerve activation in an *in vitro* model, giving further support for direct inhibition of the cough reflex, rather than effects secondary to

bronchodilation. Further clinical and pre-clinical trials need to be carried out to investigate the efficacy of methylxanthines in inhibiting excessive cough associated with disease, and to determine how these compounds are mediating their anti-tussive effects, but this class of compound could be considered a promising future anti-tussive therapy.

1.9 Models of cough

The main objective of an animal model is to provide a system in which to elucidate mechanisms and test potential therapies. Ideally, the model should parallel as closely as possible the condition in man. Development of an animal model that is predictive of the human cough reflex is difficult due to the fact that there are currently no efficacious anti-tussive drugs available for use in the clinic. Therefore there is no point of reference for which to compare the animal models to man. Despite this, both *in vitro* and *in vivo* models of cough have been developed and demonstrated to be useful in studying the underlying mechanisms behind the cough reflex, and in assessing the efficacy of potential anti-tussive treatments. Pre-clinical studies of the neural pathways involved in the cough reflex and its pharmacological regulation have been conducted in guinea pigs, rats, mice, rabbits, cats and dogs (Belvisi & Bolser, 2002). However, there are reservations about some of the models used. For example, many studies have been carried out under anaesthesia which appears to suppress neuronal conduction and activity in the CNS, and may be the source of discrepancy between several of the observations seen with RAR and C-fibre nerve responses to tussive stimuli (Belvisi & Bolser, 2002; Mazzone et al., 2005). Furthermore, there is scepticism regarding the ability of small rodents such as mice and rats to perform a cough that resembles the reflex seen in man. This is largely due to the reflexogenic origin of cough in rats, which appears to originate from the larynx rather than the tracheobronchial tree as it does in man; and the fact that mice do not have RARs or intraepithelial nerve endings, and are therefore thought not to have a fully functional cough reflex (Belvisi & Bolser, 2002). Whereas, the use of large animals such as cats, dogs and pigs involves a cost element, not only in the purchase price, but the expenses involved with maintaining the animals and the large quantities of drug required for screening purposes. Therefore, one of the most useful and convenient animal models which has been widely used in investigating the cough reflex is the guinea pig.

Results in a number of clinical trials have lead to the perception that animal models are not predictive of human cough. Although, it is difficult to know what drugs are effective in man, as few clinical trials are run under ideal conditions with cough as the primary endpoint, are not sufficiently powered, or do not have the appropriate controls. For example, neurokinin (NK) antagonists, either as selective or combined NK_1, NK_2 or NK_3 recetpor antagonists, have been shown to be effective in a number of animal models, including guinea pigs, dogs, cats and rabbits (Advenier et al., 1993; Bolser et al., 1997; Canning, 2009; Girard et al., 1995; Nasra & Belvisi, 2009). However, NK antagonists have failed to show anti-tussive activity in human trials. This lack of effect could be due to the fact that cough is rarely a primary endpoint of clinical studies, and is quantified using subjective measures. There have also been clinical trials looking at NK antagonists where cough was the main outcome measure, but the results of these trials have not been reported (Canning, 2009; Nasra & Belvisi, 2009). Furthermore, it is still not known which of the NK receptors is involved, or if a combination of these receptors contributes to cough; and according to data from animal models CNS penetration might be required for NK antagonists to be effective (Bolser et al., 1997). It is not clear whether there have been clinical studies performed with centrally acting compounds that target all three NK receptors; but there have been clinical trials using either receptor-selective or peripherally restricted NK antagonists. For example, an NK1-selective compound (CP-99,994) from Pfizer was ineffective at inhibiting cough induced by hypertonic saline (Fahy et al., 1995). And DNK333, an NK_1/NK_2 antagonist from novartis was carried through for clinical testing after some success in the guinea pig (Lewis et al., 2007). However, this was reported to be a peripherally restricted compound, and subsequently showed no anti-tussive effects in humans.

Another source of discrepancy between animal models and human cough is the limited efficacy of the opioid class of anti-tussives, which are consistently successful at inhibiting cough in animals but show variable efficacy in the clinic. This may highlight a difference in dosing, as researchers are not limited by toxicity and CNS effects (such as sedation) in pre-clinical models, but obviously doses are restricted in clinical trials. Therefore, some of the success of compounds in animal research may be due to the ability to use high doses of compound, which could subsequently be showing anti-tussive activity due to sedation of the animals, rather than an actual inhibition of the

cough reflex. Below I will discuss in further detail the animal models relevant to my PhD thesis.

1.9.1 Guinea pig isolated vagus nerve model

In vitro models assessing isolated guinea pig vagus nerve depolarisation have been shown to be predictive of cough *in vivo* (Maher et al., 2009; Patel et al., 2003). This provides us with an effective tool with which to probe the cough pathways in an *in vitro* model, saving animals, time, and cost. Another benefit of the isolated vagus model is that we can replicate key studies and directly compare responses using human vagus tissue. On the other hand, though the isolated vagus preparation provides a comprehensive pharmacological assessment tool, the data collected should be interpreted with care as the agents being tested are applied to the axon of the vagus nerve instead of the nerve endings. This means that the extracellular depolarisation signal recorded represents a summation of the changes in membrane potential of all the nerve fibres (including RARs, SARs, Aδ-fibres and C-fibres) via activation of receptors expressed in the neuronal membrane of the axon. In addition, receptor expression and signal transduction mechanisms in the axon may differ from that at the peripheral endings (Patel et al., 2003). Furthermore, the vagus nerve innervates several organs in the body (including the heart and viscera), therefore not all fibres carried in the vagus nerve terminate in the airways.

1.9.2 Mouse isolated vagus nerve model

The mouse isolated vagus preparation has not been as well characterised as an assessment tool as the guinea pig described above. It is difficult to directly correlate *in vitro* vagus nerve results from mice to functional *in vivo* outcomes, as mice to not have a fully functional cough reflex. However, the afferent arm of the cough reflex still appears to be intact in mice as we observe depolarisation of the vagus nerve to tussive stimuli which are comparable to that of human and guinea pig isolated vagus (Maher et al., 2009; in-house data). I have used the mouse vagus nerve as a tool in my investigations largely because we have access to genetically modified animals, which will allow me to substantiate the results obtained from pharmacological antagonist investigations in the guinea pig vagus.

1.9.3 Human isolated vagus nerve model

One of the major advantages of the isolated vagus preparation is that we are able to obtain human tissue. This has allowed me to verify they key data from animal tissue in an *in vitro* human model.

1.9.4 Conscious guinea pig cough model

The conscious guinea pig model of cough is generally considered to be a valid tool for studying the cough reflex, as there are a number of apparent similarities between coughing in guinea pig and man (Karlsson & Fuller, 1999). However, there are also some species differences between the effects of certain drugs. This could be due to the greater importance of peripheral afferent nerves in regulating guinea pig respiration, airway tone, and lung function in comparison to humans. Two alternative theories are that C-fibre afferents are distributed differently throughout the airways; or that stimuli activate different signalling pathways with different sensitivities to pharmacological intervention (Karlsson & Fuller, 1999). These discrepancies are likely to be due not only to species differences, but also to a lack of appropriate clinical testing (as discussed above). Therefore, despite some uncertainties, the guinea pig model has become the most frequently used animal model to test development compounds pre-clinically.

1.9.5 Isolated primary neuronal cell model

Cell bodies of vagal neurons can be isolated from the nodose and jugular ganglia, allowing subsequent analysis of the effects of both agonists and antagonists on a cellular level. TRP channels are non-selective cation channels, which allow calcium influx in to airway primary ganglia cells, thereby causing membrane depolarisation, subsequent opening of voltage-sensitive sodium channels, and the generation of action potentials. The use of fluorescent dyes allows assessment of both calcium influx in to ganglia cells and changes in membrane potential, using specialised imaging equipment. Airway-specific responses can also be determined by the use of retrograde labelling. This preparation was recently established in our labs by Dr. Eric Dubuis, using neuronal cells isolated from guinea pig ganglia. This system not only allows us to investigate the ability of tussive agonists to activate airway-specific primary vagal ganglia cells, but

also to differentiate between cells originating from the nodose or jugular ganglia, and to determine how antagonists are mediating their inhibitory effects (e.g. by blocking calcium entry, activating potassium channels, or inhibiting voltage-gated sodium channels). There is also the potential to study other species, such as neuronal cells isolated from mouse ganglia, which would allow the use of genetically modified animals (though in this species the nodose and jugular ganglia are fused and not able to be separated). It is important to note that this preparation still does not allow differentiation between the types of nerve fibre being studied (e.g. C-fibre vs Aδ-fibre). Moreover, we are unable to determine if there are any phenotypical changes induced in the primary ganglia cells during the isolation and culture process. Despite these limitations, the use of isolated primary cells can aid in our understanding of the process by which both agonists and antagonists mediate their effects on a cellular level, which could be useful in the future development of targeted and selective cough treatments.

1.10 Thesis Plan

Acute cough serves to clear the airways of unwanted material, and is an important protective reflex. However, chronic cough associated with inflammatory disease no longer has a useful purpose, and can be damaging to the airways. As described in the introduction above, current anti-tussive treatments show little efficacy, and a more in-depth understanding of the cough reflex in both healthy and disease states is required to develop better medications. Inflammatory airways diseases are associated with the enhanced release of PGE_2 and BK, and a decrease in lung pH; and these mediators can both induce coughing, as well as sensitise the cough reflex to stimulation with other tussive irritants. It is therefore possible that enhanced release of these mediators could be driving the augmented cough response associated with disease states. The aim of this thesis was to test the hypothesis that TRPA1 and TRPV1 ion channels are driving the cough response to PGE_2, BK and low pH. Identification of the ion channels mediating the tussive response to these agents could lead to the development of more effective and targeted anti-tussive treatments. To investigate this I have taken an interdisciplinary approach. Using both *in vitro* and *in vivo* models, the ion channel(s) responsible for the tussive effects of these agents were identified by:

- Utilising a range of TRPA1 and TRPV1 selective agonists and antagonists to characterise their potency, efficacy and selectivity in models of sensory nerve activity, cough and primary vagal ganglia cell stimulation.

- Determining the ability of these selective antagonists to pharmacologically inhibit *in vitro* and *in vivo* stimulation of the cough reflex with PGE_2, BK and low pH

- Using isolated vagus nerves from genetically modified mice to investigate differences in response to the tussive stimuli, to parallel pharmacological experiments above.

- Translational research, utilising human vagal nerve tissue which allowed me to parallel the above experiments, thereby confirming that the rodent models mirrored the response in human tissue.

CHAPTER 2

Methodology

2.1 Introduction

This chapter outlines the general methodology for the techniques used in this thesis. Specific details of individual experimental protocols and statistical analyses employed are given in the methods section of each chapter. All experiments were performed in accordance with the UK Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act of 1986. Drugs/reagents and appropriate vehicles used in this thesis are listed in appendix 1.

2.2 Breeding and Genotyping of Genetically Modified Mice

2.2.1 Breeding

Homozygous breeding pairs of mice genetically modified to disrupt the TRPA1 (*Trpa1*$^{-/-}$) or TRPV1 (*Trpv1*$^{-/-}$) gene were obtained from Jackson Laboratories, USA. Heterozygous breeding pairs of mice devoid of one of the ASIC1 (*Asic1*$^{+/-}$) alleles were obtained from Professor Fugger's lab (John Radcliffe Hospital, University of Oxford), and subsequently bred in-house to obtain a homozygous *Asic1*$^{-/-}$ colony. Homozygous mice devoid of the ASIC3 (*Asic3*$^{-/-}$) gene were obtained from Dr. Welsh's lab (University of Iowa).

Upon arrival at Imperial College breeding pairs were housed in individually ventilated cages at the Central Biomedical Services, Sir Alexander Fleming Building, South Kensington. Animals were placed on a transgenic diet and provided with food and water *ad libitum*. All knockouts were bred on a C57Bl/6j background, are viable and fertile. Genotyping was routinely carried out on each colony to ensure gene disruption was maintained through subsequent generations.

2.2.2 Genotyping

Tail tips were taken and genomic DNA extracted using a non-chloroform mouse tail kit (Tepnel Life Sciences, Manchester, UK). Briefly, 0.5-1 cm tail tips were placed in autoclaved 1.5 ml eppendorf tubes and lysed overnight at 55°C in 175 µl reagent M1 and 20 µl proteinase K solution (10 mg/ml in nuclease-free water). Upon removal from

the incubator 65 µl reagent M2 and 100 µl Nucleon Resin was added, the contents mixed and centrifuged at 2000 x g for 10 minutes. The aqueous phase was transferred to a fresh 1.5 ml eppendorf, taking care not to disturb the debris layer. One volume of 100% isopropanol was added and the tubes inverted several times until DNA precipitate became visible. Samples were centrifuged at 2000 x g for 5 minutes to pellet the DNA. Supernatant was discarded, and the DNA washed with 70% ethanol. Tubes were briefly vortexed to dislodge the DNA pellet, the samples re-centrifuged at 2000 x g for 2 minutes and supernatant discarded. The DNA pellet was air-dried at room temperature for 10 minutes, re-suspended in 100 µl nuclease-free water and allowed to sit at room temperature for 3 hours to re-hydrate. Samples were stored at -80°C until required for analysis. Upon removal from the freezer samples were allowed to defrost on ice. Purity and integrity of the DNA was assessed by A_{260}/A_{280} spectrophotometry using a GeneQuant RNA/DNA quantifier (Amersham Pharmacia Biotech, UK).

A neomycin cassette was inserted in to the target gene to produce the desired genotype. The neomycin cassette is a length of DNA that replaces the part of the gene that is deleted, and allows identificatiion of the cells that have undergone homologous recombination to incorporate the target vector. The cassette gives resistance to neomycin, allowing survival of mutant cells where neomycin kills the non-mutant cells. Primer sequences (Invitrogen, UK) were designed such that the wild type amplicon would span the disrupted site of the desired gene. This ensures that the wild type and KO can be easily differentiated based on the amplicon size. DNA sections in between the primer pairs were exponentially amplified using polymerase chain reaction (PCR). The PCR reaction mix contained 1x Green GoTaq Flexi Buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1.25 µl enzyme, 10 pmol forward and 10 pmol reverse primers, and a volume of sample containing 50 ng DNA (or Nuclease-free water for control). Each sample was made up to 25 µl total volume with Nuclease-free water (Promega UK Ltd.). PCR reactions were carried out using ABI PRISM 7000 software (Applied Biosystems, Warrington, Cheshire, UK). Samples were heated to 95°C for 2 minutes followed by 40 cycles of denaturing, annealing and extension steps. All samples were denatured at 95°C for 30 seconds as this is dependent on the enzyme rather than the primer pairs. The annealing temperature is specific to the primer pair, depending on factors such as melting temperature and GC content. The temperature of the extension step is also primer dependent, and the length of time that the reaction is held for depends on the

product size i.e. the larger the expected product size, the longer the time needed (approximately 1 minute per 1000bp). A final extension step of 10 minutes at the same temperature was run to ensure that all products were full length. The reaction was then stopped by cooling to 4°C for 5 minutes. Specific details for the annealing and extension steps will be given in Chapter 3.

The PCR products and a DNA ladder (Hyperladder IV, Bioline Ltd, London) were run on a 2% agarose gel in Tris Borate EDTA (TBE) buffer containing 0.05 µl/ml Safeview (NBS Biologicals Ltd, Huntingdon, UK) at 80 V for 1 hour. Finally, the gel was visualised under ultra-violet light and photographed.

2.3 Isolated Vagus Nerve Preparation

2.3.1 Nerve dissection

Male Dunkin Hartley guinea pigs (350-500g, Harlan) or Male wild type (C57Bl/6j) or genetically modified (*Trpa1*$^{-/-}$, *Trpv1*$^{-/-}$, *Asic1*$^{-/-}$ or *Asic3*$^{-/-}$) mice (18-20g) were sacrificed using 200 mg/kg sodium pentobarbitone. The neck was opened by mid-line incision to expose the trachea and thorax, and segments of vagus nerve caudal to the nodose ganglion were dissected free. The nerves were immediately placed in modified Krebs solution (mM: NaCl 118; KCl 5.9; $MgSO_4$ 1.2; $CaCl_2$ 2.5; NaH_2PO_4 1.2; $NaHCO_3$ 25.5 and glucose 5.6; pH 7.4) and bubbled with 95% O_2 / 5% CO_2. Each nerve was cleared of connective tissue and carefully desheathed prior to experimentation. Care was taken throughout to ensure that the nerve trunks remained in oxygenated Krebs solution, and that they were not stretched or damaged in any way.

Human lung samples were either obtained through a collaboration with Harefield Hospital (tissue from transplant surgery excess to clinical requirements), or purchased from the International Institute for the Advancement of Medicine (IIAM, NJ, USA). Upon arrival at Imperial College, the lungs were immediately transferred to modified Krebs solution and bubbled with 95% O_2 / 5% CO_2. Cervical vagus and branches of the recurrent laryngeal nerves were dissected away from the trachea. The epineural and perineural sheath surrounding the vagus nerve in human airways is much tougher compared to that of guinea pig nerves, and is consequently harder to remove. As such,

special care was taken when attempting to desheath the nerve and it may not have been completely removed in all circumstances. The availability of human vagus nerve is scarce and therefore limits the number of experiments that can be performed, however, where possible key experiments have been carried out. Segments of nerve approximately 20-30mm long were prepared for measurement of depolarisation. The vagus nerves of seven donors (5 Female, 2 Male; 27-72 years) with no known respiratory disease were used in these experiments.

2.3.2 Measurement of depolarisation

The desheathed nerve trunk was mounted in a 'grease-gap' recording chamber (Figure 2.1). The nerve was drawn longitudinally through a narrow channel (2mm in diameter, 10mm in length) in a Perspex block. Larger chambers were available for human vagus nerves. The centre of the channel was filled with Vaseline, injected on to the middle of the vagus through a side-arm when the nerve was in place, creating an area of high chemical and electrical resistance, thereby isolating the extracellular space between the two ends of the nerve, which did not allow the passage of ions or drugs between solutions. One end of the nerve emerging into a wider channel was constantly superfused with Krebs solution with a flow rate of approximately 2 ml/min. Ag/AgCl electrodes (Mere 2 Flexible reference electrodes, World Precision Instruments), filled with Krebs solution, made contact at either end of the nerve trunk and recorded potential difference via a DAM50 differential amplifier (World Precision Instruments). Voltages were amplified x10, filtered at 1000Hz and sampled at 5Hz.

A change in membrane potential in response to stimulation (compound potential) of the vagus nerve fibres is detected by means of ionic exchange (mainly sodium and potassium) through the cell membrane (Figure 2.2). When one end of the nerve is stimulated with an agonist, Na^+ ions present in the Krebs solution move in to the nerve cell, leaving excess Cl^- ions. These Cl^- ions interact with the AgCl electrode making contact with that end of the nerve, giving an electrical charge to the circuit. In contrast, there would be no change in the Cl^- interaction with the electrode on the 'resting' side of the nerve. This causes a difference in potential between the two electrodes, which is amplified by the differential Bio-amplifier DAM50 and recorded by the chart recorder (Lectromed Multitrace2) as depolarisation of the nerve. During repolarisation, K^+ ions

move out of the nerve cell, which cause Cl^- ions to dissociate from the AgCl electrode, leading to a loss of electrical charge from the circuit. As a result, the potential difference between the two electrodes becomes more equal, and this is recorded as repolarisation. It should be noted that this preparation does not measure firing of action potentials but changes of membrane potential (or compound potentials), and therefore cannot distinguish between a sub-threshold and supra-threshold depolarisation (Figures 2.2 & 2.3).

Temperature of the perfusate was maintained at 37^{o}C by a water bath. Two systems were run in parallel allowing experiments and measurements to be taken from two different pieces of vagus simultaneously. The superfusing medium could be changed quickly with little artefact, by means of a multi-way tap. The new solution would reach the recording site with a delay of approximately 20 seconds. Vagus nerve depolarisation was induced by 2min perfusion of a known concentration of agonist on to the exposed end of the nerve, measured in millivolts (mV) and recorded on a pen chart recorder, calibrated such that 1mm was equivalent to 0.01mV. Following stimulation the nerve was washed with Krebs solution until responses returned to baseline. Each nerve section was stimulated no more than six times. Specific compounds used in experiments will be described in the results chapters.

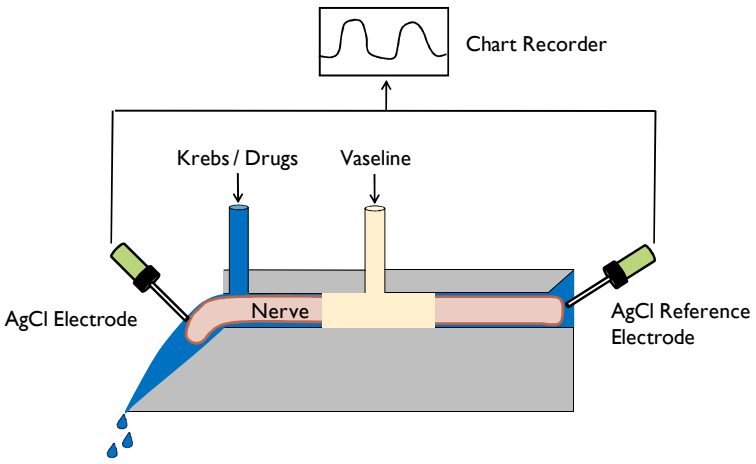


Figure 2.1. Diagram of the isolated vagus system.
The vagus nerve trunk was drawn through a narrow channel, and the two ends of the nerve electrically isolated with petroleum jelly. One end of the nerve (right) was bathed in Krebs solution and the other (left) superfused with either Krebs or the ligand being tested at a set drip rate of 2 ml/min. Electrodes made contact with either end of the nerve trunk, and measured the difference in electrical potential between the two ends. Nerve depolarisations were documented on a pen chart recorder.

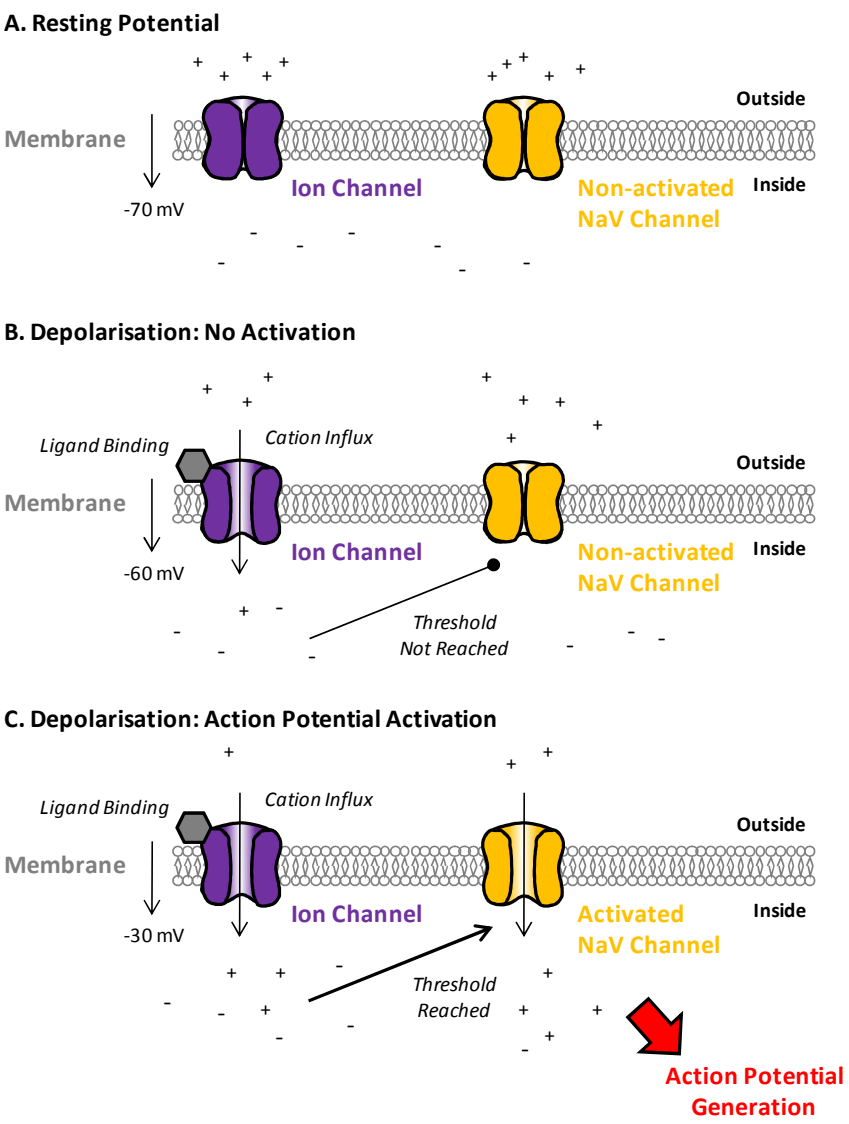
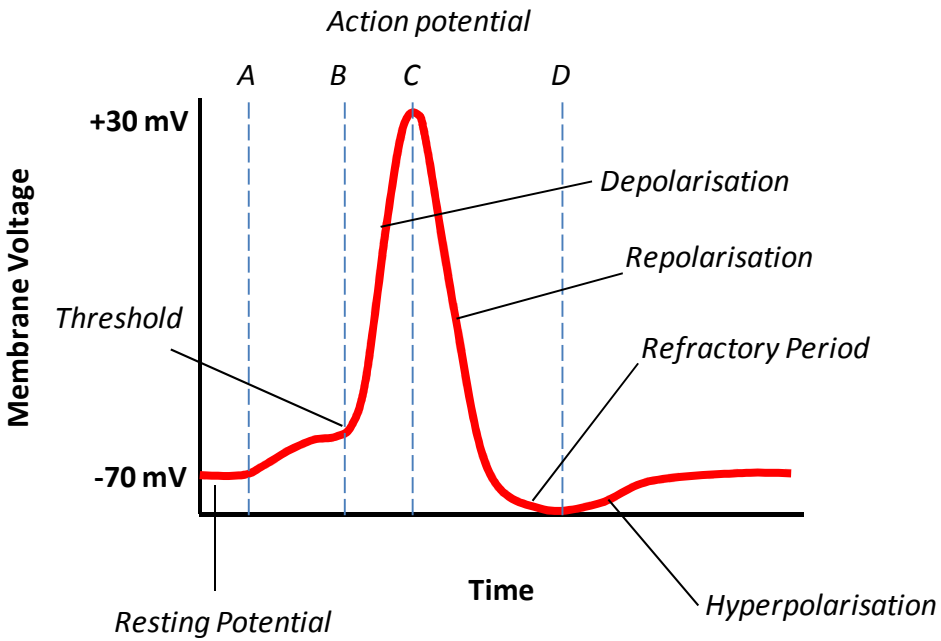


Figure 2.2. Explanation of membrane potential and depolarisation.

A. In the resting state, membrane potential is approximately -70 mV inside the cell compared to outside. This is largely due to the negative charge of proteins within the cell, and the large concentration of positively charged sodium outside the cell.

B. Activation of a ligand-gated ion channel, e.g. by binding of a ligand to its receptor, causes an influx of positively charged ions in to the cell down electrical and concentration gradients. For example, a number of the TRP channels are selective for calcium ions, but also allow the passage of other cations. This causes depolarisation, making the inside of the cell less-negative compared to the outside. In this example, the charge within the cell reaches -60 mV, which is not enough to cause the voltage-gated sodium (Na_V) channels to open (i.e. it does not reach 'threshold'). Action potentials are an all-or-none response; therefore, there is membrane depolarisation but no generation of action potentials.

C. If the membrane voltage does reach threshold, then the Na_V channels are activated, causing a massive influx of positively charged sodium ions in to the cell. This then leads to the generation of action potentials which are propagated along the nerve towards the CNS.



A. Cation Channel Opens

B. Sodium Channels Open

C. Potassium Channels Open / Sodium Channels close

D. Potassium Channels Close

Figure 2.3. Illustration of an action potential.

Action potentials are an all-or-none response, meaning that membrane depolarisation must reach a certain threshold in order to activate action potential generation. Action potentials are always the same, no matter how strong the stimulus. The strength of a stimulus is interpreted by the CNS in terms of how many action potentials are generated, and how they are spaced in time.

A. Resting membrane potential is approximately -70 mV. Opening of membrane-bound ion channels causes depolarisation.

B. If membrane depolarisation reaches threshold, voltage-gated sodium channels will open, causing a massive influx of positively charged sodium ions, which quickly depolarises the cell membrane to approximately +30 mV. This is the 'action potential' which is propagated along the nerve to the CNS.

C. At depolarised potentials, voltage-gated sodium channels inactivate, thereby stopping any further depolarisation of the cell membrane. In addition, voltage-gated potassium channels open, causing positively-charged potassium to flow down its concentration gradient, thereby repolarising the cell.

D. An action potential is always followed by an 'absolute refractory period', during which time the sodium channels are inactivated, and another action potential cannot fire. This is followed by a 'relative refractory period' during which time it is difficult to activate another action potential because potassium efflux has hyperpolarised the cell membrane. This ensures that propagation of action potentials can only go in one direction, towards the CNS. Finally, the cell membrane polarises to its resting potential.

2.3.3 Agonist and antagonist experimental protocols

Non-cumulative concentration-response curves were established for a number of known tussive stimuli. Equivalent stimulations with vehicle solutions were also tested to ensure they did not elicit nerve depolarisation of themselves. Stimuli were applied for 2 minutes, and then the tissue washed with Krebs solution until baseline was re-established. The magnitude of depolarisation (mV) for each concentration of agonist was recorded. After characterising the response to tussive agents on the vagus nerve, a sub-maximal concentration of each agonist was chosen to profile the effect of selective antagonists.

A classic pharmacological profile was used to assess the effect of an antagonist on agonist responses. Briefly, agonist was applied for 2 minutes and washed with Krebs, this was repeated to determine an average normal depolarisation. The nerve was then incubated with a concentration of antagonist for 10 minutes, immediately followed by 2 minutes incubation of agonist in the presence of antagonist and the degree of inhibition recorded. The agonist and antagonist were washed off, and a final 2-minute stimulation with agonist alone was performed to ensure nerve viability (Figure 2.4). The ability of the antagonist to inhibit agonist responses was calculated by comparing the magnitude of depolarisation induced in the presence of the antagonist with that of the initial two agonist-only stimulations, and expressed as % inhibition. Antagonist concentration-response curves were initially established against receptor-selective agonists to determine concentration-related inhibition. In addition, the effect of vehicle incubation was established to ensure that this was not having an effect on nerve stimulation. The concentrations of antagonists used in all subsequent experiments were that which produced the highest inhibition of its corresponding selective agonist without affecting the agonist for the alternate receptor.

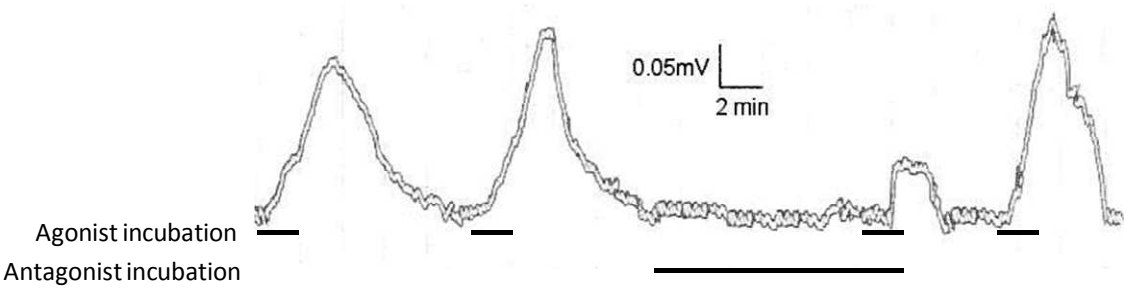


Figure 2.4. Example of a trace showing the protocol for testing antagonists.

Two 2-minute stimulations of an agonist were applied to the vagus nerve, ensuring reproducible depolarisations, this was followed by a wash-out period to re-establish baseline. An antagonist was then incubated for10-minutes, followed immediately by a 2-minute stimulation with agonist in the presence of antagonist. Both agonist and antagonist were then washed out to re-establish baseline and clear the antagonist. A final 2-minute stimulation of the vagus nerve by the agonist was applied to ensure recovery of the initial response and nerve viability.

2.4 Conscious Guinea Pig Cough Model

All experiments were performed in accordance with the U.K. Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act of 1986. Conscious, unrestrained guinea pigs (250-350 g, Harlan, UK) were placed in individual Perspex chambers (Buxco, USA). The apparatus consisted of two chambers, linked to a single nebuliser, allowing exposure of two animals to the same stimulus simultaneously. Guinea pigs were initially placed in the chambers and allowed to acclimatise for at least 5 minutes. Guinea pigs were then exposed to a nebulised aqueous solution of tussive agent or the appropriate vehicle (details for each stimulus are given in the results chapters). Aerosol was generated with an Aerogen nebuliser (Buxco, USA), and coughs counted for a total of 10 minutes. Each chamber was fitted with a microphone which amplified the cough sounds, and was connected to an external speaker. Coughs were detected by both pressure change and sound, and recorded by a Buxco Cough Analyser (Buxco, USA). A trained observer also manually counted the coughs, which were recognised by the characteristic opening of the mouth, stance and posture of the animal, the sound produced, and the flow recordings. For the antagonist studies, suspensions were dosed i.p. (maximum 10 ml/kg over two injection sites) one hour prior to agonist stimulation. Animals were monitored for any adverse events during this period. At the end of each experiment, the guinea pigs were euthanised with an overdose of sodium pentobarbitone (200 mg/kg).

2.5 Isolated Primary Vagal Ganglia Cells

2.5.1 Cell dissociation

Male Dunkin-Hartley guinea pigs (300-500g) were sacrificed by i.p. injection of sodium pentobarbitone (200 mg/kg). To dissect the vagal ganglia, the top of the skull was cut away and the brain removed. The ear bone on one side was then carefully removed to expose the jugular and nodose ganglia underneath. The surrounding membrane was cut away, the ganglia freed from adhering connective tissue and immediately placed in ice-cold Hank's balanced salt solution (HBSS; mM: 5.33 KCl, 0.441 KH_2PO_4, 138 NaCl, 0.3 Na_2HPO_4-$7H_2O$, 5.6 glucose, 5 HEPES; pH 7.4). This process was repeated for the second set of ganglia. Under a microscope in a sterile hood the nodose and jugular ganglia were cleared of any remaining connective tissue, and placed in room temperature sterile HBSS. The ganglia cells were then isolated using a two step enzymatic digestion technique. To break down the extracellular matrix and collagen, ganglia were incubated in activated papain buffer (Sigma; Papain 200 active units/ml [U/ml] in HBSS supplemented with 0.4 mg/ml L-cysteine, 0.5 mM EDTA and 1.5 mM $CaCl_2$) for 30 minutes at 37°C, with gentle agitation every 5 minutes. After centrifugation for 2 min at 1400 rpm, the papain buffer was disposed of, and the ganglia incubated at 37°C for 40 minutes Ca^{2+}-free and Mg^{2+}-free HBSS containing type 4 collagenase (CLS4, Worthington, 320 U/ml) and Dispase II (Roche; 1.92 U/ml at 37°C), with gentle agitation every 5-10 minutes. The cells were again centrifuged at 1400 rpm for 2 min, and the collagenase solution carefully removed. The cells were dissociated from remaining tissues, axons and satellite cells by tituration in 1 ml HBSS at room temperature with fire-polished glass Pasteur pipettes of decreasing tip pore size from 1 mm to 0.3 mm. Cells were separated from the remaining tissue by centrifugation for 9 min at 22°C (1400 rpm) in L15 medium containing 20% Percoll (v/v). The Percoll was washed off using 2 ml L15 medium, and after centrifugation (2300 rpm for 3 min at 22°C) the cells were resuspended in complete F-12 medium containing 10% FBS and 1% penicillin/streptomycin (Sigma Aldrich; final solution 2 U/ml penicillin and 10 mg/ml streptomycin). The cells were then plated on poly-d-lysine/Laminin (Sigma; 22.5 µg/ml) coated fluorodishes. After the suspended primary ganglia cells had been allowed to adhere for 2h in a 37°C, 95% O_2 / 5% CO_2 environment, the cell-attached fluorodishes were gently flooded with 2 ml of complete F12 medium. The plates were used for experimentation within 24h.

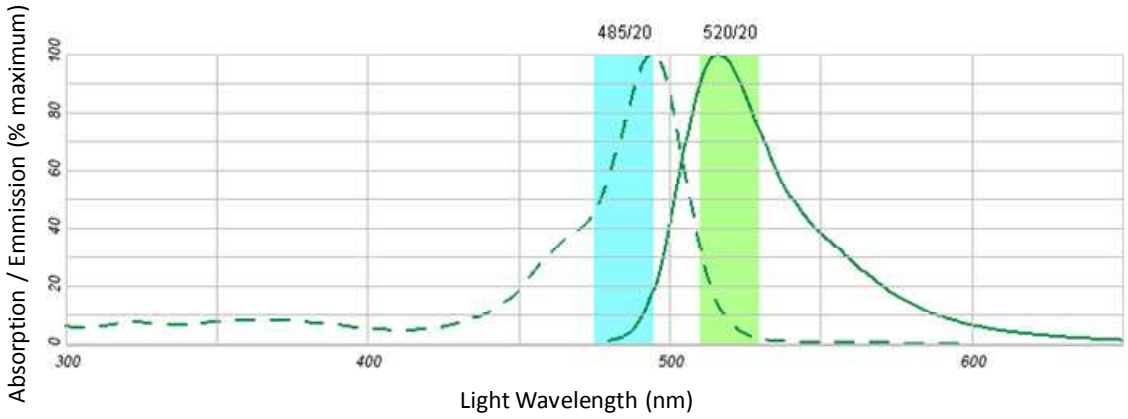
2.5.2 Loading cells with fluorescent dyes

Changes in intracellular calcium were determined by imaging of a dye which fluoresces upon binding to calcium. For initial experiments, fluorodishes were loaded with Fluo-4 AM (6 μM; Invitrogen) in extracellular solution (ECS; containing in mM: KCl 5.4, NaCl 136, $MgCl_2$ 1, $CaCl_2$ 1.8, NaH_2PO_4 0.33, D-Glucose 10, HEPES 10; pH adjusted with NaOH to 7.4 at 37°C) and incubated for 40 min in the dark at 25°C. The fluorodishes were then washed with ECS and the Fluo-4 AM dye was allowed to de-esterify to Fluo-4 for 30 min in the dark at 25°C before use. This was later changed for the ratiometric dye Fura-2 AM (3 μM; Invitrogen), using the same loading protocol, because Fura-2 is a ratiometric dye it is more suitable for longer experiments (such as the antagonist studies). Using a ratio of calcium-bound versus calcium-free signals avoids issues associated with photo bleaching of the dye, which causes a decrease in the subsequent signal when non-ratiometric dyes are used. AM dyes were used as a means of loading the hydrophilic Fluo-4 and Fura-2 dyes into the ganglia cells. AM esters make the dyes sufficiently hydrophobic that they are able to passively pass through the cell membrane by simply adding them to the extracellular medium. Intracellular esterases then cleave the AM group, and trap the dye inside the cell (Paredes et al., 2009).

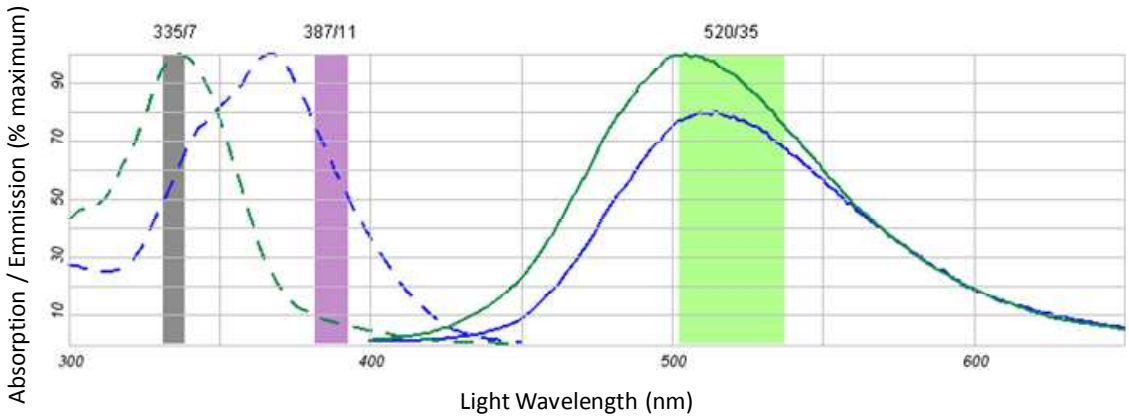
To record membrane potential changes, primary ganglia cell membranes were bound to the potentiometric dye 4-[2-[6-(dioctylamino)-2-naphthalenyl]ethenyl]-1-(3-sulfopropyl)-pyridinium (Di-8-ANEPPS; Invitrogen). Di-8-ANEPPS is a fast-response probe which exhibits a uniform change in fluorescence of approximately 7.5% per 100 mV change in membrane voltage (Hardy et al., 2006). Cells were loaded with Di-8-ANEPPS in ECS in the dark, during de-esterification of the calcium dye, loading time did not exceed 20 min and the cells were used immediately. During loading, temperature was maintained between 10-20°C to prevent internalisation of the dye and maximise localisation within the cell membrane.

Fluo-4 is excited by a Xenon light at λ=485 nm and emitted fluorescent light is recorded at λ=520 nm. Fura2 is excited at λ=340 nm and λ=380 nm and emitted fluorescence recorded at λ=520 nm. Di-8-ANEPPS is excited at λ=470 nm and recorded at λ=700 nm (Figure 2.5).

A. Fluo-4



B. Fura-2



C. Di-8-ANNEPS

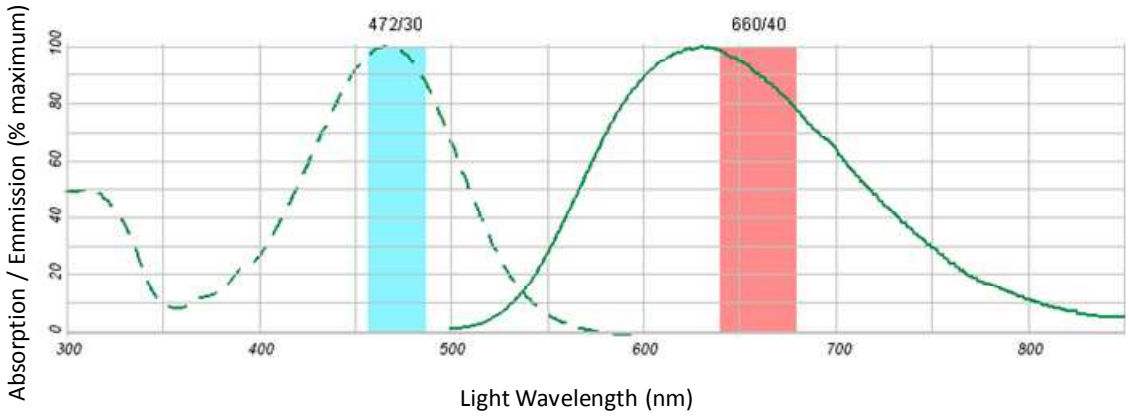


Figure 2.5. Absorption spectra, emission spectra and filters for each of the fluorescent dyes used in imaging experiments.

Dashed lines represent absorption spectra, and solid lines represent emission spectra. Coloured bands represent the filter and bandwidth used to excite and record each fluophore. (b) *Green traces denote calcium-bound Fura-2 and blue traces denote calcium-free Fura-2.*

2.5.3 Intracellular calcium and membrane voltage imaging

Intracellular free calcium ($[Ca^{2+}]_i$) measurements and membrane voltage changes were performed in dissociated jugular and nodose cells. Each fluorodish was placed in a full incubation chamber mounted at 37°C on the stage of a widefield inverted microscope Zeiss Axiovert 200 (Carl Zeiss Inc., NY, USA) equipped with an Hamamatsu EM-CCD C9100-02 camera for ultrafast low light imaging run by Simple PCI software. The cells were constantly superfused with 37°C ECS buffer using a house-designed pressurised solution-changing perfusion system allowing complete bath (600 µl volume) replacement in 3s (Figure 2.6). Prior to experiments, the cells were superfused for 5-10 min with ECS-only. Cells were excited and signal recorded using a Xenon gas Arc lamp Cairn ARC Optosource Illuminator, a Quad filter set Ex 485-20 Bs 475-495 Em 510-53120x and a LD Plan-Neofluar AIR Korr objective.

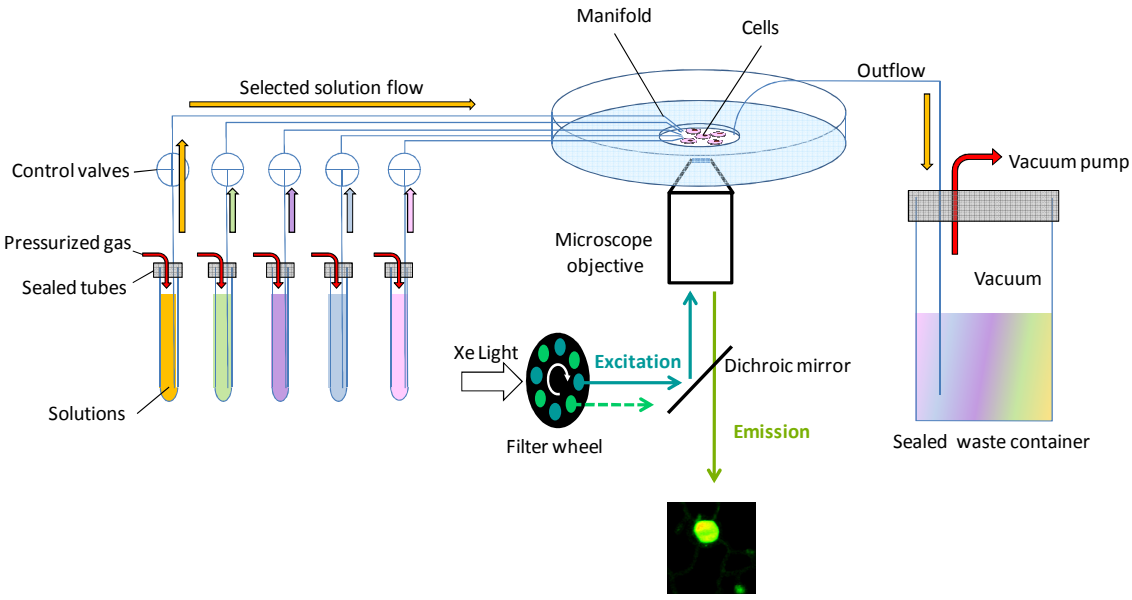


Figure 2.6. Diagram of the pressurised solution-changing perfusion system.

Pressurised gas is used to force the selected solution in to the imaging plate. Changes in fluorescence (from the calcium or voltage dyes) induced by perfusion of the solution is imaged via an epifluorescence microscope equipped with an appropriate set of filters changed via a filter wheel. Still images are captured by a camera every 1-20 seconds, and displayed on a computer screen. Excess solution is drawn out of the imaging plate by a negative pressure vaccum pump, with waste collected in a sealed container.

Potassium chloride in modified ECS solution (K50; containing in mM: 50 KCl, 91.4 NaCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 0.33 NaH_2PO_4, 10 glucose, 10 HEPES; adjusted to pH 7.4) was applied at the start and end of each experiment for 10s to assess cell viability and allow for normalisation of subsequent agonist signals. Concentrations of agonist were applied for 20-60s, and antagonists for 60s. After each application of agonist, perfusion was switched back to ECS until complete recovery to baseline of the calcium level. The same pharmacological antagonist protocol was used as the isolated vagus preparation (described in section 2.3.3), with the only variation being that the antagonists were incubated for a shorter period (60s) prior to agonist stimulation. To take into account the multiphasic responses obtained in some cells, $[Ca^{2+}]_i$ data were measured as total area under curve (A.U.C; total elevation of calcium above resting level over time), and expressed as % of the K50 A.U.C response to normalise the data between experiments. Only ganglia cells producing a fast response to K50 stimulation which was washable within 5min, and that had diameter of over 20 μm were analysed. In contrast, peak amplitude change in fluorescence of Di-8-ANNEPS was used to measure membrane voltage changes, expressed as % of K50 peak amplitude to normalise the data. In the results sections for all imaging experiments, n-numbers will be expressed as 'N' = number of animals and 'n' = total number of cells recorded from.

2.5.4 Retrograde labelling of airway primary vagal ganglia cells

Conscious male Dunkin Hartley guinea pigs (250-300g) were dosed i.n. with 1 ml/kg of a lipophilic carbocyanine dye DiIC18(3),1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Invitrogen). DiI has a very low cytotoxicity, and is highly fluorescent and photostable when incorporated into membranes. This dye uniformly labels neuronal cells *via* lateral diffusion in plasma membranes at a rate of 6mm a day *in vivo* due to an active dye transport process. DiI exhibits maximum excitation and emission fluorescence wavelengths at approximately λ=520-550 nm and λ=570 nm, respectively (Figures 2.7 & 2.8). A stock solution of 12.5 mg/ml DiI was made up in 100% ethanol. The solution was kept covered in aluminium foil to avoid bleaching of the dye in light. Immediately before use, the stock solution was diluted in 1/50 with 0.9% saline to make a final working concentration of 0.25 mg/ml DiI and 2% ethanol in saline.

Fourteen days after i.n. dosing with DiI (1 ml/kg), the guinea pigs were sacrificed by i.p. injection of pentobarbitone (200 mg/kg), the primary vagal ganglia cells isolated, and calcium imaging experiments carried out as described above. This allowed sufficient time for the DiI tracer to travel to the vagal ganglia and stain airway cells (Undem et al., 2004).

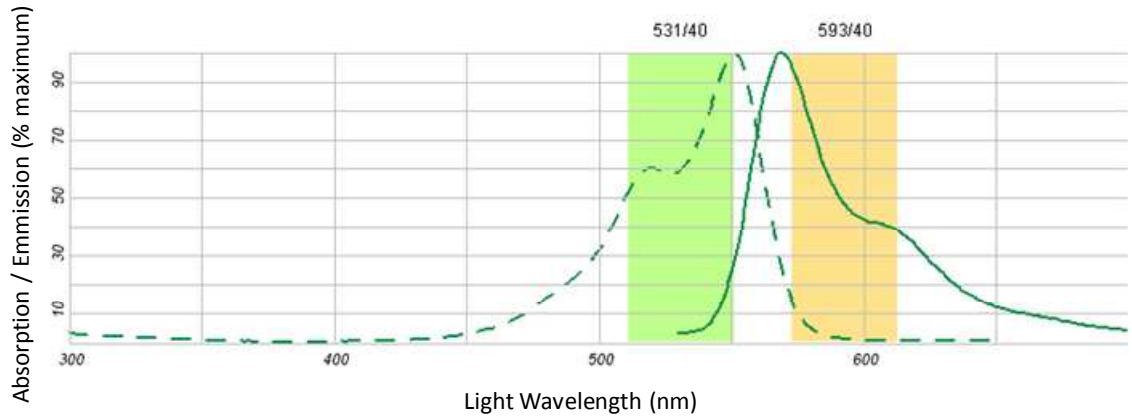


Figure 2.7. Absorption spectra, emission spectra and filters for DiI.

Dashed lines represent absorption spectra, and solid lines represent emission spectra. Coloured bands represent the filter and bandwidth used to excite and record the fluophore.

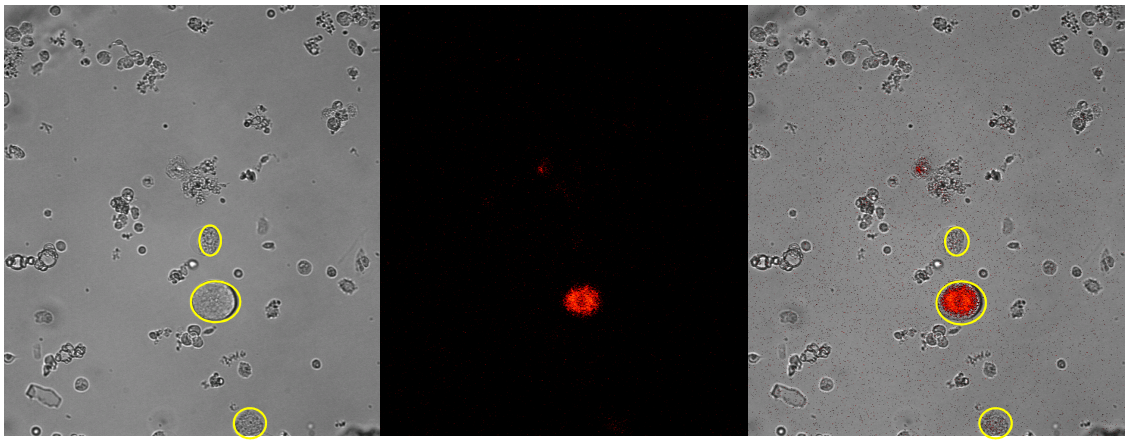


Figure 2.8. Fluorescent staining of airway primary vagal ganglia cells.

Isolated sensory cells collected 14 days after i.n. dosing of guinea pigs with DiI. Staining is shown in the middle panel. Primary ganglia cells are outlined in yellow on bright field images (left) and on combined bright field / DiI fluoresence images (right). In this example, 1 out of 3 cells are stained.

CHAPTER 3

Characterisation of Models and Pharmacological Tools

3.1 Rationale

Both capsaicin and resiniferatoxin are well-established TRPV1-selective agonists that have been shown to induce coughing in humans and animals (Karlsson & Fuller, 1999; Lalloo et al., 1995; Morice et al., 2007, 2001; Trevisani et al., 2004). However, the role that other receptors play in the cough reflex has yet to be studied in detail. In this chapter I test the hypothesis that, due to its co-expression on nociceptive neurons and ability to bind a multitude of known noxious stimuli, the TRPA1 ion channel is another potential tussive mediator. These studies are important because many environmental and endogenous irritants are known to be TRPA1 or TRPV1 activators, and as such these receptors could play a role in cough hypersensitivity associated with inflammatory airways disease. In particular, a decrease in airways pH and an increase in the release of endogenous inflammatory mediators such as PGE_2 and BK have been associated with airways disease. All three of these mediators induce coughing of themselves, and can also sensitise the cough response to other tussive mediators (Choudry et al., 1989; Costello et al., 1985; Katsumata et al., 1991; Lalloo et al., 1995; Maher et al., 2009). Low pH directly gates the TRPV1 ion channel, and PGE_2 is thought to activate TRPV1 downstream of its G protein-coupled receptor. Furthermore, BK mediates some of its nociceptive effects via activation of the TRPA1 or TRPV1 ion channels through the intracellular phospholipase C pathway. Due to their activation by such a wide variety of compounds, it is possible that TRPA1 and TRPV1 could be mediating the cough induced by these endogenous irritants.

In order to investigate a role for TRPA1 and TRPV1 in mediating the tussive effects of low pH, PGE_2 and BK, it was initially necessary to characterise the *in vitro* and *in vivo* models of cough that I would be using. I began with an *in vitro* isolated vagal tissue preparation, which is a well-established model in our lab, and has been shown to be predictive of the cough reflex *in vivo* (Maher et al., 2009; Patel et al., 2003; Usmani et al., 2005). The isolated vagus nerve system provides an ideal opportunity to pharmacologically assess TRP receptor agonists and antagonists, while avoiding potential pharmacokinetic issues which are inherent in *in vivo* models. This system also allows for comprehensive characterisation with lesser quantities of compound compared to *in vivo*, and reduces both the number of animals and cost. Moreover, a number of species can be used. Guinea pigs, unlike rats and mice, possess a functional cough reflex and were therefore the species of choice for the *in vitro* model. Mouse vagus nerves can also be used in this system; though mice do not possess a cough

reflex *per se*, the afferent arm of the reflex still appears to be intact and behaves similarly to guinea pig and human vagus nerves (Birrell et al., 2009; Maher et al., 2009). This permits the use of genetically modified animals which have had the TRPA1 or TRPV1 gene disrupted, allowing investigation in to the importance of a particular receptor without pharmacological intervention. I also had access to human vagus nerves obtained from donor tissue surplus to clinical requirement and donated for scientific research purposes, thereby allowing verification of the key results in a human model (for patient details refer to Chapter 2). The initial aims of this chapter were, therefore, to assess the ability of TRPA1 and TRPV1 agonists to depolarise isolated vagus nerves; to determine the ability of TRPA1 and TRPV1 antagonists to inhibit this response; and to establish the selectivity of these compounds for their reported receptors.

Though many pharmacological compounds show promise *in vitro*, their effects do not always translate *in vivo* due to pharmacokinetic and pharmacodynamic issues. Guinea pigs are the only small animal to exhibit a functional cough reflex which resembles human cough. These animals provide the opportunity to assess a functional cough reflex at a much lower cost than, for example, the cat or dog. TRPV1 agonists are already known to induce coughing in guinea pigs, and both capsaicin and citric acid have been established as reliable tussive agonists in our lab. Therefore, the second aim of this chapter was to verify the *in vitro* findings on the vagus nerve in a conscious guinea pig cough model. That is, to establish the ability of TRPA1 agonists to induce cough, and the efficacy of the TRP-selective antagonists to inhibit cough *in vivo*.

3.2 Methods

3.2.1 Genotyping of Genetically Modified Mice

Breeding pairs of mice devoid of the *Trpa1*, *Trpv1*, *Accn2* (ASIC1) or *Accn3* (ASIC3) genes were backcrossed on to the C57Bl/6j background. Homozygous breeding pairs of *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$ mice were purchased from Jackson Laboratories (USA) and *Asic3*$^{-/-}$ mice were kindly provided by Dr. Welsh (University of Iowa). Heterozygous *Asic1*$^{+/-}$ mice were provided by Professor Fugger (John Radcliffe Hospital, University of Oxford) and subsequently bred to obtain a homozygous knockout colony. Breeding colonies were maintained at Imperial College, London.

Following DNA extraction and quantification, the DNA sections in between the primer pairs were exponentially amplified using PCR (see chapter 2, section 2.2.2 for details). Samples were heated to the denaturing temperature for 2 minutes, followed by 40 cycles of denaturing, annealing and extension steps (specified in Table 3.1). A final 10 minutes at the same temperature as the extension step was run to ensure that all products were full length. The reaction was then stopped by cooling to 4°C for 5 minutes. The PCR products and a DNA ladder (Hyperladder IV, Bioline Ltd, London) were run on a 2% agarose gel in Tris Borate EDTA (TBE) buffer containing 0.05 µl/ml Safeview (NBS Biologicals Ltd, Huntingdon, UK) at 80 V for 1 hour. Finally, the gel was visualised under ultra-violet light and photographed. Expected base pairs (bp) for the wild type and knockout primers are detailed in Table 3.1. Wild type and knockout primers for the TRPA1, TRPV1 and ASIC3 genes were run and visualised in the same reaction, whereas, primers for the ASIC1 gene were run in separate reactions.

Table 3.1. Genotyping PCR conditions and expected primer bands for wild type and genetically modified mice.

Mouse Type	PCR Conditions			Expected Primer Bands	
	Denaturing	Annealing	Extension	Wild Type	Knockout
TRPA1	30 s / 95°C	30 s / 68°C	60 s / 72°C	317 bp	184 bp
TRPV1	30 s / 95°C	60 s / 64°C	60 s / 72°C	984 bp	600 bp
ASIC1	30 s / 94°C	30 s / 65°C	60 s / 75°C	267 bp	280 bp
ASIC3	30 s / 94°C	30 s / 59°C	60 s / 68°C	400 bp	600 bp

Abbreviations: bp = base pairs; s = seconds

3.2.2 Characterisation of the isolated vagus nerve preparation

3.2.2.1 Guinea pig vagus nerve

Non-cumulative concentration-response (CR) curves were established for a number of TRPV1- and TRPA1-selective stimuli. In the guinea pig, CRs were established for the TRPV1 agonists capsaicin (0.1, 1, 10, 100 µM) and resiniferatoxin (1, 3, 10, 30 nM); and the TRPA1 agonists acrolein (0.1, 0.3, 1, 3 mM), cinnamaldehyde (0.1, 0.3, 1, 3 mM), and allyl-isothiocyanate (0.1, 0.3, 1, 3 mM). An equivalent stimulation with vehicle (0.1% DMSO v/v for all agonists) was also tested to ensure this did not elicit nerve depolarisation of itself. Stimuli were applied for 2 minutes, and then the tissue washed with Krebs solution until recovery of baseline. After characterisation, sub-maximal concentrations of agonist (1 µM capsaicin and 300 µM acrolein) were chosen to profile the effect of TRPA1- and TRPV1-selective antagonists.

A concentration of antagonist was applied to the vagus in combination with the agonist to measure degree of inhibition in depolarisation (detailed in chapter 2, section 2.3.3). CRs for the TRPV1 antagonists capsazepine (1, 3, 10 µM) and JNJ17203212 (0.1, 1, 10, 100 µM) were established against 1 µM capsaicin; and the TRPA1 antagonist HC-030031 (3, 10, 30 µM) against 300 µM acrolein. In addition, the effect of vehicle (0.1% DMSO v/v for all antagonists) was established to ensure that this was not having an effect on nerve stimulation. The concentration of antagonist which produced maximal inhibition was then tested against

the alternate agonist to demonstrate that there was no off-target effect at the selected concentration. Therefore, 10 µM capsazepine and 100 µM JNJ172032121 were tested against 300 µM acrolein; and 10 µM HC-030031 was tested against 1 µM capsaicin.

3.2.2.2 Mouse vagus nerve

Non-cumulative CR curves in wild type C57Bl/6j mice were established for capsaicin (0.1, 1, 10, 100 µM), resiniferatoxin (1, 3, 10, 30 nM), acrolein (0.1, 0.3, 1, 3 mM), cinnamaldehyde (0.1, 0.3, 1, 3 mM) and allyl-isothiocyanate (0.3, 1, 3, 10 mM), or vehicle (0.1% DMSO v/v) as described for the guinea pig above. After characterising the response to tussive agents on the vagus nerve, sub-maximal concentrations of agonist (1 µM capsaicin and 300 µM acrolein) were chosen for further experiments. The ability of these agonists to activate vagus nerves from *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$ mice was compared to wild type (C57Bl/6j), thereby giving an indication of their selectivity at the concentration chosen.

TRPA1- and TRPV1-selective antagonist CRs were then established for the mouse. The TRPV1 antagonists capsazepine (1, 3, 10 µM) and JNJ17203212 (1, 10, 100 µM) were tested against 1 µM capsaicin; and the TRPA1 antagonist HC-030031 (3, 10, 30 µM) was tested against 300 µM acrolein. In addition, the effect of vehicle (0.1% DMSO v/v) was established to ensure that this was not having an effect on nerve stimulation. The concentration of each antagonist which produced maximal inhibition was then tested against the alternate agonist to demonstrate the selectivity of the antagonist at this concentration. Therefore, 10 µM capsazepine and 100 µM JNJ172032121 were tested against 300 µM acrolein; and 10 µM HC-030031 was tested against 1 µM capsaicin.

3.2.2.3 Human vagus nerve

Due to the scarcity of human vagus tissue, only selected concentrations of agonist and antagonist were tested, based on effective concentrations in the guinea pig experiments. Therefore, the ability of 1 µM capsaicin and 300 µM acrolein to activate human vagal tissue was verified. Following this, the ability of vehicle (0.1% DMSO v/v), JNJ17203212 (100 µM) or HC-030031 (10 µM) to antagonise capsaicin and acrolein responses was determined.

3.2.3 Characterisation of the conscious guinea pig cough model

Conscious, unrestrained guinea pigs (300-400g, Harlan, UK) were individually placed in a closed chamber. Animals were exposed to a 5 minute aerosol of capsaicin (15, 30, 60, 90 µM), acrolein (10, 30, 100, 300 mM), or appropriate vehicle (1% ethanol plus 1% tween 80 in saline for capsaicin; 0.9% saline for acrolein). Each guinea pig was exposed to only one concentration of agonist. The number of coughs was recorded during the 5 minute agonist stimulation and for a further 5 minutes post-stimulation (10 minutes in total) as described in chapter 2, section 2.4.

Once a suitable concentration of agonist which elicited robust coughing had been determined, dose-responses for the selective antagonists HC-030031 (30, 100, 300, 100 mg/kg; TRPA1) or JNJ17203212 (30, 100, 300, 1000 mg/kg; TRPV1) or appropriate vehicle (0.5% methyl cellulose in sterile saline for HC-030031; 15% solutol in 5% dextrose solution for JNJ17203212) were established as described in chapter 2, section 2.4. Antagonists were injected i.p. one hour prior to aerosol stimulation with either acrolein (100 mM) or capsaicin (60 µM). In addition, at the dose which maximally inhibited its own receptor (300 mg/kg HC-030031 and 100 mg/kg JNJ17203212) the antagonists were tested against the alternate agonist to demonstrate that there was no off-target effect at the selected dose. Agonists were aerosolised for 5 minutes, and the number of coughs counted for 10 minutes.

3.2.4 Data Analysis

Antagonism of agonist-induced vagal sensory nerve activation was analysed by paired t-test, comparing responses in the same nerve before and after antagonist incubation. Antagonism of agonist-induced cough was analysed using the Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison post-hoc test comparing all groups to vehicle. Significance was set at $p < 0.05$, and all data were plotted as mean ± s.e.m. of *n* observations.

3.3 Results

3.3.1 Genotyping of genetically modified mice

As discussed in section 3.1, though mice do not possess a functional cough response, the isolated vagus nerves from wild type mice display similar depolarising responses to irritant stimuli in comparison to both guinea pig and human nerves (Birrell et al., 2009; Maher et al., 2009). It therefore appears that the afferent arm of the cough reflex is intact in these animals, and they are hence a very useful model to study cough as we are able to manipulate their genetic template. Pairs of mice genetically modified to remove the gene for the TRPA1, TRPV1, ASIC1 or ASIC3 receptor were obtained, and breeding colonies maintained at Imperial College. Each colony was genotyped before and sporadically during experimentation to confirm knockdown of the appropriate gene. Only male homozygous knockout animals were used in the isolated vagus experiments. All of the genetically modified mice were bred on a C57BL/6j background, and as such wild type C57BL/6j mice were used as the appropriate control.

All animals from the *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$ breeding colonies were confirmed to be homozygous knockouts. Knockdown of the TRPA1 and TRPV1 gene was successfully maintained in these colonies. Primers and probes were designed so that a single band at 184 base pairs (bp) or 600 bp indicates a homozygous TRPA1 or TRPV1 knockout animal, respectively. Whereas, a single band at 317 bp or 984 bp indicates the associated wild type gene (Figure 3.1).

Mice for the ASIC1 colony were received as heterozygous *Asic1*$^{+/-}$ animals, which were bred together. Subsequent generations were genotyped until a full knockout colony was established (Figure 3.2A). Because the number of bp for the wild type and knockout genes is very similar, the primers and probes were run in separate reactions. A single band at 280 bp indicates a homozygous ASIC1 knockout animal, whereas, a single band at 267 bp indicates a wild type animal. DNA from animals showing two bands (one for each reaction) indicates a heterozygous animal. Once established, knockdown of the ASIC1 gene was successfully maintained in this colony.

Mice for the ASIC3 colony were received as homozygous knockouts, and this was confirmed via genotyping (Figure 3.2B). A single band at 600 bp indicates a homozygous ASIC3 knockout animal, whereas, a single band at 400 bp indicates a wild type animal. Knockdown of the ASIC3 gene was successfully maintained in this colony.

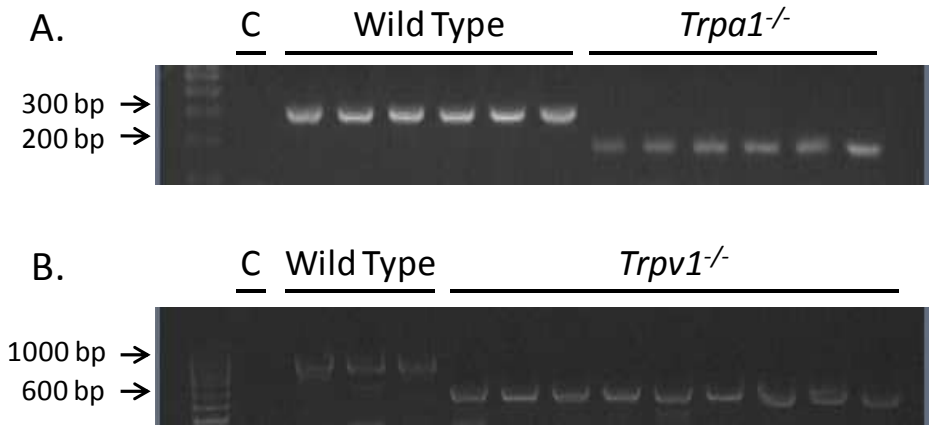


Figure 3.1. Genotyping of DNA extracted from wild type and genetically modified mice with the TRPA1 or TRPV1 gene disrupted.

These gels show DNA extracted from wild type (C57BL/6j) and genetically modified animals. (a) The primers for the wild type TRPA1 gene produce a band at 317 bp and the primers for the disrupted gene produce a band at 184 bp. (b) The primers for the wild type TRPV1 gene produce a band at 984 bp and the primers for the disrupted gene produce a band at 600 bp. Primers and probes for both the wild type and disrupted gene were run in the same reaction. Only single bands were observed, indicating that the colonies consisted of only wild type or homozygous knockout animals, and no heterozygotes. C = water (negative control).

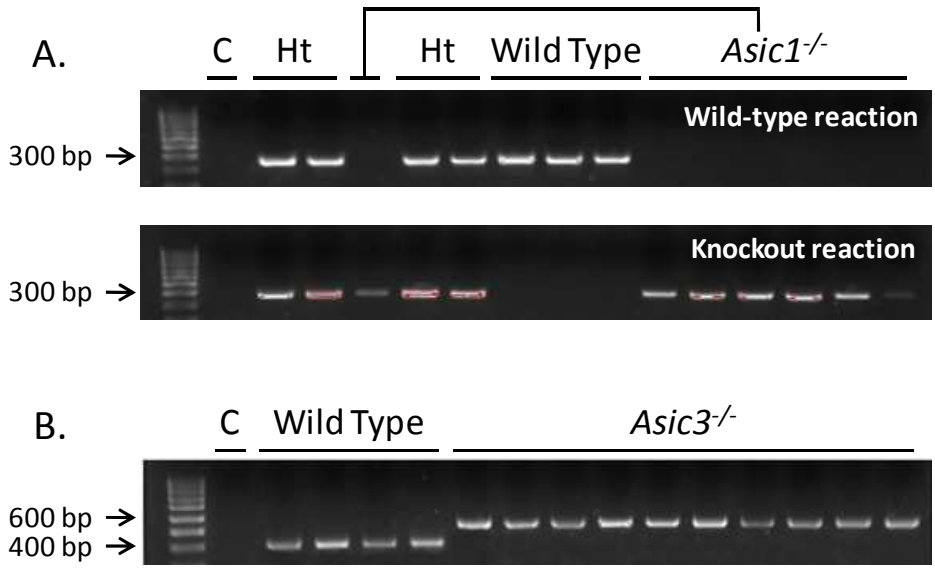


Figure 3.2. Genotyping of DNA extracted from wild type and genetically modified mice with the ASIC1 or ASIC3 gene disrupted.

These gels show DNA extracted from wild type (C57BL/6j), and genetically modified animals. (a) The primers for the wild type ASIC1 gene produce a band at 267 bp and the primers for the disrupted gene produce a band at 280 bp. (b) The primers for the wild type ASIC3 gene produce a band at 400 bp and the primers for the disrupted gene produce a band at 600 bp. Primers and probes for the ASIC1 wild type or disrupted gene were run in separate reactions, whereas primers and probes for the ASIC3 wild type or disrupted gene were run in the same reaction. Single bands indicate homozygous wild type or knockout animals, whereas double bands indicate heterozygous (Ht) animals. C = water (negative control).

3.3.2 Characterisation of isolated vagus nerve responses

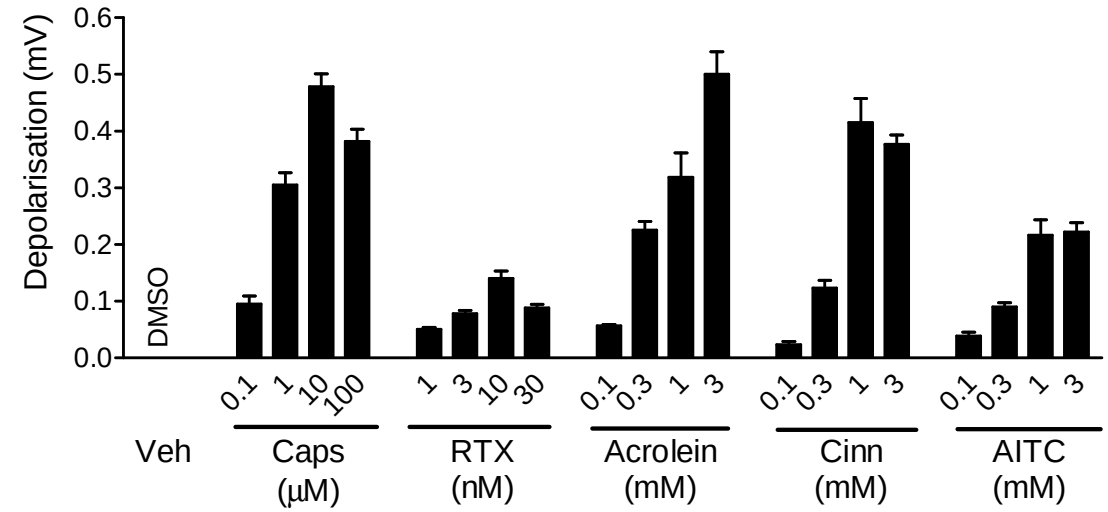
Our lab has previously established the isolated vagus preparation as a reliable *in vitro* model for cough, having shown that stimulants which activate the vagus nerve also induce coughing in both animals and humans (Belvisi et al., 2008; Birrell et al., 2009; Maher et al., 2009). I began by determining concentration-responses for known TRPA1- and TRPV1-selective agonists and antagonists in guinea pig and mouse isolated vagus nerves. I then went on to ascertain the selectivity of these compounds for their reported receptor at the concentrations which were chosen for further experimentation. Key experiments were repeated in human vagal tissue.

3.3.2.1 Sensory nerve activation by TRP-selective agonists

Depolarisation, which is a measure of sensory nerve activity, was recorded while perfusing a concentration of agonist over a 2 minute period. Vehicle (0.1% DMSO) had no effect on the nerves, whereas all other stimuli caused concentration-dependent increases in magnitude of depolarisation in both the guinea pig and mouse vagus nerves (Figures 3.3A & 3.4A). Selected concentrations of capsaicin (1 µM) and acrolein (300 µM) also induced depolarisation in human isolated vagus nerves (Figure 3.5). During experimentation it was observed that multiple stimulations with a TRPA1 ligand could desensitise subsequent nerve responses in guinea pig and mouse vagal tissue (Figures 3.3B & 3.4B). This was investigated further, as the pharmacological antagonist profile requires multiple reproducible stimulations with an agonist. After ascertaining these effects, it was decided to move ahead with a concentration of 300 µM acrolein for future vagus studies, because this concentration induced an acceptable magnitude of depolarisation in both the guinea pig and mouse nerves without causing desensitisation. A 1 µM concentration of capsaicin was also chosen for further experiments because this TRPV1 agonist evokes a large and reproducible magnitude of depolarisation, in contrast to resiniferatoxin responses which tend to be smaller, and difficult to wash out.

Having determined an appropriate concentration of TRPA1 and TRPV1 agonists with which to move forward, I then proceeded to establish the selectivity of these agonists for their reported receptor. At 300 µM, the TRPA1 agonist acrolein induced normal responses in both wild type and *Trpv1*$^{-/-}$ mouse vagus nerve, but failed to cause depolarisation in *Trpa1*$^{-/-}$ tissue. Conversely, 1 µM of the TRPV1 agonist capsaicin induced normal responses in wild type and *Trpa1*$^{-/-}$ nerves, but failed to cause depolarisation in *Trpv1*$^{-/-}$ tissue (Figure 3.6).

A.



B.

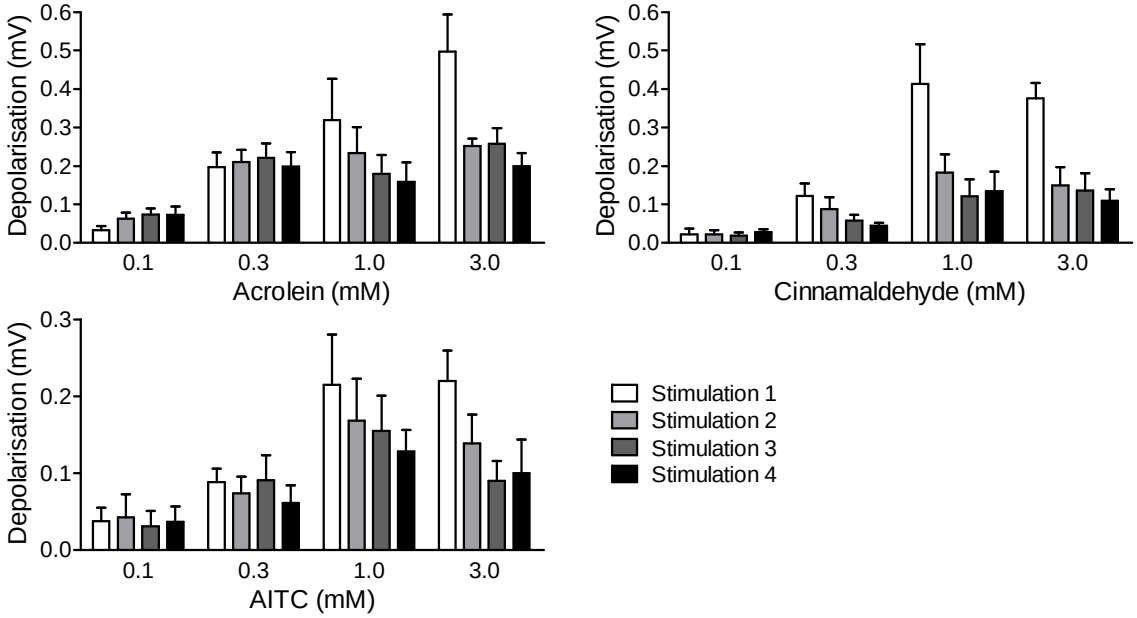
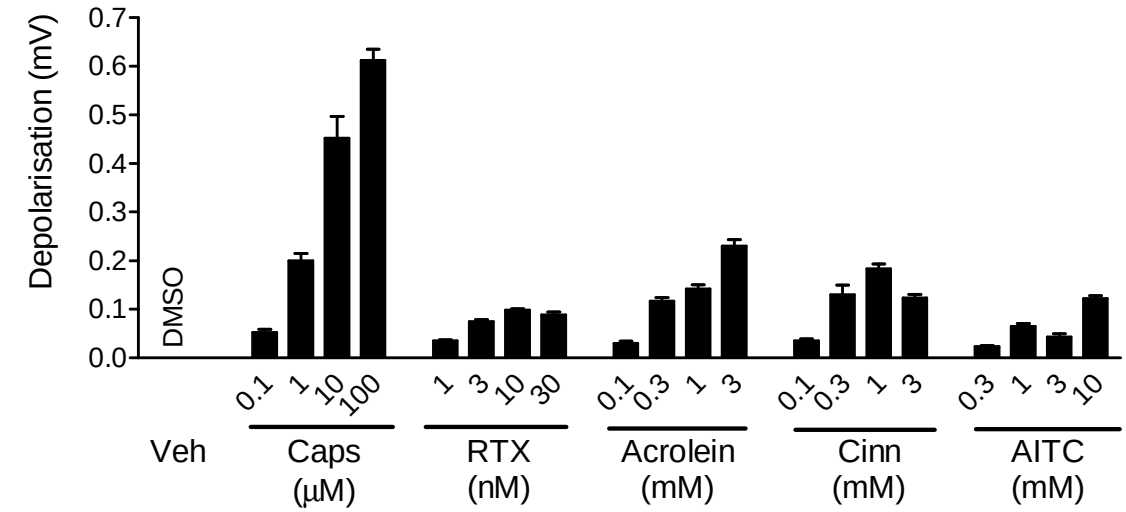


Figure 3.3. Characterising TRPV1 and TRPA1 selective agonists on the guinea pig isolated vagus nerve.

Agonists were applied to guinea pig isolated vagus nerves for 2 minutes in a random order. (a) *Concentration responses were established for TRPV1 (capsaicin [Caps], resiniferatoxin [RTX]) and TRPA1 (acrolein, cinnamaldehyde [Cinn], allyl isothiocyanate [AITC]) agonists, and vehicle (0.1% DMSO [Veh]).* (b) *TRPA1 agonists were found to desensitise the vagus nerve with consecutive stimulations. Magnitude of depolarisation (mV) was recorded, and data plotted as mean ± s.e.m. of n = 6 observations.*

A.



B.

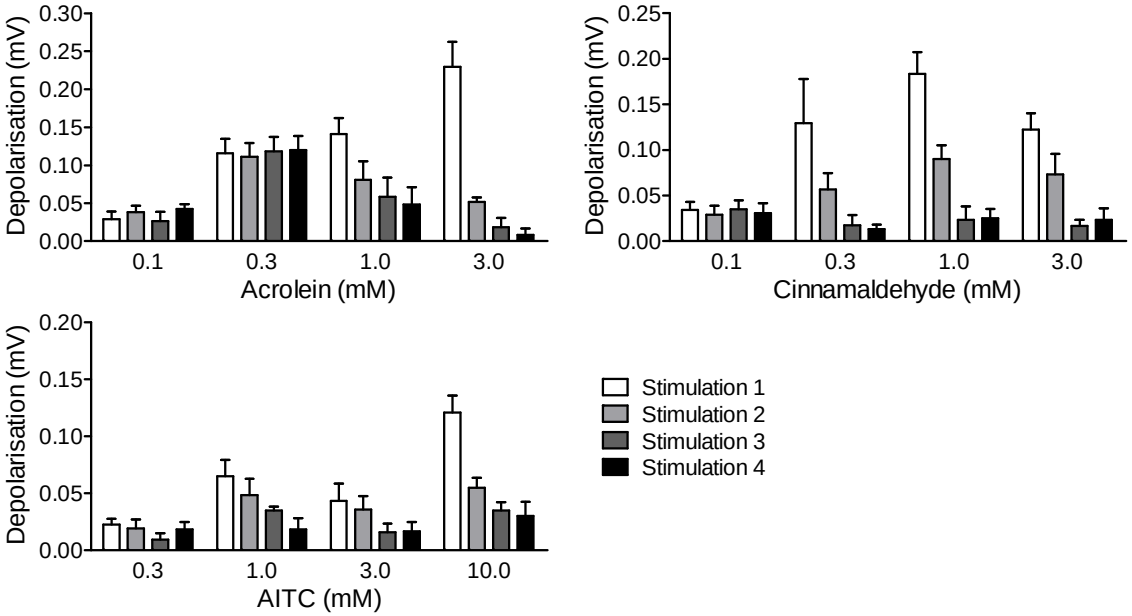


Figure 3.4. Characterising TRPV1 and TRPA1 selective agonists on the mouse isolated vagus nerve.

Agonists were applied to wild type mouse (C57BL/6j) isolated vagus nerves for 2 minutes in a random order. (a) *Concentration responses were established for TRPV1 (capsaicin [Caps], resiniferatoxin [RTX]) and TRPA1 (acrolein, cinnamaldehyde [Cinn], allyl isothiocyanate [AITC]) agonists, and vehicle (0.1% DMSO [Veh]).* (b) *TRPA1 agonists were found to desensitise the vagus nerve with consecutive stimulations. Magnitude of depolarisation (mV) was recorded, and data plotted as mean ± s.e.m. of n = 6 observations.*

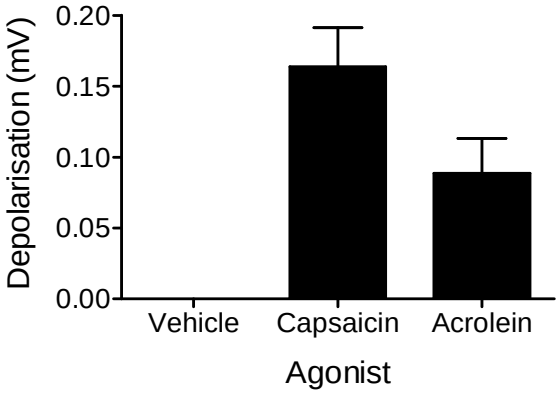


Figure 3.5. TRPV1 and TRPA1 selective agonists activate human isolated vagus nerves.

Agonists were applied to human isolated vagus nerves for 2 minutes. Both TRPV1 (1 µM capsaicin) and TRPA1 (300 µM acrolein) selective agonists induced sensory nerve depolarisation. In contrast, vehicle (0.1% DMSO) did not activate human vagus nerves. Magnitude of depolarisation (mV) was recorded, and data plotted as mean ± s.e.m. of n = 4-8 observations.

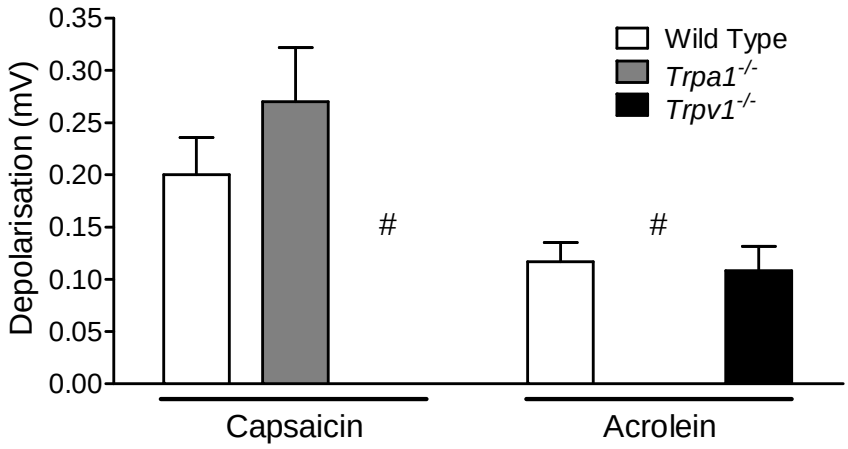


Figure 3.6. Establishing the selectivity of TRPV1 and TRPA1 agonists on the mouse isolated vagus nerve.

Capsaicin (1 µM) or acrolein (300 µM) was applied to wild type (C57BL/6j), Trpa1$^{-/-}$ or Trpv1$^{-/-}$ mouse isolated vagus nerves for 2 minutes. The selectivity of these agonists for their reported receptor was confirmed by the lack of response in the corresponding knockout mouse, while showing normal magnitudes of depolarisation (mV) in wild type and tissue from the alternate knockout mouse. Data are plotted as mean ± s.e.m. of n = 6 observations. # indicates a lack of depolarising response to agonist stimulation.

3.3.2.2 Inhibition of sensory nerve activation by TRP-selective antagonists

After confirming that the TRP-selective ligands induced depolarisation, and that they were indeed selective for their reported receptor, I went on to establish concentration responses for the associated TRP-selective antagonists. The TRPV1 selective antagonists capsazepine and JNJ17203212 both concentration-dependently inhibited the TRPV1-selective agonist capsaicin in guinea pig (Figure 3.7) and mouse (Figure 3.8) isolated vagus nerves. At 10 µM and 100 µM, respectively, capsazepine and JNJ17203212 blocked capsaicin responses by 100% in both species ($p < 0.05$). Moreover, 100 µM JNJ17203212 inhibited capsaicin responses by 100% in human tissue ($p < 0.05$). In contrast, at these concentrations, neither capsazepine nor JNJ17203212 had an off-target inhibitory effect on acrolein-induced nerve depolarisation. Alternately, the TRPA1 selective antagonist HC-030031 concentration-dependently inhibited acrolein-induced responses in guinea pig (Figure 3.7) and mouse (Figure 3.8) vagus nerves. At 10 µM, HC-030031 inhibited acrolein responses by 90 ± 7% in guinea pig, 91 ± 7% in mouse, and 93 ± 10% in human tissue ($p < 0.05$). HC-030031 showed no off-target effect on capsaicin-induced nerve depolarisation at this concentration.

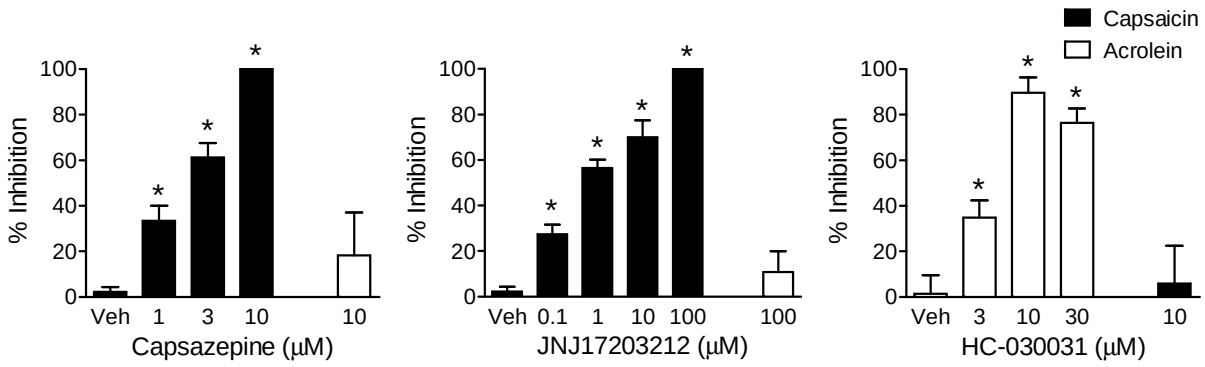


Figure 3.7. Characterising TRPV1- and TRPA1-selective antagonists on the guinea pig isolated vagus nerve.

*Concentration responses were established for TRPV1 (capsazepine, JNJ17203212) and TRPA1 (HC-030031) selective antagonists or vehicle (Veh, 0.1% DMSO) against TRPV1 (1 µM capsaicin, black bars) and TRPA1 (300 µM acrolein, white bars) selective agonists. The concentration which maximally inhibited its own receptor was then tested against the alternate agonist to establish selectivity at the chosen concentration. Data represent mean ± s.e.m of n = 6 observations. * indicates statistical significance ($p < 0.05$; paired t-test), comparing responses in the same nerve before and after antagonist incubation.*

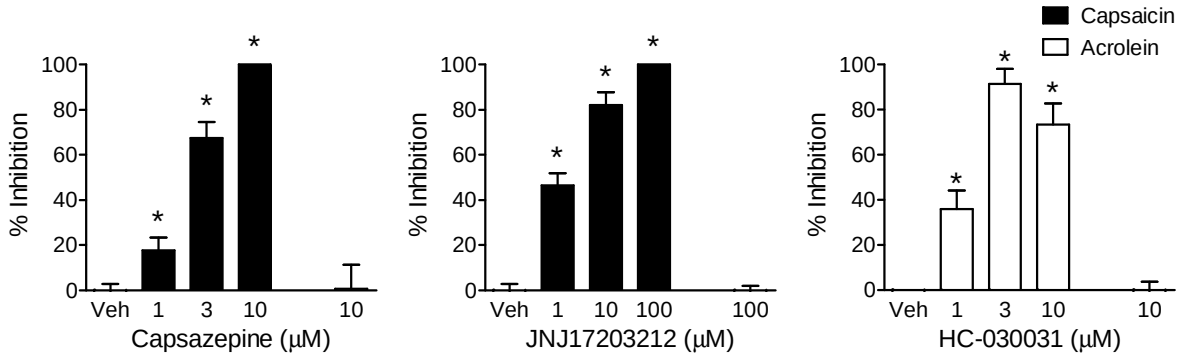


Figure 3.8. Characterising TRPV1- and TRPA1-selective antagonists on the mouse isolated vagus nerve.

*Concentration responses were established for TRPV1 (capsazepine, JNJ17203212) and TRPA1 (HC-030031) selective antagonists or vehicle (0.1% DMSO [Veh]) against TRPV1 (1 μM capsaicin, black bars) and TRPA1 (300 μM acrolein, white bars) selective agonists. The concentration which maximally inhibited its own receptor was then tested against the alternate agonist to establish selectivity at the chosen concentration. Data represent mean ± s.e.m of n = 6 observations. * indicates statistical significance (p < 0.05; paired t-test), comparing responses in the same nerve before and after antagonist incubation.*

A. **B.**

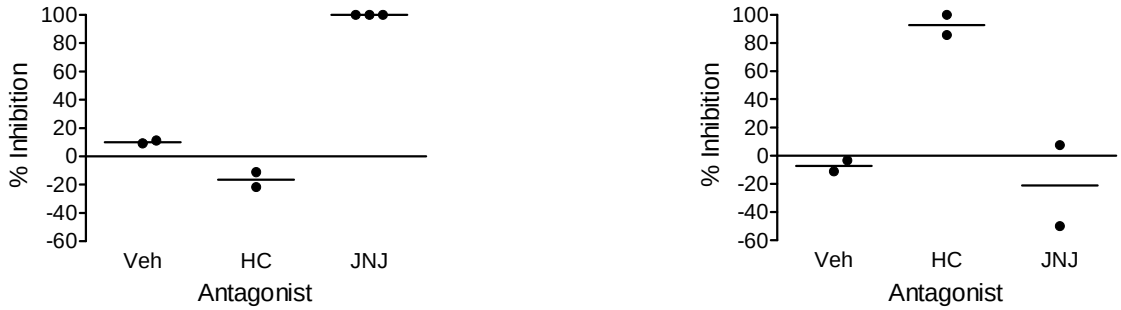


Figure 3.9. Characterising TRPV1- and TRPA1-selective antagonists on human isolated vagus nerve.

(a) *Capsaicin-induced sensory nerve depolarisation was inhibited by the TRPV1-selective antagonist JNJ17203212 (JNJ; 100 μM), but was unaffected by either the TRPA1-selective antagonist HC-030031 (HC; 10 μM) or vehicle (Veh; 0.1% DMSO).* (b) *Acrolein-induced sensory nerve depolarisation was inhibited by HC, but not JNJ or vehicle. Data represent mean ± s.e.m of n = 2-3 observations.*

3.3.3 Characterisation of the conscious guinea pig cough model

Capsaicin is a well-established tussive ligand, and is known to induce robust cough in both human and animal models via activation of the TRPV1 receptor. At the time of these experiments TRPA1 had not yet been established as a tussive mediator; however we have subsequently published some of the following results in guinea pigs, and corroborated the findings in human volunteers (Birrell et al., 2009). Another group has also observed coughing with TRPA1 stimulation in a guinea pig model (Andrè et al., 2009).

In the above experiments, I verified that the agonists acrolein (TRPA1) and capsaicin (TRPV1) are selective for their reported receptor *in vitro*. This section establishes concentration-responses for TRPA1- and TRPV1-selective agonists and antagonists in an *in vivo* guinea pig cough model. The selectivity of the antagonists was also determined at the concentration which was chosen for further experimentation.

3.3.3.1 TRP-selective agonist-induced cough

Concentration-responses for capsaicin and acrolein-induced cough were established. The vehicles elicited no response, whereas both capsaicin and acrolein concentration-dependently induced large, single explosive coughs (Figures 3.10 and 3.11). Concentrations of 60 µM capsaicin and 100 mM acrolein, which caused substantial coughing, were chosen for use in future experiments.

3.3.3.2 Inhibition of cough by TRP-selective antagonists

In the isolated vagus nerve, the TRPV1-selective antagonists capsazepine and JNJ17203212 were both shown to abolish capsaicin-induced nerve depolarisation. Capsazepine is a well-established TRPV1 antagonist that has previously been shown to inhibit capsaicin-induced cough responses (Lalloo et al., 1995; Trevisani et al., 2004). However, capsazepine has a poor pharmacokinetic profile *in vivo* (Valenzano et al., 2003). The JNJ17203212 compound is a new-generation TRPV1 inhibitor which is reported to have a good pharmacokinetic profile *in vivo*, and has also been shown to inhibit capsaicin and citric acid cough (Bhattacharya et al., 2007). JNJ17203212 was used in these *in vivo* studies to inhibit the TRPV1 ion channel. Though a number of TRPV1-selective antagonists have now been

discovered, there are currently very few TRPA1-selective antagonists available. Of the ones that have been established, the HC-030031 compound has been the most widely used, and has been shown to be effective in *in vivo* models of pain (Eid et al., 2008; McNamara et al., 2007). Moreover, in the *in vitro* isolated vagus model, I have established that HC-030031 blocks acrolein-induced nerve responses by approximately 90%. In agreement with the *in vitro* findings, JNJ17203212 concentration-dependently inhibited capsaicin-induced guinea pig cough. Coughing was completely abolished at a dose of 100 mg/kg and above ($p < 0.05$), but at this dose acrolein-induced cough was not affected (Figure 3.12A). Conversely, HC-030031 pre-treatment dose-dependently inhibited acrolein-induced cough, but had no effect on capsaicin stimulation (Figure 3.12B). Maximum inhibition was associated with pre-treatment of 300 mg/kg HC-030031, which reduced the number of acrolein-induced coughs to 0.4 ± 0.3 in comparison to 8.5 ± 1.2 coughs with vehicle pre-treatment ($p < 0.05$).

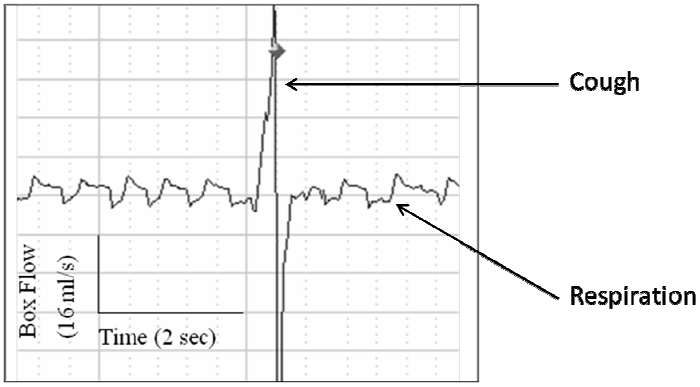
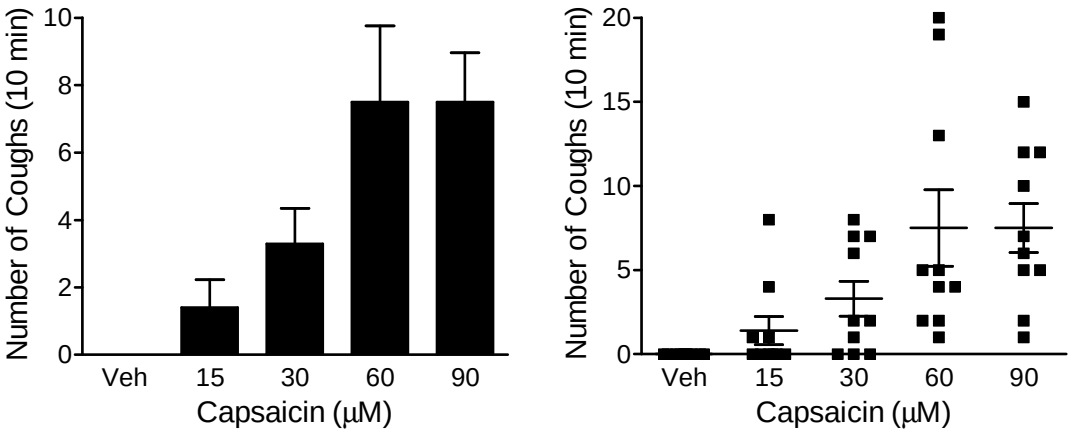


Figure 3.10. Example of a cough trace recorded in response to capsaicin stimulation in guinea pigs.

Inhalation of aerosolised capsaicin or acrolein caused large, single, explosive coughs. The occurrence of a cough was distinguished from other respiratory events (e.g. sneezes) by analysis software (Buxco, USA) as well as the characteristic posture and sound produced by the guinea pigs during a tussive effort. This image of a cough response to capsaicin was acquired by screen capture during real time data replay.

A.



B.

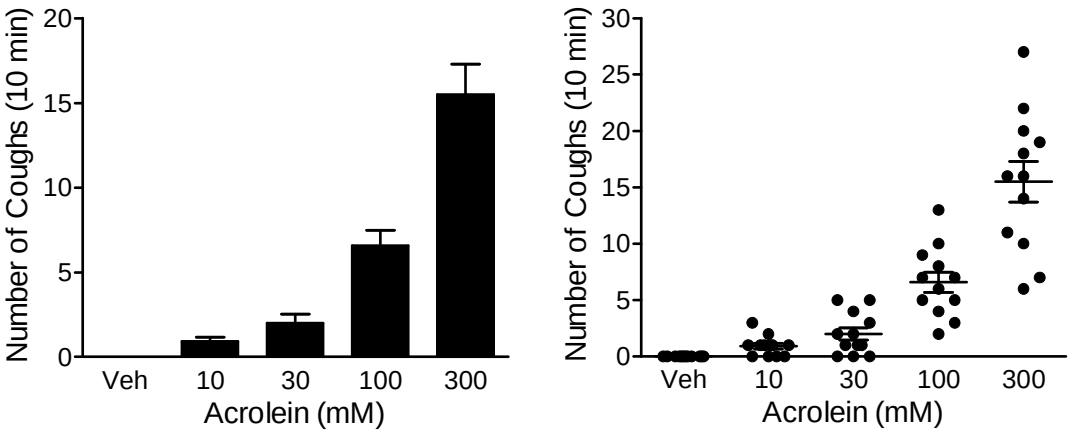
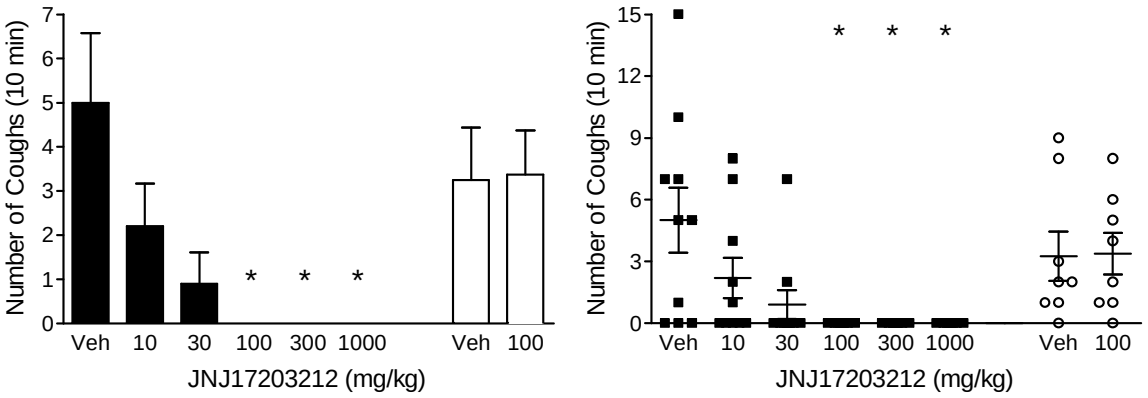


Figure 3.11. Characterising TRPV1 and TRPA1 selective agonists in a conscious guinea pig cough model.

Guinea pigs were exposed to aerosolised agonist or vehicle (Veh) for 5 minutes, with the number of coughs was counted during this period and for a further 5 minutes (10 minutes total). Concentration-responses were established for (a) *capsaicin and* (b) *acrolein. Data are plotted in both bar chart and scatter-graph format as mean ± s.e.m of n = 10-12 observations.*

A.



B.

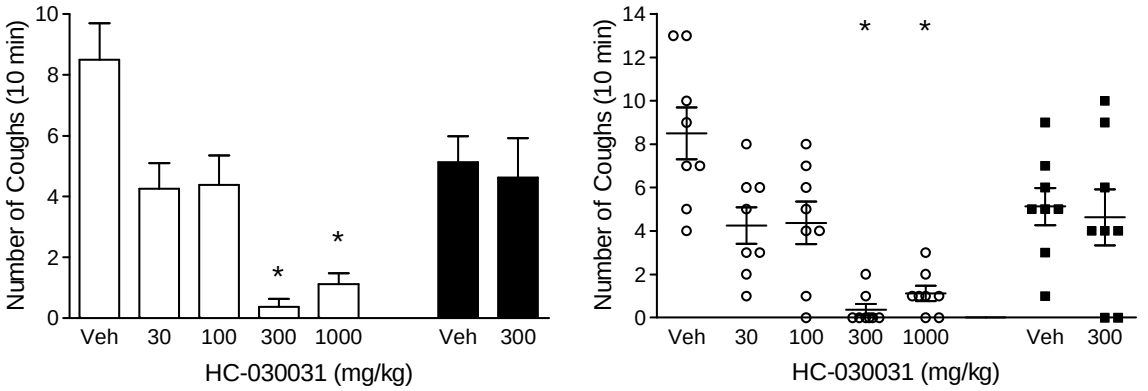


Figure 3.12. Characterising the effect of selective TRPV1 and TRPA1 antagonists in the conscious guinea pig cough model.

Guinea pigs were exposed to aerosolised TRPV1 (60 µM capsaicin; black bars & filled squares) or TRPA1 (100 mM acrolein; white bars & open circles) agonists for 5 minutes, and the number of coughs counted for 10 minutes. Concentration-responses were established for (a) *TRPV1 (JNJ17203212) and* (b) *TRPA1 (HC-030031) selective antagonists against their selective agonist. The dose which maximally inhibited its own receptor was then tested against the alternate agonist to establish selectivity at the chosen dose. Antagonists were injected i.p. 1 hour prior to stimulation. Veh = antagonist vehicle. Data are plotted in both bar chart and scatter graph format as mean ± s.e.m of n=8-10 observations. * indicates significance compared to vehicle control ($p < 0.05$; Kruskal Wallis one-way ANOVA with Dunn's multiple comparison post-hoc test).*

3.4 Discussion

Discovery of the TRPV1 receptor as a binding site for tussive stimuli has lead to a greater understanding of the mechanisms driving cough in both health and disease. Capsaicin and citric acid, both classical TRPV1 agonists, have been extensively used to study the cough reflex in a wide range of models, from *in vitro* single-fibre and isolated vagus analysis; to *in vivo* models of cough in animals both conscious and under anaesthesia; to human trials investigating cough in healthy individuals and those suffering from various chronic cough pathologies (including asthma, COPD, gastro-esophageal reflux, and idiopathic cough). The breakthrough discovery of a specific ion channel directly involved in causing cough brought with it the possibility of developing novel targeted anti-tussive therapies, which have the potential to inhibit coughing without the associated side-effects of current treatments such as opioids. The more recent discovery of the TRPA1 ion channel seems even more promising, as it is an extremely promiscuous receptor that binds a wide range of both environmental and endogenous compounds that are associated with respiratory irritation. If this receptor is found to play a significant role in cough, and excessive cough states associated with disease, it opens up another area of research that could ultimately lead to the development of more efficacious anti-tussive therapies.

This chapter outlines the development of two models for investigating the role of TRPA1 and TRPV1 ion channels in sensory nerve activation and cough using receptor-selective pharmacological tools. Furthermore, breeding colonies of genetically modified animals with the TRPA1, TRPV1, ASIC1 and ASIC3 gene disrupted were established, and knockdown of the appropriate receptor was confirmed using genotyping. These mice are a valuable asset to this research, as they allow non-pharmacological assessment of the importance of these ion channels in mediating sensory nerve activation. I began by using these animals to confirm the selectivity of the TRPA1 and TRPV1 agonists for their reported receptor, which conferred confidence in moving forward with these tools to investigate the associated receptor-selective antagonists.

In this chapter I have described an *in vitro* model of sensory afferent activation in the isolated vagus nerve, which has been previously shown to be predictive of tussive effects *in vivo* (Maher et al., 2009; Patel et al., 2003; Usmani et al., 2005). The vagus nerves supply the majority of sensory afferent fibres to the airway, therefore, depolarisation of the isolated

vagus nerve from both guinea pig and mouse was characterised using a number of TRPA1- and TRPV1-selective pharmacological tools. The guinea pig was chosen because these animals are capable of evoking a cough reflex, and I could therefore repeat key experiments in response to tussive stimuli in an *in vivo* model (Maher et al., 2009; Patel et al., 2003). Whereas, a mouse model was used as we have access to receptor knockout animals. Although mice do not cough *per se*, they do still appear to possess the afferent arm of the cough reflex, and thus provide an invaluable tool for assessment. Furthermore, it has been established that the vagus nerves in both of these species respond in a similar manner to that of man (Belvisi et al., 2008; Maher et al., 2009). Key experiments were also replicated in human vagus tissue, to confirm that the findings in the animal models are relevant to man.

Using the isolated vagus nerve preparation, I investigated the ability of a number of TRPA1- and TRPV1-selective agonists to activate the vagus nerves. All TRPA1 and TRPV1 agonists induced depolarisation, and acrolein and capsaicin were chosen for further experimentation. I was able to confirm that acrolein was indeed a selective TRPA1 agonist, and capsaicin a TRPV1 selective agonist by showing a lack of response in *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$ mouse vagal tissue. These compounds were therefore suitable to use in profiling TRP-selective antagonists. The TRPA1-selective antagonist HC-030031 was shown to inhibit acrolein responses, but not capsaicin; conversely, the TRPV1-selective antagonists capsazepine and JNJ17203212 inhibited capsaicin responses, but not acrolein. Thus, the selectivity of the chosen antagonists was established, and a concentration chosen for future experiments. Key experiments were repeated in human isolated vagus tissue to corroborate the findings in a human model. Both acrolein and capsaicin induced depolarisation in human sensory nerves, which were blocked by their corresponding but not alternate antagonist. The above results illustrate that the effects of these compounds can be translated across species.

In vitro assessment of sensory nerve depolarisation provides a reliable alternative to *in vivo* cough experiments, which are associated with inherent and complex pharmacokinetic issues; are expensive; require the use of a large number of animals; and a large quantity of test compound. The isolated vagus nerve preparation thus allowed assessment of the potential role of the TRPA1 and TRPV1 ion channels with compounds that may not have an ideal pharmacokinetic profile *in vivo*. However, the data from the isolated vagus nerve preparation should be interpreted with some caution, as the tussive stimuli and compounds are applied to the trunk of the nerve. Therefore, the signal that is recorded extracellularly in these

experiments represents a summation of the changes in membrane potential of all of the axons within the vagus nerve, including all types of nerve fibre (e.g. RARs, Aδ nociceptors and C-fibres) and non-airway fibres. Furthermore, the expression and transduction mechanisms of the receptors situated on the membrane of the axon may not represent their behaviour at the peripheral nerve endings (Patel et al., 2003). An alternative method that is widely used in the research community is the single fibre technique, where agents are applied directly to the nerve ending in the airway, and action potentials recorded from a single fibre in the nerve trunk (Fox et al., 1997, 1996; Kollarik & Undem, 2002). However, this technique is less robust, has a lower throughput and requires specialised equipment. Nevertheless, agents that activate sensory nerves, using either the isolated vagus preparation or the single fibre technique, also induce cough *in vivo* (Birrell et al., 2009; Fox et al., 1997, 1996; Kollarik & Undem, 2002; Lalloo et al., 1995; Maher et al., 2009; Patel et al., 2003; Usmani et al., 2005).

To demonstrate that the *in vitro* results do in fact translate to *in vivo* cough responses, both agonist and antagonist concentration-responses were repeated in a conscious guinea pig model of cough. Measuring tussive responses in conscious, unrestrained animals allows analysis (by a trained observer) of the cough to incorporate stance and posture as well as sound produced during the cough reflex, and does not place undue stress on the animal by using restraints. Furthermore, anaesthesia has been implicated in inhibition of the C-fibre response to tussive stimuli, and some anaesthetics are thought to activate the TRPA1 receptor (Belvisi & Hele, 2006; Eilers et al., 2010; Gallos & Flood, 2010; Karlsson & Fuller, 1999). An automated system was also in place to count the coughs induced by capsaicin and acrolein (Buxco, USA), which agreed closely with counts from the trained observer. Tussive responses in the *in vivo* cough model mirrored previous findings in both the isolated vagus preparation and neuronal imaging experiments. That is, both capsaicin and acrolein induced concentration-related coughing; and these tussive responses were dose-dependently inhibited by the corresponding TRPA1 (HC-030031) and TRPV1 (JNJ17203212) antagonists. Conversely, coughing induced by a selected concentration of acrolein or capsaicin was not affected by the alternate antagonist, demonstrating that the antagonists were not acting off-target at the doses selected.

In summary, I have shown here that TRPA1- and TRPV1-selective agonists activate human, guinea pig and mouse vagal sensory nerves *in vitro*, and cause cough *in vivo*. Furthermore, these effects can be blocked by selective antagonists at a concentration which does not

display off-target actions. Having established these tools, I went on to probe the role of the TRPA1 and TRPV1 ion channels in mediating sensory nerve activation and tussive responses to the endogenous irritants PGE_2, BK and low pH.

CHAPTER 4

Endogenous Mediators: Prostaglandin E_2

4.1 Rationale

PGE_2 is derived from the metabolism of arachadonic acid by cyclo-oxygenase (COX) enzymes: COX-1 which is costitutively expressed, and COX-2 which is inducible. PGE_2 elicits coughing when inhaled, and pre-treatment with PGE_2 sensitises airway afferent nerves and the cough reflex to stimulation with other tussive agonists (Choudry et al., 1989; Costello et al., 1985; Gauvreau et al., 1999; Ho et al., 2000; Kawakami et al., 1973). The production of PGE_2 is known to be upregulated in response to airways inflammation, and as such PGE_2 could be implicated in the pathogenesis of chronic cough associated with airways diseases such as asthma, COPD and post-viral cough. It has been postulated that this mediator may sensitise patients who suffer from chronic cough to environmental tussive irritants; or it could be inducing cough itself if concentrations within the airways reach sufficient levels to directly activate airway sensory nerves.

There is a particular interest in determining how PGE_2 induces cough, as this lipid mediator has also been shown to have beneficial anti-inflammatory and bronchodilatory effects in the lungs, and has thus been suggested as a novel therapy for asthmatics (Gauvreau et al., 1999; Kawakami et al., 1973; Melillo et al., 1994; Pavord et al., 1993). PGE_2 preferentially binds to the EP receptor (Bos et al., 2004). Because there are four isoforms of the EP receptor, it is possible that the GPCR through which PGE_2 elicits cough could be different from that through which it has beneficial properties. It was recently established that PGE_2 induces sensory nerve depolarisation in human, guinea pig and mouse vagal tissue, and causes cough in guinea pigs via the EP_3 receptor (Maher et al., 2009). However, the signalling mechanisms through which PGE_2 mediates its tussive effects downstream of EP_3 receptor activation have yet to be determined.

Our understanding of the mechanisms behind PGE_2-induced cough is limited. Signalling downstream of initial G-protein activation must ultimately lead to gating of an ion channel in order to cause a net change in membrane potential and subsequent action potential generation. TRPV1 has been associated with the role of PGE_2 in oedema (Claudino et al., 2006). It is possible that cough elicited by PGE_2 is also at least partially mediated via activation of TRPV1, an ion channel that is already known to mediate the tussive effects of citric acid and capsaicin (Lalloo et al., 1995; Trevisani et al., 2004). This hypothesis is supported by the fact that prior exposure to PGE_2 can enhance cough and neuronal responses

to capsaicin stimulation (Choudry et al., 1989; Ho et al., 2000). In addition, TRPA1 is another promising candidate for mediating the tussive effects of PGE_2, due to its co-expression with TRPV1 on sensory neurons that innervate the airways. TRPA1 has also been implicated in the detection of noxious stimuli, and is known to mediate the response to numerous irritant gases, dusts, vapours and chemicals linked to cough (Bandell et al., 2004; Bautista et al., 2006, 2005; Jordt et al., 2004; Simon & Liedtke, 2008; Story et al., 2003; Taylor-Clark et al., 2008a, 2008b). In Chapter 3 I showed that acrolein, a TRPA1-selective agonist, is able to induce coughing in a guinea pig model. This data has been published alongside a clinical collaboration with Professor Morice's group showing that inhalation of aerosolised cinnamaldehyde can induce coughing in healthy human volunteers (Birrell et al., 2009), thus confirming a role for TRPA1 in the tussive reflex.

In Chapter 3, the relevant *in vitro* and *in vivo* models were characterised; and the efficacy and selectivity of TRPA1- and TRPV1-selective agonists and antagonists was determined. The aim of this chapter was to examine the role of TRPA1 and TRPV1 ion channels in PGE_2-induced sensory nerve activation and cough. I began by characterising responses to PGE_2 stimulation using guinea pig, mouse and human isolated vagus nerves. The ability of TRPA1 and TRPV1-selective antagonists to inhibit these responses was then assessed, using the concentrations established in Chapter 3. Finally, these results were verified in an *in vivo* model of PGE_2-induced cough using conscious guinea pigs.

4.2 Methods

4.2.1 PGE_2-induced sensory nerve activation

Non-cumulative CR curves were established for vehicle (0.1% ethanol) or PGE_2 on guinea pig (3, 10, 30, 100 µM) and wild type mouse (0.1, 1, 10, 100 µM) isolated vagus nerves. Stimuli were applied for 2 minutes in a random order, and then the tissue washed with Krebs solution until baseline was re-established. The ability of 10 µM PGE_2 to activate human, *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$ mouse vagus nerves was also assessed.

After characterising the response to PGE_2, a sub-maximal concentration (10 µM) was chosen to profile the effect of antagonists. Initially, to address the question of whether application of PGE_2 induces production of more prostanoids downstream of arachadonic acid via the cyclooxygenase pathway, which could alter the PGE_2-induced sensory nerve response, the effect of a cyclooxygenase inhibitor (10 µM indomethacin) was determined. Indomethacin was perfused on to guinea pig or wild type mouse isolated vagus nerves for 10 minutes, and the effect of this inhibitor on PGE_2 responses was assessed as described in chapter 2, section 2.3.3. Indomethacin was chosen as this is an established cyclooxygenase inhibitor, which has been successfully used in other functional preparations at a concentration of 10 µM to block the production of prostanoids (Belvisi et al., 1992; Patel et al., 1995; Takahashi et al., 1994; Ward et al., 1995).

In chapter 3, appropriate concentrations were established for the TRPA1- (10 µM HC-030031) and TRPV1-selective (10 µM capsazepine, 100 µM JNJ17203212) antagonists, which maximally inhibited their own receptor but did not exhibit an off-target effect on the alternate receptor. These antagonists were assessed for their ability to inhibit PGE_2-induced activation in guinea pig, mouse (wild type, *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$) and human isolated vagus nerves.

4.2.2 PGE_2-induced guinea pig cough

Conscious, unrestrained male Dunkin Hartley guinea pigs (300-400 g, Harlan, UK) were individually placed in a plethysmography chamber. For the initial CR, animals were exposed to 10 minutes of aerosolised vehicle (0.1 M phosphate buffer) or 30, 100 or 300 µg/ml PGE_2.

The number of coughs was recorded during the 10 minute stimulation as previously described in section 2.4.

For the antagonist study, guinea pigs were dosed i.p. with a combination of either: (a) vehicle (0.5% methyl cellulose in sterile saline) plus vehicle (15% solutol in 5% dextrose solution); (b) vehicle (15% solutol in 5% dextrose solution) plus HC-030031 (300 mg/kg); (c) vehicle (0.5% methyl cellulose in sterile saline) plus JNJ17203212 (100 mg/kg); or (d) HC-030031 (300 mg/kg) plus JNJ17203212 (100 mg/kg). Guinea pigs were then exposed to an aerosol of 300 µg/ml PGE_2 for 10 minutes, and the number of coughs counted as previously described.

4.2.3 Data Analysis

Antagonism of PGE_2-induced vagal sensory nerve activation was analysed by paired t-test, comparing responses in the same nerve before and after antagonist incubation. Comparison of the magnitude of PGE_2-induced responses in *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$ tissue was analysed using the Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison post-hoc test comparing all groups to wild type responses. Antagonism of PGE_2-induced cough was also analysed using the Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison post-hoc test comparing all groups to vehicle. Significance was set at $p < 0.05$, and all data were plotted as mean ± s.e.m. of *n* observations.

4.3 Results

In chapter 3, I confirmed that stimulation of the TRPA1 and TRPV1 ion channels induce both sensory nerve activation *in vitro*, and cough *in vivo*. I also established appropriate concentrations of selective antagonists to use in the models of cough employed in this thesis, which maximally inhibited the TRPA1 or TRPV1 receptors without showing off-target effects. In the following chapters I proceeded to investigate the role of TRPA1 and TRPV1 receptors in mediating the tussive effects of endogenous mediators, beginning with PGE_2. PGE_2 is an endogenous inflammatory mediator which is upregulated in airways disease, and is associated with both initiation of cough, and sensitisation of the cough reflex to capsaicin stimulation (Choudry et al., 1989; Costello et al., 1985; Gauvreau et al., 1999; Ho et al., 2000; Kawakami et al., 1973).

4.3.1. PGE_2-induced sensory nerve activation

PGE_2 provoked concentration-related increases in magnitude of depolarisation in both guinea pig and mouse isolated vagus nerves. A concentration of 10 µM, which elicits submaximal nerve activation in both species, was used in subsequent experiments. Incubation with 10 µM PGE_2 also successfully stimulated human isolated vagus nerves. In contrast, vehicle (0.1% ethanol) had no effect in any of these species (Figure 4.1).

To rule out the possibility that the PGE_2 response is being potentiated due to downstream production of other prostanoids, the cyclooxygenase inhibitior indomethacin (10 µM) was tested against PGE_2 stimulation. Indomethacin failed to alter PGE_2-induced depolarisiation of either the guinea pig or mouse vagus nerves, indicating that the observed response was not being augmented by subsequent production of other prostanoids (Figure 4.2).

The magnitude of depolarisation induced by 10 µM PGE_2 in *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$ vagus nerves was approximately half that seen in wild type tissue (Figure 4.3), thus indicating a role for both of these ion channels in mediating PGE_2 sensory nerve activation. This role was confirmed using TRPA1- (HC-030031) and TRPV1-selective (capsazepine, JNJ17203212) antagonists. Either TRPA1 or TRPV1 antagonism partially inhibited PGE_2-induced depolarisation in guinea pig, wild type mouse and human isolated vagus. When used in combination, both TRPA1 and TRPV1 antagonism virtually abolished nerve responses in

these species (Figure 4.4). To confirm that these results were not due to unusual pharmacological effects of combining the two antagonists, TRPA1 or TRPV1 antagonists were used to inhibit PGE_2 activtion in vagal tissue taken from the alternate genetically modified mouse i.e. TRPA1 antagonist with *Trpv1*$^{-/-}$ tissue; and TRPV1 antagonist with *Trpa1*$^{-/-}$ tissue. Results from these studies corroborated the earlier observations, showing almost complete loss of PGE_2-induced sensory nerve activation (Figure 4.4B).

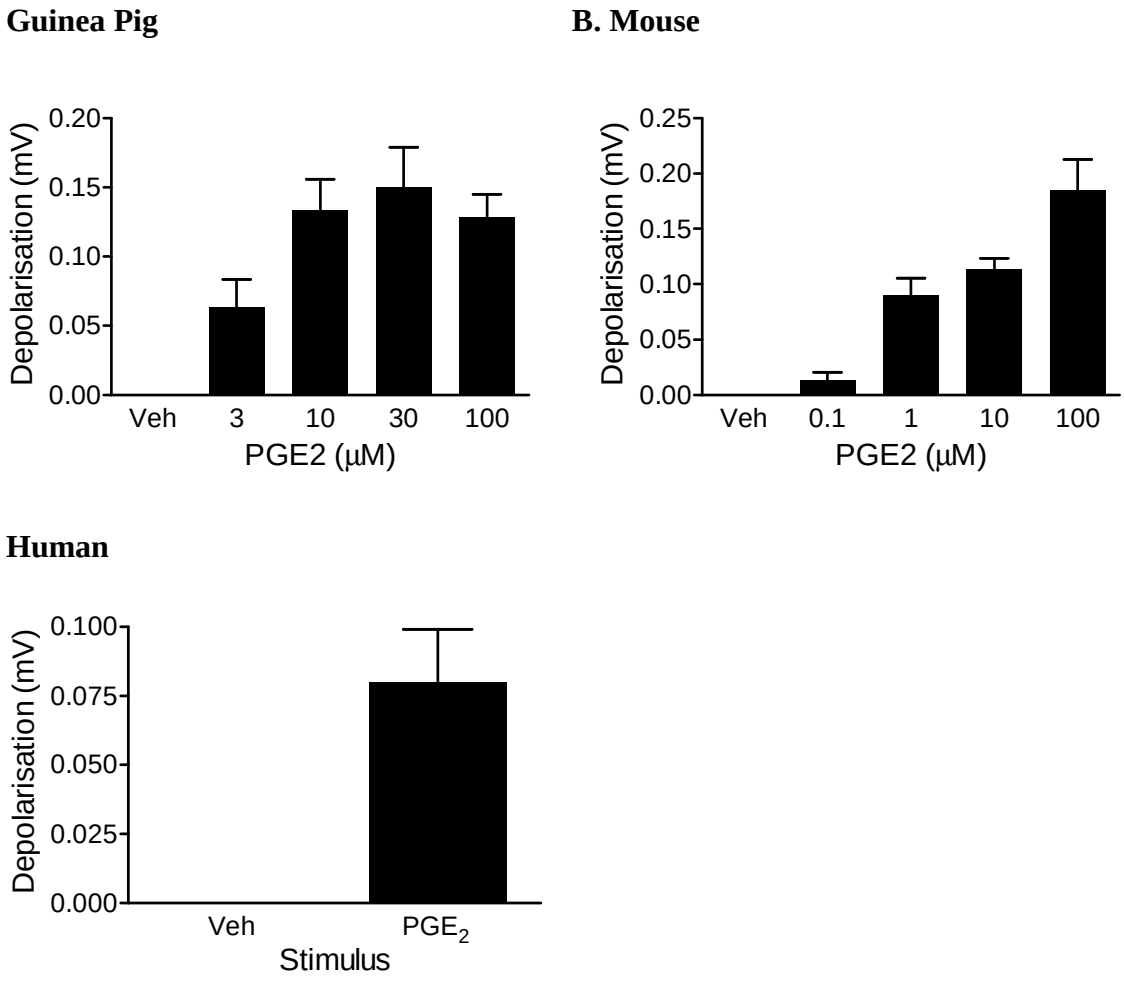


Figure 4.1. Characterisation of the isolated vagus nerve response to PGE_2 stimulation.
Concentration responses were established for PGE_2 on (a) *guinea pig and* (b) *wild type mouse isolated vagus nerve.* (c) *human vagus nerve also responded to PGE_2 (10 µM) but not vehicle stimulation. Veh = vehicle (0.1% ethanol). Data are presented as mean ± s.e.m of n = 6 observations for guinea pig and mouse; and n = 2-6 for human experiments.*

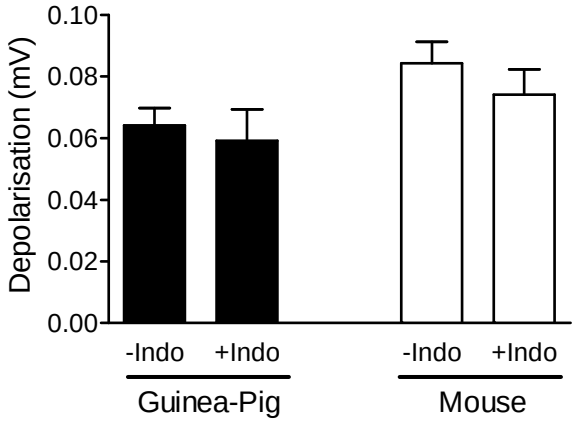


Figure 4.2. Inhibition of cyclooxygenase does not alter PGE_2-induced vagus nerve stimulation.
Isolated vagus nerves were stimulated with 10 µM PGE_2 for 2 minutes, followed by 10 minutes incubation with the cyclooxygenase inhibitor indomethacin (Indo, 10 µM), and stimulation with PGE_2 in the presence of indomethacin. Indomethacin did not alter PGE_2-induced sensory nerve depolarisation in either the guinea pig or mouse isolated vagus, determined by paired t-test comparing responses in the same nerve before and after antagonist incubation (significance set at $p < 0.05$). Data are presented as mean ± s.e.m. of n = 6 observations.

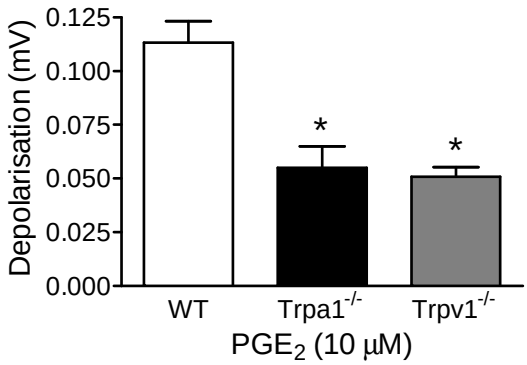


Figure 4.3. Determining a role for TRPA1 and TRPV1 ion channels in PGE_2-induced isolated vagus nerve responses.
*PGE_2 (10 µM) was applied to wild type (C57Bl/6j), $Trpa1^{-/-}$ or $Trpv1^{-/-}$ mouse isolated vagus nerves for 2 minutes. The magnitude of depolarisation (mV) in the $Trpa1^{-/-}$ and $Trpv1^{-/-}$ tissue was approximately half that seen in wild type, indicating a role for both of these ion channels in PGE_2-induced nerve activation. * indicates statistical significance compared to wild type control ($p < 0.05$, Kruskal Wallis one-way ANOVA with Dunn's multiple comparison post-hoc test). Data are presented as mean ± s.e.m. of n = 6 observations.*

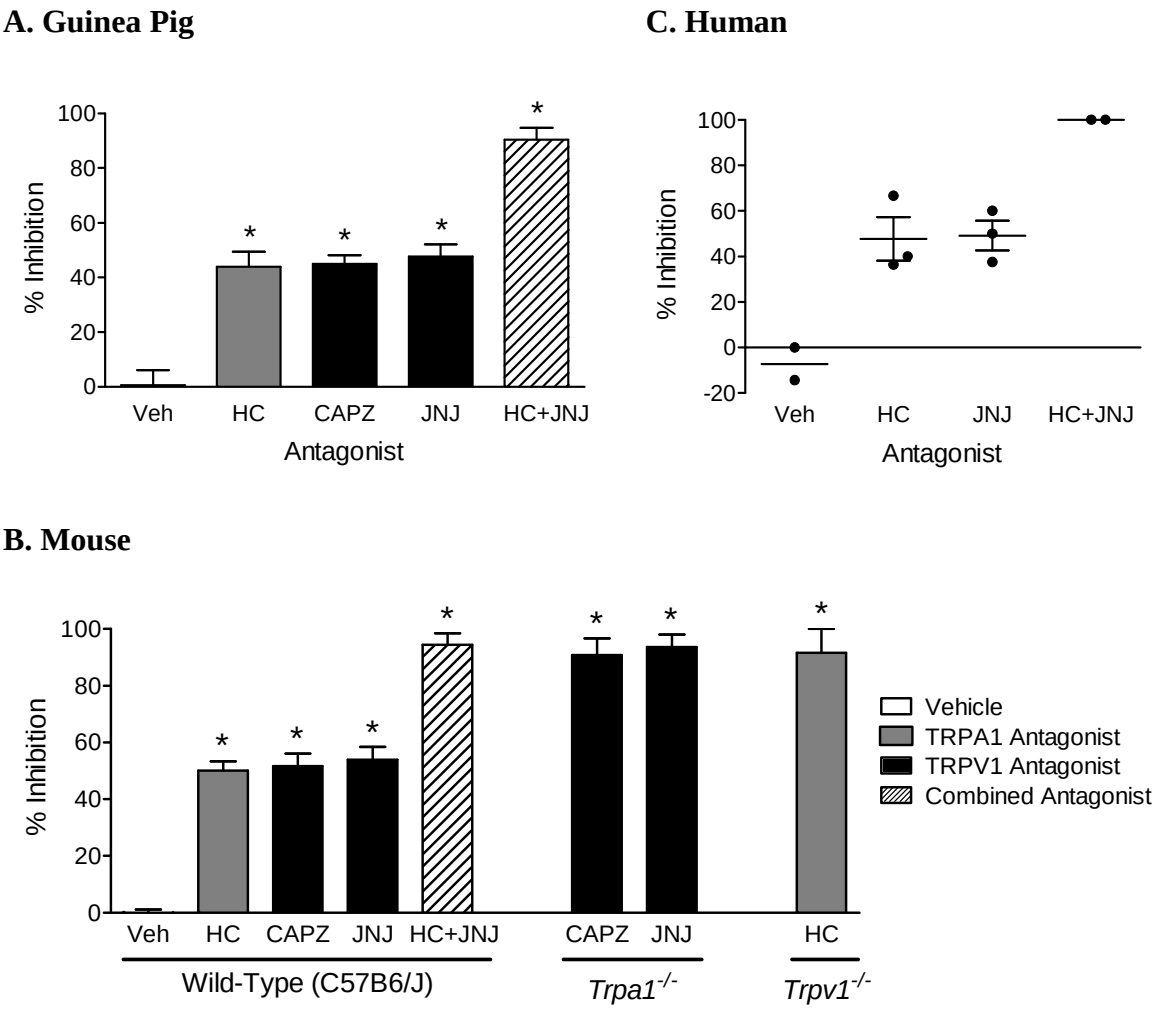


Figure 4.4. Pharmacologically determining a role for TRPA1 and TRPV1 ion channels in PGE_2-induced isolated vagus nerve responses.

Isolated vagus nerves were incubated with a selective TRPA1 antagonist (10 µM HC-030031, HC); a selective TRPV1 antagonist (10 µM capsazepine, CAPZ; 100 µM JNJ17203212, JNJ); a combination of both TRPA1 and TRPV1 antagonists (HC+JNJ); or vehicle (0.1% DMSO, Veh) for 10 minutes prior to PGE_2 (10 µM) stimulation. Antagonism of either TRPA1 or TRPV1 partially inhibited PGE_2-induced responses in (a) *Guinea pig,* (b) *Mouse and* (c) *Human vagus nerves. Whereas, a combination of both TRPA1 and TRPV1 antagonists, or tissue taken from $Trpa1^{-/-}$ or $Trpv1^{-/-}$ mice incubated with the alternate antagonist, virtually abolished PGE_2 stimulation. * indicates statistical significance ($p < 0.05$; paired t-test), comparing responses in the same nerve before and after antagonist incubation. Data are presented as mean ± s.e.m. of n = 6 observations for guinea pig and mouse; and n = 2-3 for human experiments.*

4.3.2 PGE_2-induced guinea pig cough

In contrast to capsaicin and acrolein, which evoke single loud coughs (refer to Figure 3.10), PGE_2 elicited trains of smaller coughs, often with multiple bouts occurring during one 10 minute exposure (Figure 4.5). A concentration-response for PGE_2-induced cough in the conscious guinea pig model had been previously established in our lab. Neither vehicle nor 30 µg/ml PGE_2 elicited any coughing; 100 µg/ml induced coughing in one animal; whereas 300 µg/ml caused robust coughing in most animals (Figure 4.6). Due to the significant costs involved in testing PGE_2 at higher concentrations, 300 µg/ml was chosen for use in the antagonist study.

The ability of TRPA1- (300 mg/kg HC-030031) and TRPV1-selective (100 mg/kg JNJ17203212) antagonists to inhibit the PGE_2-induced cough response was then assessed. When pre-treated with vehicle only, PGE_2 induced on average 16 ± 4 coughs. Pre-treatment with either TRPA1 or TRPV1 antagonists partially inhibited the cough response. Interestingly, either antagonist inhibited the number of coughs by over half, i.e. PGE_2 stimulation elicited 2 ± 2 and 4 ± 2 coughs with TRPA1 or TRPV1 antagonists, respectively ($p < 0.05$). Furthermore, pre-treatment with both TRPA1 and TRPV1 antagonists completely abolished the cough response to PGE_2 stimulation (0 ± 0 coughs; Figure 4.7). This corroborates the *in vitro* vagal sensory nerve data presented above, and provides strong evidence for activation of both TRPA1 and TRPV1 ion channels by PGE_2 downstream of the EP_3 receptor.

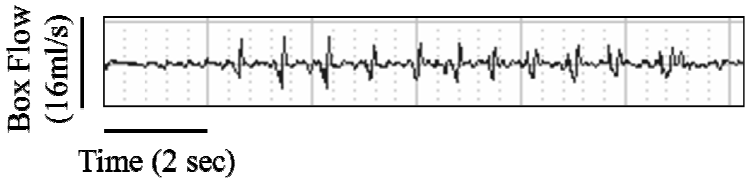


Figure 4.5. Example of a cough trace recorded in response to PGE_2 stimulation in guinea pigs.
Inhalation of aerosolised PGE_2 caused bouts of small coughs. These coughs were not always detected by the analysis software (Buxco, USA), and thus the trained observer was crucial in counting the number of coughs during stimulation. Coughs were distinguished by the characteristic posture and sound produced by guinea pigs during a tussive effort. This image of a cough response to PGE_2 was acquired by screen capture during real time data replay.

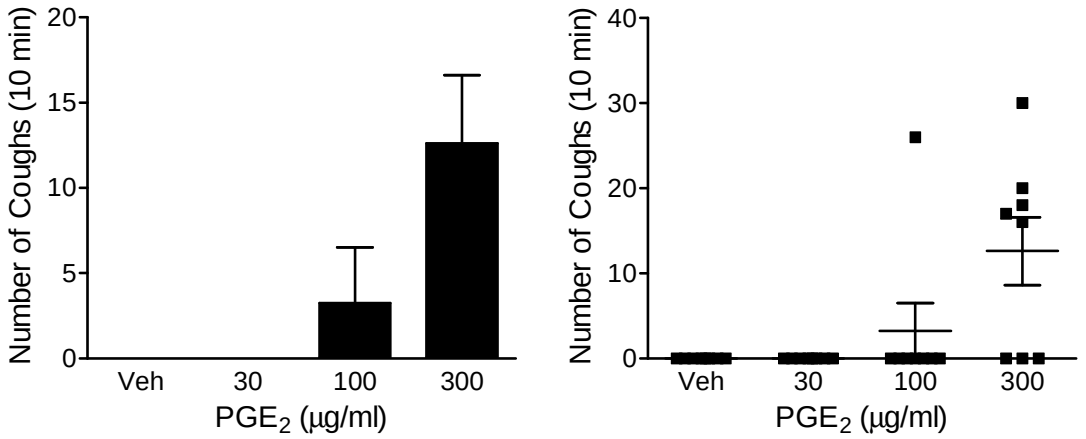


Figure 4.6. Characterising PGE_2-induced cough in the conscious guinea pig model.

A concentration response for PGE_2-induced cough was established. PGE_2 was aerosolised for 10 minutes, and the number of coughs counted during this period. Data are plotted in both bar chart and scatter-graph format as mean ± s.e.m. of n=8 observations. Veh = vehicle.

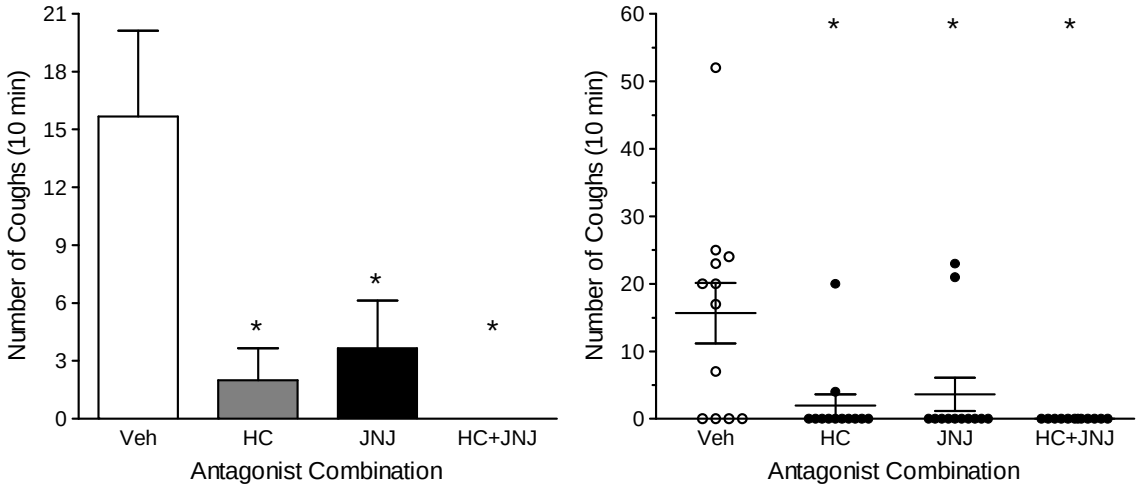


Figure 4.7. Determining a role for TRPA1 and TRPV1 ion channels in PGE_2-induced cough.

*Guinea pigs were injected i.p. 1 hour prior to aerosolised PGE_2 (300 µg/ml) stimulation with: TRPA1 vehicle plus TRPV1 vehicle (Veh); TRPA1 antagonist (300 mg/kg HC-030031) plus TRPV1 vehicle (HC); TRPV1 antagonist (100 mg/kg JNJ17203212) plus TRPA1 vehicle (JNJ); or TRPA1 antagonist plus TRPV1 antagonist (HC+JNJ). Data are plotted in both bar chart and scatter graph format as mean ± s.e.m. of n=12 observations. * indicates statistical significance compared to vehicle control ($p < 0.05$; Kruskal Wallis one-way ANOVA with Dunn's multiple comparison post-hoc test).*

4.4 Discussion

It has been established that PGE_2 causes sensory nerve depolarisation and cough in guinea pigs via the EP_3 GPCR (Maher et al., 2009). Downstream of GPCR activation, opening of one or more ion channels must subsequently occur in order to induce a tussive response. The aim of this chapter was to investigate the ion channel(s) responsible for PGE_2-induced tussive responses using the isolated vagus preparation and an *in vivo* cough model.

It is possible that stimulation with PGE_2 may cause production of other prostanoids downstream of the arachadonic acid / cyclooxygenase pathway. This could implicate other GPCRs and therefore other pathways in the sensory nerve effects elicited by stimulation with PGE_2. I investigated this theory in the *in vitro* isolated vagus preparation by incubating vagus nerves with the cyclooxygenase inhibitor indomethacin, using a normal antagonist profile. Indomethacin did not alter PGE_2-induced sensory nerve responses in either guinea pig or wild type mouse tissue. This provides evidence against the theory that downstream production of prostanoids act to enhance the PGE_2-induced response in sensory nerves. Therefore, I could confidently move ahead with investigating the role of TRPA1 and TRPV1 in the PGE_2-induced tussive response.

It is well known that irritants which activate the TRPV1 ion channel cause sensory nerve activation and cough. In chapter 3, I confirmed these effects and also established a role for TRPA1 irritants in inducing sensory nerve activation and cough. Both the TRPA1 and TRPV1 ion channels have been shown to be activated via the phospholipase C pathway (Claudino et al., 2006; Wang et al., 2008), and PGE_2 is known to activate this pathway downstream of the EP_3 receptor (Claudino et al., 2006); therefore one or both of these ion channels may mediate the tussive effects of PGE_2. I proceeded to investigate this hypothesis using the concentrations of TRPA1- and TRPV1-selective antagonists established in chapter 3, and genetically modified *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$ mice. Pharmacological inhibition of either receptor with the selective antagonists reduced PGE_2 responses by approximately 50%; and when used in combination all-but abolished sensory nerve depolarisation in guinea pig, mouse and human vagus nerves. Furthermore, knockdown of either receptor in genetically modified mice halved the magnitude of depolarisation induced by PGE_2 stimulation in comparison to wild type control; and when used in combination with the alternate antagonist, again vagus nerve activation was virtually abolished. This provided strong *in vitro* evidence

that PGE_2-induced tussive responses are mediated approximately 50/50 by the TRPA1 and TRPV1 ion channels downstream of EP_3 activation, and that this effect is translated across species.

Having implicated a role for both TRPA1 and TRPV1 in PGE_2-induced sensory nerve activation, I proceeded to confirm these results in the *in vivo* cough model. Appropriate doses of TRPA1 (HC-030031) and TRPV1 (JNJ17203212) inhibitors had been established in Chapter 3. Pre-treatment with either TRPA1 or TRPV1 antagonists partially inhibited guinea pig cough responses to PGE_2 stimulation compared to vehicle control; whereas pre-treatment with a combination of both antagonists abolished coughing altogether. Interestingly, cough was inhibited 87% by TRPA1 and 77% by TRPV1 antagonists alone, in contrast to the approximate 50% inhibition observed in the *in vitro* models. Furthermore, though treatment with both TRPA1 and TRPV1 antagonists completely abolished PGE_2-induced cough *in vivo*, there was still a small depolarisation effect observed on the vagus nerve *in vitro*. I postulate that this disparity in outcomes could be due to the different end-points being measured between the *in vitro* and *in vivo* preparations. That is, in the isolated vagus nerve preparation, I was measuring any change in membrane potential, leading to cell membrane depolarisation. However, this depolarisation needs to reach a certain threshold in order to generate action potential firing, which informs the CNS that there is a need to cough. As such, 50% inhibition of the PGE_2 response on the axon of the vagus nerve may prevent the stimulus reaching threshold in more than half of the nerve fibres, therefore fewer action potentials are generated, and the information relayed to the CNS causes substantially fewer coughs.

To summarise, this chapter has established that PGE_2-induced sensory nerve activation in the guinea pig, mouse and human vagus nerves is equally mediated by opening of the TRPA1 and TRPV1 ion channels. This was confirmed in an *in vivo* guinea pig cough model, whereby inhibition of either TRPA1 or TRPV1 substantially reduced the tussive effects of PGE_2, and a combination of both antagonists completely abolished PGE_2 associated cough.

CHAPTER 5

Endogenous Mediators: Bradykinin

5.1. Rationale

The production of BK is upregulated during inflammation, and mediates a wide range of effects in the airways (Ellis & Fozard, 2002). Like PGE_2, BK causes cough in both humans and animals when inhaled in aerosolised form (Choudry et al., 1989; Herxheimer & Stresemann, 1961; Katsumata et al., 1991; Kaufman et al., 1980), and sensitises sensory nerve responses to capsaicin *in vitro* (Fox et al., 1996). In addition, inhibition of the breakdown of BK with ACE inhibitors has been linked to cough hypersensitivity associated with this therapy for cardiovascular disease (Fox et al., 1996). As such, BK could also be involved in the pathogenesis of chronic cough associated with airways pathologies.

BK binds to one of two GPCR isoforms, the B_1 and B_2 receptors. In general, the B_2 receptor is constitutively expressed, whereas the B_1 receptor is inducible (Calixto et al., 2000; Ellis & Fozard, 2002; Vianna & Calixto, 1998). It has therefore been assumed that the acute tussive effects of BK are due to activation of the B_2 receptor. Indeed, the enhanced cough reflex observed with ACE inhibitor treatment, which is caused by accumulation of BK due to a reduction in breakdown, was shown to be sensitive to pre-treatment with a B_2 antagonist (Fox et al., 1996). However, the GPCR associated with the tussive effects of BK has never been explicitly investigated. In addition, a number of the effects associated with BK are indirect, and include the release of other endogenous mediators such as prostaglandins (Calixto et al., 2000; Ellis & Fozard, 2002). The first aim of this chapter was to determine the GPCR through which BK activates sensory nerves, and establish if BK-induced sensory nerve stimulation is due to the subsequent release of tussive prostanoids.

Despite the above suggestion that BK may act indirectly to cause cough, it is also known to mediate some nociceptive effects via the activation of TRPA1 and/or TRPV1 ion channels, probably through the intracellular phospholipase C signalling pathway (Bautista et al., 2006; Wang et al., 2008). It is therefore possible that binding of BK to one of the bradykinin receptors induces the release of intracellular secondary messengers which then phosphorylate and activate ion channels to cause airway irritation. The second aim of this chapter was to determine whether TRPA1 and TRPV1 are involved in guinea pig, mouse and human sensory nerve activation induced by BK stimulation. The final aim was to verify the above results in an *in vivo* model of conscious guinea pig cough.

5.2 Methods

5.2.1 BK-induced sensory nerve activation

Non-cumulative CR curves were established for vehicle (0.1% dH_2O) or BK on guinea pig (1, 3, 10, 30 µM) and wild type mouse (0.1, 1, 10, 100 µM) isolated vagus nerve. Stimuli were applied for 2 minutes in a random order, and the tissue washed with Krebs solution until baseline was re-established. The ability of 3 µM BK to activate human, or 1 µM BK to activate *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$ vagus nerves was also assessed. After characterising the response to BK on the vagus nerve, a sub-maximal concentration (3 µM for guinea pig and human, or 1 µM for mouse) was chosen to profile the effect of antagonists.

The GPCR through which BK induces coughing has not yet been definitively established. Therefore, I initially investigated the ability of B_1-selective (1 µM R715) and B_2-selective (10 µM WIN 64338) antagonists to inhibit BK-induced sensory nerve depolarisation in guinea pig and mouse isolated vagus nerves. These were based on concentrations 10-fold higher than the reported affinity for the mouse B_1 (Regoli et al., 1998) or guinea pig B_2 receptors (Scherrer et al., 1995). A concentration 10-fold higher than the reported affinity is normally sufficient to inhibit responses in the isolated vagus nerve, allowing for the fact that we are testing the axon of the nerve (not at the nerve endings); and that not all of the myelin sheath may have been removed, which could reduce the availability of the drug to the nerve fibres. I observed an inhibitory effect of the B_1 receptor antagonist on BK-induced vagus activation in mouse tissue, which was unexpected as the dogma states that B_1 receptors are inducible (not constitutively expressed), therefore, in order to confirm these results I went on to test the ability of a B_1-selective agonist to concentration-dependently induce depolarisation in the mouse (0.03, 0.1, 0.3, 1 µM Lys-[Des-Arg^9]Bradykinin).

To address the question of whether stimulation with BK induces production of prostanoids via the cyclooxygenase pathway (which could alter sensory nerve activation), the effect of a cyclooxygenase inhibitor (10 µM indomethacin) was determined. Indomethacin was perfused on to guinea pig or wild type mouse isolated vagus nerves for 10 minutes, and the effect of this inhibitor on BK responses was assessed as described in chapter 2, section 2.3.3. Indomethacin was chosen as this is a well-established cyclooxygenase inhibitor, which has been successfully used in other functional preparations at a concentration of 10 µM to block

the production of prostanoids (Belvisi et al., 1992; Patel et al., 1995; Takahashi et al., 1994; Ward et al., 1995).

In chapter 3, appropriate concentrations were established for TRPA1- (10 µM HC-030031) and TRPV1-selective (10 µM capsazepine, 100 µM JNJ17203212) antagonists, which maximally inhibited their own receptor but did not exhibit off-target effects. These antagonists were assessed for their ability to inhibit BK-induced activation in guinea pig, mouse (wild type, *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$) and human isolated vagus nerves.

5.2.2 BK-induced guinea pig cough

Conscious, unrestrained male Dunkin Hartley guinea pigs (300-400 g, Harlan, UK) were individually placed in a plethysmography chamber. For the initial CR, animals were exposed to 10 minutes of aerosolised vehicle (0.9% saline) or 0.3, 1, 3 or 10 mg/ml BK. The number of coughs was recorded during the 10 minute stimulation as previously described in section 2.4.

For the antagonist study, guinea pigs were dosed i.p. with a combination of either: (a) vehicle (0.5% methyl cellulose in sterile saline) plus vehicle (15% solutol in 5% dextrose solution); (b) vehicle (15% solutol in 5% dextrose solution) plus HC-030031 (300 mg/kg); (c) vehicle (0.5% methyl cellulose in sterile saline) plus JNJ17203212 (100 mg/kg); or (d) HC-030031 (300 mg/kg) plus JNJ17203212 (100 mg/kg). Guinea pigs were then exposed to an aerosol of 3 mg/ml BK for 10 minutes, and the number of coughs counted as previously described.

5.2.3 Data Analysis

Antagonism of BK-induced sensory nerve activation was analysed by paired t-test, comparing responses in the same nerve before and after antagonist incubation. Comparison of the magnitude of BK-induced responses in *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$ tissue was analysed using the Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison post-hoc test comparing all groups to wild type responses. Antagonism of BK-induced cough was also analysed using the Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison post-hoc test comparing all groups to vehicle. Significance was set at $p < 0.05$, and all data were plotted as mean ± s.e.m. of *n* observations.

5.3 Results

BK is an endogenous inflammatory mediator that is upregulated in airway disease, and is associated with both initiation of cough and sensitisation of the cough reflex to capsaicin stimulation (Choudry et al., 1989; Fox et al., 1996; Katsumata et al., 1991; Kaufman et al., 1980). Indeed, BK has been implicated in cough hypersensitivity observed with patients taking ACE inhibitors, which are prescribed to help with cardiovascular disorders (Fox et al., 1996). In this instance, ACE inhibitors reduce the degradation of endogenous BK, thereby causing accumulation, sensitisation and subsequently an enhanced cough response. Though it is yet to be definitively established how the tussive effects of BK are being mediated, including which GPCR and ion channels are involved.

5.3.1. BK-induced sensory nerve activation

BK provoked concentration-related increases in magnitude of depolarisation of both guinea pig and mouse isolated vagus nerves. Submaximal concentrations of 3 µM for guinea pig and 1 µM for mouse were used for subsequent antagonist experiments. Incubation with 3 µM BK also successfully stimulated human isolated vagus nerves. In contrast, vehicle (0.1% dH_2O) had no effect in any of these species (Figure 5.1).

BK binds to one of two known GPCRs: the B_1 receptor, which is thought to be inducible rather than constitutively expressed; and the B_2 receptor, which is constitutive. It has therefore generally been assumed that the B_2 receptor mediates the acute tussive effects of BK. However, this has never been definitively investigated. I began by determining which GPCR BK binds to stimulate vagal sensory nerves. In guinea pig and human isolated vagus nerves, a B_1-selective antagonist (1 µM R715) had no inhibitory effect on BK stimulation; whereas, a B_2-selective antagonist (10 µM WIN 64338) abolished BK-induced responses (Figure 5.2A & 5.2C). In contrast, BK-induced mouse vagus nerve stimulation was largely mediated via the B_1 receptor, with a smaller but significant role for B_2 (Figure 5.2B). Neither the B_2 antagonist in guinea pig and human vagus, nor the B_1 or B_2 antagonists in mouse vagus had an inhibitory effect on acrolein or capsaicin-induced responses, indicating that these compounds were not acting off-target on the TRPA1 or TRPV1 ion channels. To confirm the unexpected results observed in mice, a B_1-selective agonist (Lys-(Des-Arg^9)Bradykinin) was tested on wild type mouse isolated vagus nerves. Depolarisation

occurred in a concentration-dependent manner, indicating that B_1 was indeed causing a stimulatory effect in mouse sensory nerves (Figure 5.3). This indicates that there are species differences in the way BK effects are mediated in mice compared to guinea pigs and humans.

It has been suggested that BK may induce its tussive effects via downstream production of prostanoids through the cyclooxygenase pathway (Ellis & Fozard, 2002). I tested this hypothesis by incubating sensory nerves with the general cyclooxygenase inhibitor indomethacin (10 µM). Indomethacin did not alter BK-induced sensory nerve activation either in mouse or guinea pig tissue (Figure 5.4), providing evidence against a role for prostanoid production in BK-induced cough.

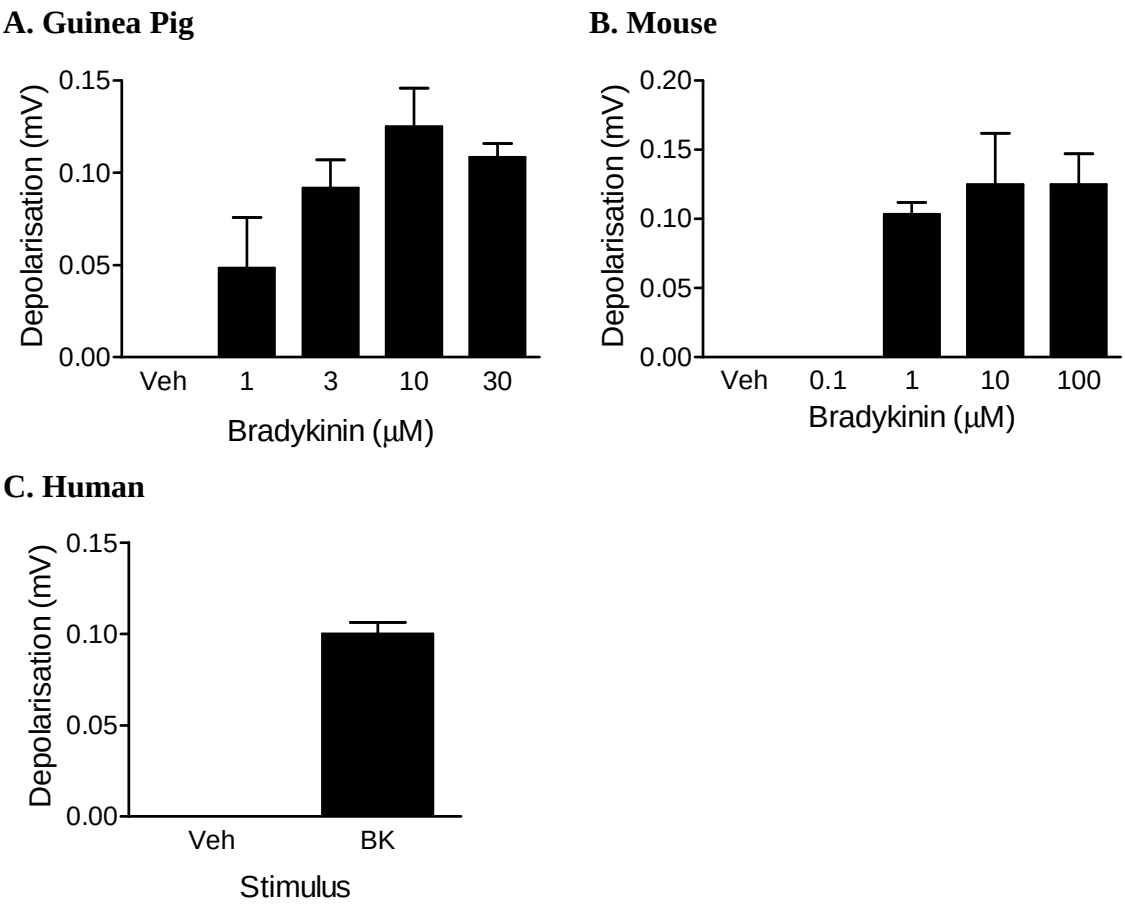
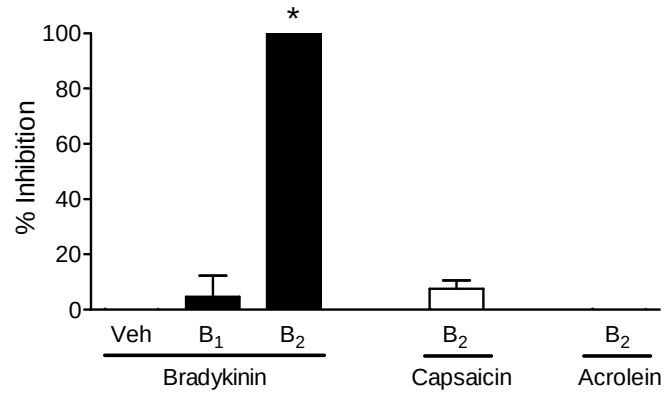


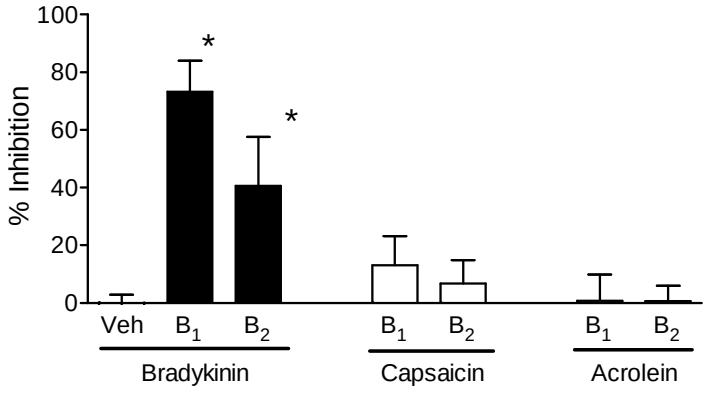
Figure 5.1. Characterisation of the isolated vagus nerve response to BK stimulation.

A concentration response was established for BK on (a) *guinea pig and* (b) *wild type mouse isolated vagus nerve.* (c) *Human vagal tissue also responded to BK stimulation (3 µM). Veh = vehicle (0.1% dH2O). Data are presented as mean ± s.e.m of n = 6 observations for guinea pig and mouse; and n = 4-5 for human experiments.*

A. Guinea Pig



B. Mouse



C. Human

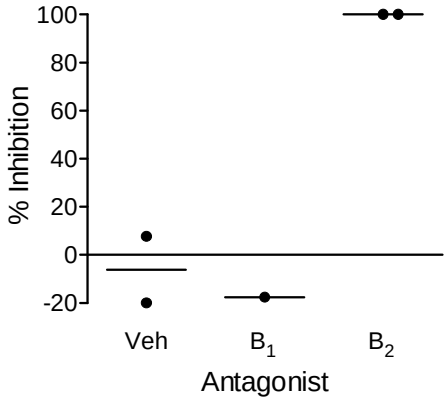


Figure 5.2. Determining the G protein-coupled receptor responsible for BK-induced sensory nerve stimulation.

Isolated vagus nerves were incubated with selective B_1 (1 µM R715) or B_2 (10 µM WIN 64338) inhibitors or vehicle (Veh, 0.1% DMSO) for 10 minutes prior to stimulation with an agonist. (a) *Guinea pig and* (c) *human nerve responses stimulated by BK (3 µM) were abolished by B_2 antagonism, but not inhibited by B_1 antagonism or vehicle.* (b) *Mouse nerve responses stimulated by BK (1 µM) were partially inhibited by either B_1 or B_2 antagonists, but not affected by vehicle. Neither B_1 nor B_2 antagonism inhibited capsaicin (1 µM) or acrolein (300 µM) responses in guinea pig or mouse tissue. * indicates statistical significance ($p < 0.05$; paired t-test), comparing responses in the same nerve before and after antagonist incubation. Data are presented as mean ± s.e.m. of n = 6 observations for guinea pig and mouse; and n=1-2 for human experiments.*

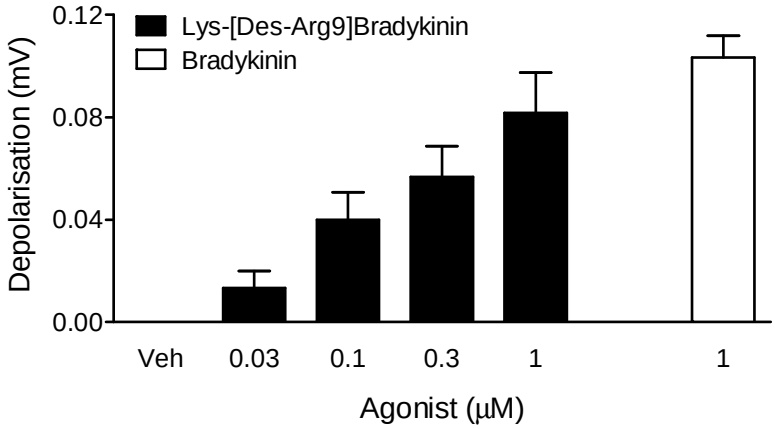


Figure 5.3. A selective B1 agonist activates mouse isolated vagus nerves.

A concentration-response curve was established for the B_1-selective agonist Lys-[Des-Arg9]Bradykinin (black bars) in isolated mouse vagus nerves to corroborate constitutive expression of the B_1 receptor in this species. The magnitude of depolarisation is compared to the concentration of bradykinin used in antagonist experiments (1 µM; white bar). Veh = vehicle (0.1% DMSO). Data are presented as mean ± s.e.m. of n=6 observations.

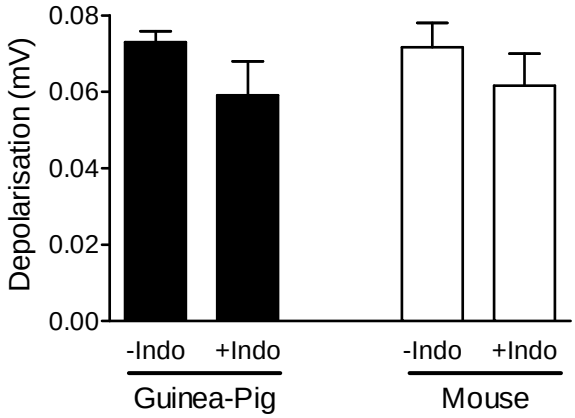


Figure 5.4. Inhibition of cyclooxygenase does not alter BK-induced vagus nerve stimulation.

Isolated vagus nerves were stimulated with BK (3 µM guinea pig; 1 µM mouse) for 2 minutes, followed by10 minutes incubation with the cyclooxygenase inhibitor indomethacin (Indo, 10 µM), and stimulation with BK in the presence of indomethacin. Indomethacin failed to alter BK-induced sensory nerve depolarisation in either the guinea pig or mouse isolated vagus, determined by paired t-test comparing responses in the same nerve before and after antagonist incubation (significance set at $p < 0.05$). Data are presented as mean ± s.e.m. of n = 6 observations.

Having established that BK mediates sensory nerve depolarisation via the B_2 GPCR in guinea pigs and humans, and both B_1 and B_2 GPCRs in mice, I went on to investigate whether TRPA1 and TRPV1 were the ion channels stimulated downstream of GPCR activation. The magnitude of depolarisation induced by 1 µM BK in *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$ vagus nerve was approximately half that seen in wild type (Figure 5.5), indicating a role for both of these ion channels in mediating the BK response in mouse tissue. The importance of TRPA1 and TRPV1 ion channels in mice as well as guinea pigs and humans was confirmed using TRPA1- (HC-030031) and TRPV1-selective (capsazepine, JNJ17203212) antagonists. Either TRPA1 or TRPV1 antagonism partially inhibited BK-induced sensory nerve activation in guinea pig, wild type mouse and human isolated vagus nerves. When used in combination, both TRPA1 and TRPV1 antagonism virtually abolished nerve responses in all of these species (Figure 5.6). To confirm that these results were not due to unusual pharmacological effects of combining two antagonists, TRPA1 or TRPV1 antagonists were used to inhibit BK activation in vagal tissue taken from the alternate genetically modified mouse i.e. TRPA1 antagonist with *Trpv1*$^{-/-}$ tissue; and TRPV1 antagonist with *Trpa1*$^{-/-}$ tissue. Results from these studies corroborated the earlier observations, showing almost complete loss of BK-induced sensory nerve responses (Figure 5.6B)

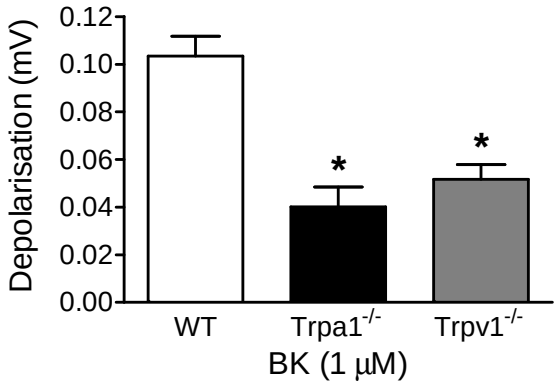


Figure 5.5. Determining a role for TRPA1 and TRPV1 ion channels in BK-induced isolated vagus nerve responses.

BK (1 µM) was applied to wild type (C57Bl/6j), Trpa1$^{-/-}$ *or Trpv1*$^{-/-}$ *mouse isolated vagus nerves for 2 minutes. The magnitude of depolarisation (mV) in Trpa1*$^{-/-}$ *and Trpv1*$^{-/-}$ *tissue was approximately half that seen in wild type, indicating a role for both of these ion channels in BK-induced nerve activation. * indicates statistical significance compared to wild type control (p < 0.05, Kruskal Wallis one-way ANOVA with Dunn's multiple comparison post-hoc test). Data are presented as mean ± s.e.m. of n = 6 observations.*

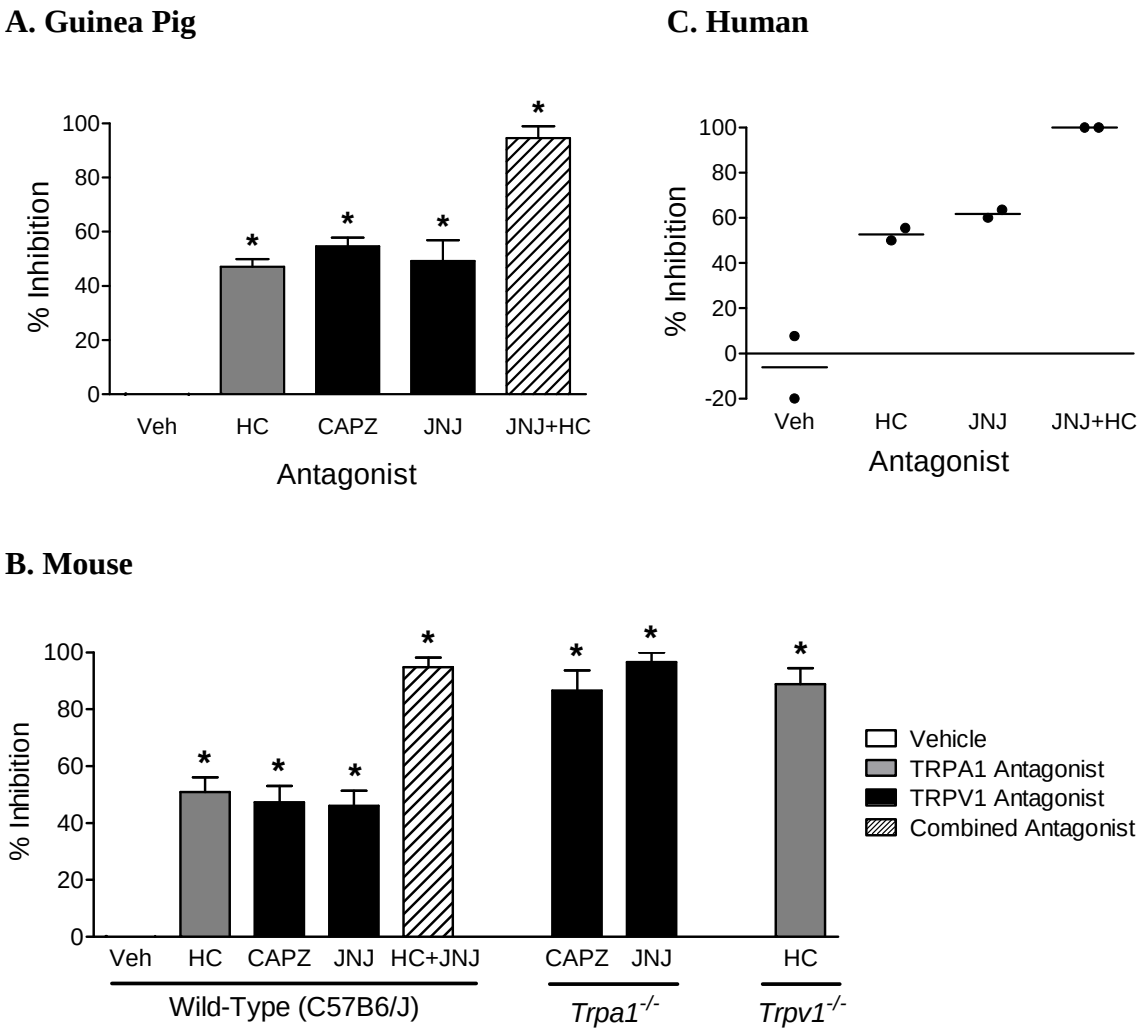


Figure 5.6. Pharmacologically determining a role for TRPA1 and TRPV1 ion channels in BK-induced isolated vagus nerve responses.

Isolated vagus nerves were incubated with a selective TRPA1 antagonist (10 µM HC-030031, HC); a selective TRPV1 antagonist (10 µM capsazepine, CAPZ; 100 µM JNJ17203212, JNJ); a combination of both TRPA1 and TRPV1 antagonists (HC+JNJ); or vehicle (0.1% DMSO, Veh) for 10 minutes prior to BK (3 µM guinea pig and human; 1 µM mouse) stimulation. Antagonism of either TRPA1 or TRPV1 partially inhibited BK-induced responses in (a) *Guinea pig,* (b) *Mouse and* (c) *Human vagus nerves. Whereas, a combination of both TRPA1 and TRPV1 antagonists, or tissue taken from Trpa1$^{-/-}$ or Trpv1$^{-/-}$ mice incubated with the alternate antagonist, virtually abolished BK stimulation. * indicates statistical significance ($p < 0.05$; paired t-test), comparing responses in the same nerve before and after antagonist incubation. Data are presented as mean ± s.e.m. of n = 6 observations for guinea pig and mouse; and n = 2 for human experiments.*

5.3.2 BK-induced guinea pig cough

To investigate BK-induced coughing in conscious guinea pigs, I began by establishing a CR to determine an appropriate concentration of aerosolised BK with which to test the antagonists. Aerosolisation of vehicle produced no coughs, and 30 mg/ml induced few coughs. In contrast, 1, 3 and 10 mg/ml BK elicited robust coughing in most or all guinea pigs (Figure 5.7). Similar to PGE_2, BK elicited trains of smaller coughs, often with multiple bouts occurring during one 10 minute exposure (refer to Figure 4.5).

Following the results obtained in the isolated vagus model, the ability of TRPA1- (300 mg/kg HC-030031) and TRPV1-selective (100 mg/kg JNJ17203212) antagonists to inhibit the BK-induced cough response was assessed. When pre-treated with vehicle only, BK induced on average 12 ± 4 coughs. Pre-treatment with either TRPA1 or TRPV1 antagonists partially inhibited the cough response. As observed with PGE_2-induced cough, either TRPA1 or TRPV1 antagonists inhibited the number of BK-induced coughs by over half. BK stimulation elicited 1 ± 0.2 ($p < 0.05$ in comparison to vehicle control) and 3 ± 2 coughs with TRPA1 or TRPV1 antagonists, respectively. The cough response with TRPV1 antagonist pre-treatment did not reach significance in comparison to vehicle control, likely because of variability introduced in the data due to one animal in the TRPV1 antagonist group that coughed 22 times. Pre-treatment with both TRPA1 and TRPV1 antagonists completely abolished the cough response to BK stimulation (0 ± 0 coughs; Figure 5.8). This data corroborates the *in vitro* sensory nerve results presented above, and provides strong evidence for activation of both TRPA1 and TRPV1 ion channels by BK downstream of the B_2 receptor in guinea pigs.

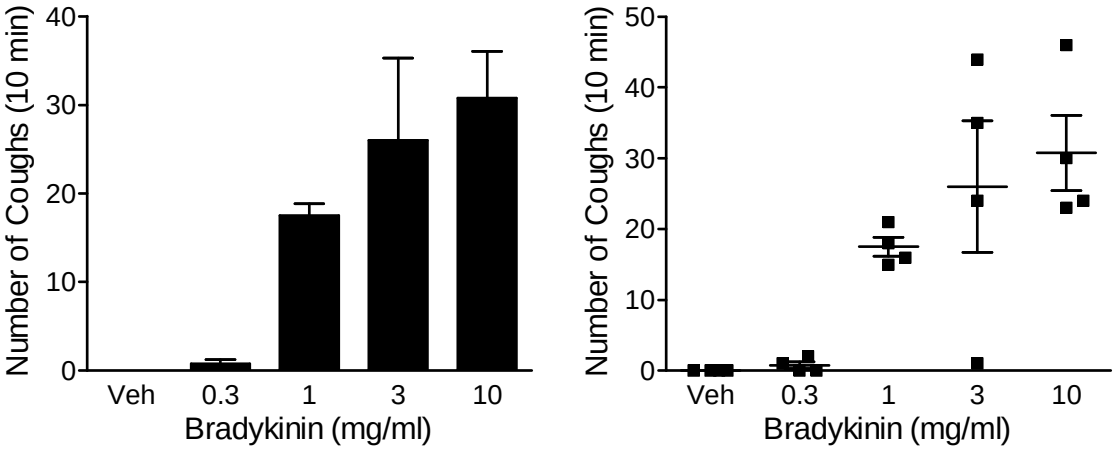


Figure 5.7. Characterising BK-induced cough in the conscious guinea pig model.

A concentration response for BK-induced cough was established. BK was aerosolised for 10 minutes and the number of coughs counted during this period. Data are plotted in both bar chart and scatter-graph format as mean ± s.e.m. of n=4 observations. Veh = vehicle (0.9% saline).

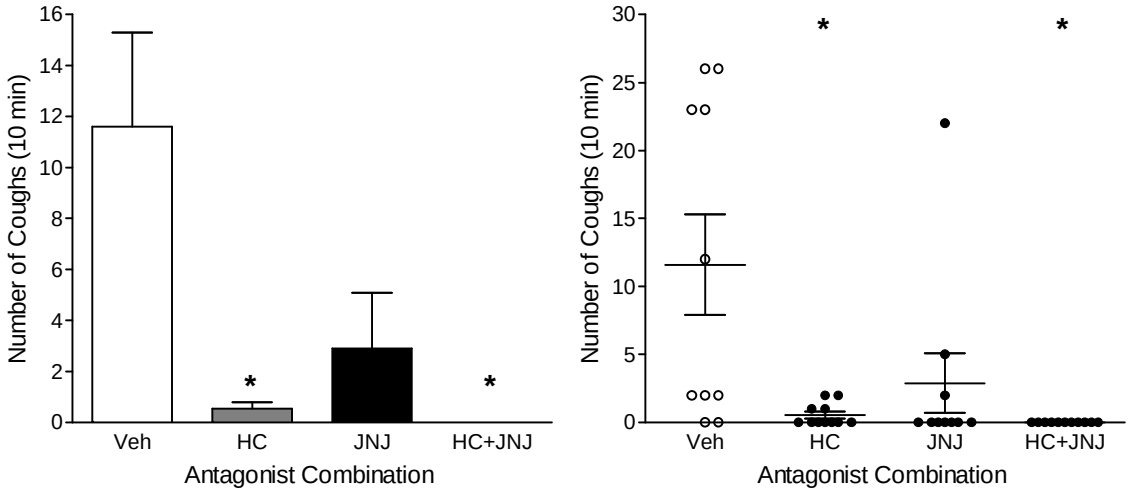


Figure 5.8. Determining a role for TRPA1 and TRPV1 ion channels in BK-induced cough.

*Guinea pigs were injected i.p. 1 hour prior to aerosolised BK (3 mg/ml) stimulation with: TRPA1 vehicle plus TRPV1 vehicle (Veh); TRPA1 antagonist (300 mg/kg HC-030031) plus TRPV1 vehicle (HC); TRPV1 antagonist (100 mg/kg JNJ17203212) plus TRPA1 vehicle (JNJ); or TRPA1 antagonist plus TRPV1 antagonist (HC+JNJ). Data are plotted in both bar chart and scatter graph format as mean ± s.e.m. of n=10-11 observations. * indicates statistical significance compared to vehicle control ($p < 0.05$; Kruskal Wallis one-way ANOVA with Dunn's multiple comparison post-hoc test).*

5.4 Discussion

Inhaled BK induces coughing in both humans and animals (Choudry et al., 1989; Herxheimer & Stresemann, 1961; Katsumata et al., 1991; Kaufman et al., 1980). Inhibition of the breakdown of BK with ACE inhibitors has also been linked to cough hypersensitivity associated with this therapy for cardiovascular disease, which was postulated to be via activation of the B_2 GPCR (Fox et al., 1996). Indeed, activation of the B_2 isoform of the bradykinin receptor is generally believed to mediate the tussive effects of BK. But there has never been a comprehensive investigation in to which GPCR, or downstream ion channels, are associated with the tussive effects of BK. The aims of this chapter were, therefore to determine both the GPCR and ion channels responsible for mediating the tussive effects of BK using the isolated vagus preparation and an *in vivo* cough model.

BK binds to one of two GPCR isoforms, the B_1 and B_2 receptors. In general, the B_2 receptor is constitutively expressed, whereas, the B_1 receptor is thought to be inducible. However, it has been suggested that there may be some constitutive expression of the B_1 isoform in the mouse (Vianna & Calixto, 1998). Using the isolated vagus nerve preparation I have established that BK-induced responses are indeed mediated entirely by the B_2 receptor in guinea pig and human tissue. In contrast, both B_1 and B_2 isoforms play a role in BK activation of the mouse sensory nerves, with B_1 mediating the majority of the response. To confirm these findings, I tested a B_1-selective agonist on mouse isolated vagus, and observed a concentration-related increase in sensory nerve depolarisation. These results corroborate the findings of Vianna & Calixto (1998), where they also proposed constitutive expression of B_1 in the mouse. This indicates a difference in the way that BK mediates its airway irritant effects in the mouse in comparison to guinea pigs and humans. Despite this, the subsequent signalling pathways downstream of GPCR binding appear to activate the same ion channels in these species. In addition to their effects on BK-induced vagus nerve depolarisation, both the B_1 and B_2 antagonists were assessed for their ability to inhibit capsaicin and acrolein responses. Neither antagonist was shown to have an effect, indicating that they were not having off-target inhibitory outcomes on the TRPA1 or TRPV1 ion channels, and therefore confirming the action of BK on its own GPCRs.

A number of the outcomes associated with BK have been found to be via indirect effects, including the release of other endogenous mediators such as prostaglandins downstream of arachadonic acid metabolism (Ellis & Fozard, 2002). It is well-known that PGE_2 activates

airway sensory nerves, causing cough; and it is feasible that other prostanoids are capable of tussive effects as well. I therefore investigated the possibility that BK-induced sensory nerve activation was an indirect effect due to the downstream release of irritant prostanoid compounds. Vagus nerves were incubated with the general cyclooxygenase inhibitor indomethacin. Indomethacin did not have any effect on BK-induced nerve depolarisation in either the guinea pig or mouse, indicating that BK does not act via this pathway to stimulate the vagus nerve.

Despite the above suggestion that BK may act indirectly, it is also known to mediate some nociceptive effects via the activation of TRPA1 and/or TRPV1 ion channels, probably through the intracellular phospholipase C signalling pathway (Bautista et al., 2006). It is therefore possible that binding of BK to one of the bradykinin receptors induces the release of intracellular secondary messengers which then phosphorylate ion channels to cause airway irritation. This hypothesis was investigated using the concentrations of TRPA1 and TRPV1-selective antagonists established in Chapter 3, as well as genetically modified *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$ mice. Pharmacological inhibition of either TRPA1 or TRPV1 receptors with selective antagonists reduced BK responses by approximately 50%; and when used in combination virtually abolished sensory nerve depolarisation in guinea pig, mouse and human vagus nerves. Furthermore, knockdown of either the TRPA1 or TRPV1 receptor in genetically modified mice halved the magnitude of depolarisation induced by BK stimulation in comparison to wild type controls. When used in combination with the alternate antagonist, again, vagus nerve activation was all-but abolished in *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$ tissue. These results provided strong *in vitro* evidence that BK-induced tussive responses are mediated approximately 50/50 by the TRPA1 and TRPV1 ion channels, and that this effect is translated across species.

Having implicated a role for both TRPA1 and TRPV1 in BK-induced sensory nerve activation, I proceeded to confirm these results in the *in vivo* cough model. Appropriate doses of TRPA1 (HC-030031) and TRPV1 (JNJ17203212) inhibitors had been established in chapter 3. Pre-treatment with either TRPA1 or TRPV1 antagonists partially inhibited the guinea pig cough response to BK stimulation compared to vehicle control, whereas pre-treatment with a combination of both antagonists abolished cough altogether. As with PGE_2, BK-induced cough was inhibited by over half with either TRPA1 (95%) or TRPV1 (75%) antagonists compared to vehicle control, which is contrary to findings in the *in vitro* isolated

vagus model, whereby either antagonist inhibited BK responses by around 50%. Similarly, though treatment with both TRPA1 and TRPV1 antagonists completely abolished BK-induced cough, there was still a small depolarisation effect observed in the vagus nerve preparation. As discussed in section 4.4 in relation to PGE_2-induced responses, this could be due to the difference in end-points being measured by these two models. That is, the isolated vagus nerve preparation measures compound membrane depolarisation of the sensory nerves, which does not necessarily lead to action-potential generation and subsequent coughing. This is because action potentials are an all-or-none response, and if membrane depolarisation of the nerves does not reach threshold, there is no signal sent to the brain. As such, 50% inhibition of the BK response on the vagus nerve may prevent the stimulus reaching threshold in more than half of the nerve fibres, therefore fewer action potentials are generated, and the information relayed to the CNS causes substantially fewer coughs.

In summary, this chapter has established that BK-induced sensory nerve activation in guinea pig and human vagus nerves is through the B_2 GPCR; whereas, the majority of BK-mediated activation in mouse sensory nerves is through B_1, with a smaller role for the B_2 receptor. Despite this, signalling downstream in all three species appears to activate both TRPA1 and TRPV1 ion channels, which are equally involved in mediating the irritant effects of BK. Furthermore, results generated from the *in vivo* guinea pig cough model indicat that inhibition of either TRPA1 or TRPV1 could substantially reduce the tussive effects of BK over-and-above the 50% blockade seen on the axon of the vagus nerve *in vitro*; and that a combination of both antagonists completely abolishes BK associated cough.

CHAPTER 6

Endogenous Mediators: Low pH

6.1. Rationale

The balance of pH within the body is maintained within a narrow range in healthy individuals. Accordingly, cells possess mechanisms in which to sense deviations from normal pH levels. Low pH is an established and effective tussive agent which is commonly used in human and animal cough research, and to assess cough hypersensitivity in disease states (Karlsson & Fuller, 1999; Morice et al., 2007, 2001). Indeed, patients with inflammatory airways disease have been observed to exhibit lower lung pH than healthy individuals (Hunt et al., 2000; Kostikas et al., 2002), and as such low pH could play a role in the pathogenesis of chronic cough associated with these diseases. It is known that sensory nerve activation and tussive effects of low pH are partially mediated via the TRPV1 ion channel, though we do not currently understand how acidic solutions cause channel opening (Kollarik & Undem, 2002; Lalloo et al., 1995). We also do not know what other ion channel(s) are involved in mediating the low pH response.

TRPA1 has also been implicated in detecting changes in pH. In DRG neurons, and models of pain using both wild type and *Trpa1*$^{-/-}$ mice, TRPA1 has been demonstrated to play a role in sensing high pH (alkalinity) (Dhaka et al., 2009; Fujita et al., 2008). Moreover, TRPA1 was recently found to be activated by decreases in intracellular pH (Wang et al., 2011). A role for TRPA1 in low pH-induced cough has not yet been investigated. The first aim of this chapter was, therefore, to confirm a role for TRPV1 in our models, and determine whether TRPA1 plays a role in low pH-induced sensory nerve activation *in vitro*, and guinea pig cough *in vivo*.

In addition, the Acid Sensing Ion Channels (ASICs) have been suggested to play a role in low pH-induced cough. However, research investigating these channels is problematic due to the poor selectivity of the available pharmacological tools. Moreover, this area of research is further complicated by the propensity of ASIC channels to form heteromultimeric channels. As such, there is yet to be a comprehensive investigative effort in to the role these ion channels play in the tussive response. Current evidence suggests that the ASIC1 or ASIC3 receptors may be involved, based on their expression in rat DRG neurons (Alvarez de la Rosa et al., 2002), and the rapidly inactivating profile of the TRPV1-independent acid sensing mechanisms in sensory neurons (Kollarik & Undem, 2002). Therefore, ASIC1 and/or ASIC3 may be involved in the response either as homodimers or heterodimers, in combination with

one or more of the other ASIC subtypes. The second aim of this chapter was to determine whether ASIC ion channels also play a role in low pH-induced sensory nerve activation using a non-selective ASIC channel inhibitor, and tissue from *Asic1*$^{-/-}$ and *Asic3*$^{-/-}$ mice.

6.2 Methods

6.2.1 Low pH-induced sensory nerve activation

Low pH activates TRPV1 ion channels at room-temperature with a pH ≤ 6 (Jordt et al., 2000). A solution of pH5 had previously been established to activate guinea pig vagus nerves within our lab, and I proceeded to confirm this in mouse (wild type, *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$*)* and human tissue. In these experiments I used a Krebs-based solution altered to pH5 (mM: NaCl 118.5; KCl 1.7; KH_2PO_4 6.6; $MgSO_4$ 1.2; $CaCl_2$ 2.5; NaH_2PO_4 0.1; and glucose 10), because low pH generated with citric acid (which is normally used to assess low pH-induced cough *in vivo*) cannot be made up in a physiologically buffered solution. Low pH or vehicle (0.1% dH_2O) was applied to the nerve for 2 minutes, then the tissue washed with unaltered Krebs solution until baseline was re-established. After determining that low pH depolarised vagal sensory nerves, TRPA1- (10 µM HC-030031) and TRPV1-selective (10 µM capsazepine, 100 µM JNJ17203212) antagonists were assessed for their ability to inhibit low pH-induced sensory nerve activation in guinea pig, mouse (wild type, *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$) and human isolated tissue.

The role of the ASIC ion channels in low pH-induced stimulation was also investigated using the isolated vagus nerve preparation. It has been suggested in the literature that the ASIC1 or ASIC3 ion channels may play a role in low pH responses (Kollarik & Undem, 2002), and thus I tested the ability of low pH to induce nerve depolarisation in *Asic1*$^{-/-}$ and *Asic3*$^{-/-}$ mouse tissue compared to wild type (C57Bl/6j). Following this, I investigated the ability of a non-selective ASIC channel inhibitor (100 µM amiloride) to inhibit nerve responses in the guinea pig, wild type mouse and *Trpv1*$^{-/-}$ mouse tissue; and the ability of TRPV1-selective (10 µM capsazepine and 100 µM JNJ17203212), TRPA1-selective (10 µM HC-030031) and non-selective ASIC antagonists (100 µM amiloride) to inhibit responses in *Asic1*$^{-/-}$ and *Asic3*$^{-/-}$ tissue. Amiloride (10 µM) is a non-selective blocker which inhibits ASIC1-like, ASIC2-like and ASIC3-like currents in rat DRG neurons elicited by stimulation with pH4.5 (Dubé et al., 2005). A concentration 10-fold higher than this (100 µM) was chosen for experiments on the isolated vagus nerve, as is common practice in this preparation. Until recently, amiloride and amiloride-related compounds were the only known small molecule blockers of ASIC channels. A more selective non-amiloride inhibitor of ASIC channels has been developed (Dubé et al., 2005), but is expensive to buy due to a multi-step chemical synthesis.

6.2.2 Citric acid-induced guinea pig cough

Conscious, unrestrained male Dunkin Hartley guinea pigs (300-400 g, Harlan, UK) were individually placed in a plethysmography chamber. For the initial CR, animals were exposed to 10 minutes of aerosolised vehicle (0.9% saline) or 0.1, 0.2, 0.3 or 0.4 M citric acid. The number of coughs was recorded during the 10 minute stimulation as previously described in chapter 2, section 2.4.

Pre-treatment with the vehicle for JNJ17203212 (15% solutol in 5% dextrose solution) was previously observed to inhibit citric acid cough responses. Therefore, the vehicle for JNJ17203212 in this instance was changed to the same as that for HC-030031 i.e. 0.5% methyl cellulose in 0.9% saline. For the antagonist study, guinea pigs were dosed i.p. with a combination of either (a) vehicle (0.5% methyl cellulose in 0.9% saline); (b) vehicle plus HC-030031 (300 mg/kg); (c) vehicle plus JNJ17203212 (100 mg/kg); or (d) HC-030031 (300 mg/kg) plus JNJ17203212 (100 mg/kg). Guinea pigs were then exposed to an aerosol of 0.3 M citric acid for 10 minutes, and the number of coughs counted as previously described.

6.2.3 Data Analysis

Antagonism of low pH-induced sensory nerve activation was analysed using paired t-test comparing responses in the same nerve before and after antagonist incubation. Comparison of the magnitude low pH-induced responses in *Trpa1*$^{-/-}$, *Trpv1*$^{-/-}$, *Asic1*$^{-/-}$ or *Asic3*$^{-/-}$ tissue was analysed using the Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison post-hoc test comparing all groups to wild type responses. Antagonism of citric acid-induced cough was also analysed using the Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison post-hoc test comparing all groups to vehicle. Significance was set at $p < 0.05$, and all data were plotted as mean ± s.e.m. of *n* observations.

6.3 Results

Citric acid is a well-known tussive ligand that is commonly used in clinical trials to assess cough sensitivity, and has been shown to activate the TRPV1 ion channel in both *in vitro* and *in vivo* models of cough. Indeed, TRPV1 mediates responses to acidic solutions of pH $\leq$ 6 (Jordt et al., 2000). However, antagonism of TRPV1 only partially inhibits the tussive effects of citric acid, implicating at least one other ion channel in this response. Patients suffering from inflammatory airways disease exhibit a decrease in lung pH, and it has been postulated that this could be important in the development of excessive cough seen in disease states. It is therefore important to establish what other receptors are mediating the irritant effects of acidity in the airways. TRPA1 is a potential target for sensing acidic solutions, and has already been discovered to sense alkaline-induced pain (Fujita et al., 2008) and intracellular changes in pH (Wang et al., 2011). Alternately, ASIC ion channels have been postulated to play a role in sensing acidity in the airways (Kollarik & Undem, 2002).

6.3.1 Low pH-induced sensory nerve activation

Low pH 5 provoked reliable and robust depolarisation of the guinea pig, mouse and human isolated vagus nerves (Figure 6.1). This level of acidity was chosen as it is known to activate TRPV1 ion channels. In contrast, vehicle (0.1% dH_2O) had no effect on nerve stimulation in any of these species. The magnitude of depolarisation induced by low pH in *Trpv1*$^{-/-}$ mouse vagus was approximately half that seen in wild type tissue, but was unaffected in *Trpa1*$^{-/-}$ tissue (Figure 6.2). These results were confirmed using TRPA1- (HC-030031) and TRPV1-selective (capsazepine, JNJ17203212) antagonists. Whereby, TRPV1 antagonism partially inhibited low pH-induced sensory nerve activation in guinea pig, wild type mouse and human isolated vagus; TRPA1 antagonism had no effect on the magnitude of low pH responses. When used in combination, both TRPA1 and TRPV1 antagonism did not inhibit low pH responses any more than a TRPV1 antagonist alone (Figure 6.3). This confirms a role for TRPV1 in mediating low pH-induced sensory nerve activation, but does not implicate TRPA1 ion channels in the response.

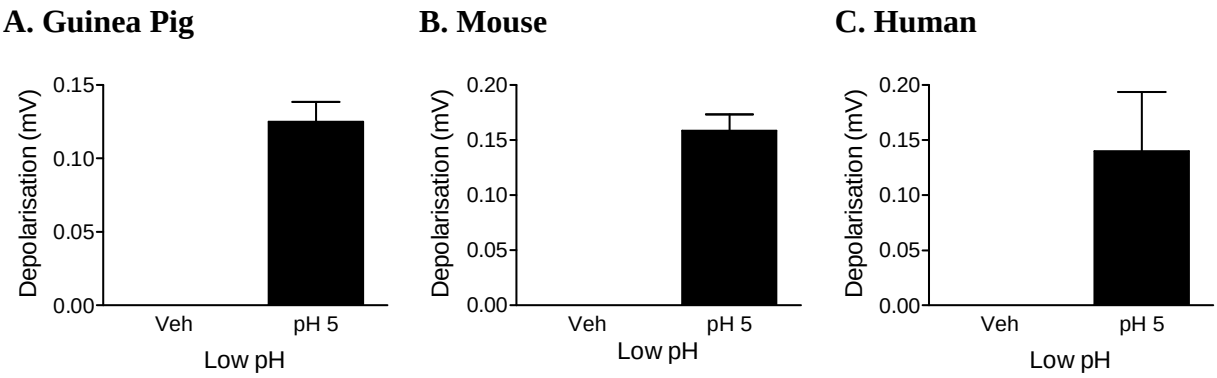


Figure 6.1. Characterisation of the isolated vagus nerve response to low pH stimulation.

The ability of low pH (Krebs solution altered to pH 5) to stimulate sensory nerve fibres was assessed on (a) *guinea pig,* (b) *wild type mouse and* (c) *human isolated vagus nerve. Veh = vehicle (0.1% dH2O). Data are presented as mean ± s.e.m. of n = 6 observations for guinea pig and mouse; and n = 4-6 for human experiments.*

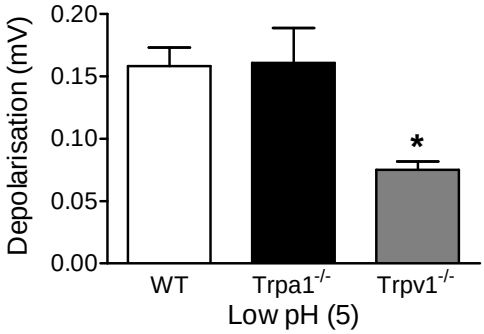
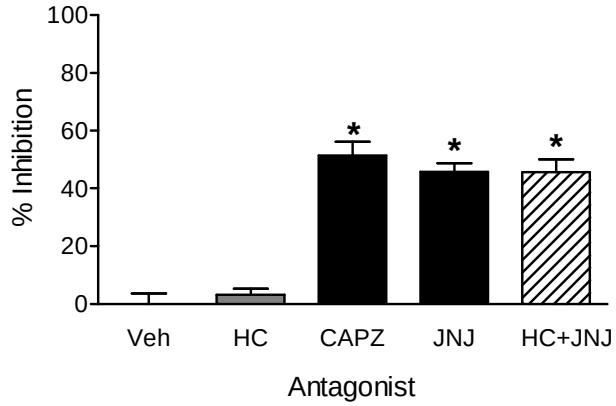


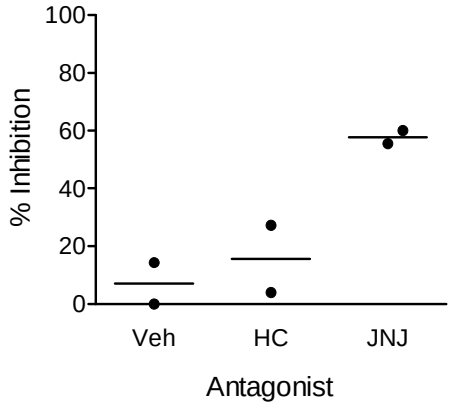
Figure 6.2. Determining a role for TRPA1 and TRPV1 ion channels in low pH-induced isolated vagus nerve responses.

*pH 5 was applied to wild type (C57Bl/6j), Trpa1$^{-/-}$ or Trpv1$^{-/-}$ mouse isolated vagus nerves for 2 minutes. The magnitude of depolarisation (mV) in the Trpv1$^{-/-}$ tissue was approximately half that seen in wild type, indicating a role for this ion channel in low pH-induced sensory nerve activation. In contrast, the magnitude of depolarisation in Trpa1$^{-/-}$ tissue was not significantly different to wild type control. * indicates statistical significance compared to wild type control ($p < 0.05$, Kruskal Wallis one-way ANOVA with Dunn's multiple comparison post-hoc test). Data are presented as mean ± s.e.m. of n = 6 observations.*

A. Guinea Pig



C. Human



B. Mouse

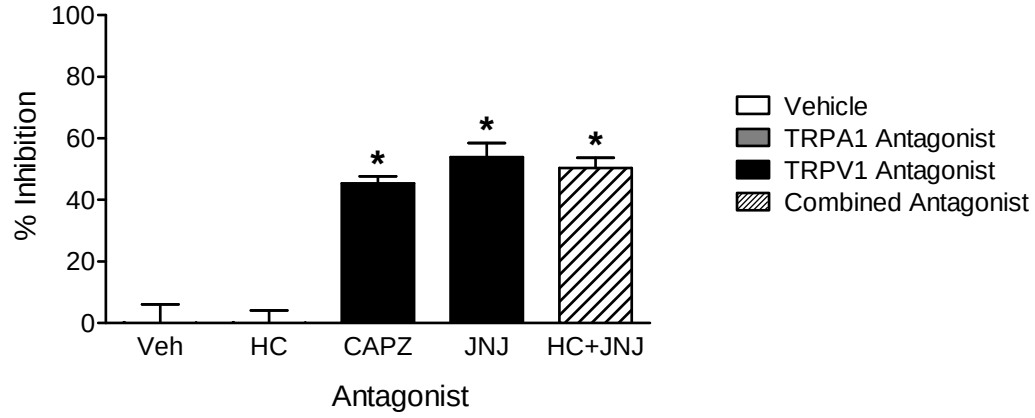
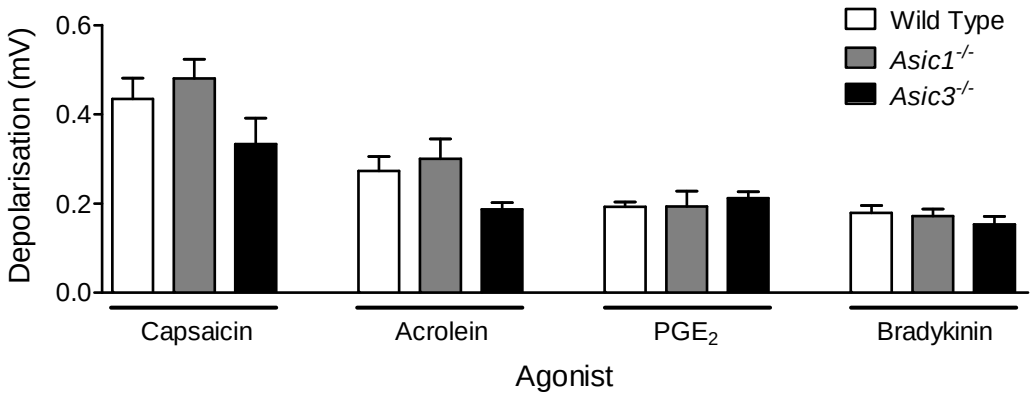


Figure 6.3. Pharmacologically determining a role for TRPA1 and TRPV1 ion channels in low pH-induced isolated vagus nerve responses.

Isolated vagus nerves were incubated with a selective TRPA1 antagonist (10 µM HC-030031, HC); a selective TRPV1 antagonist (10 µM capsazepine, CAPZ; 100 µM JNJ17203212, JNJ); a combination of both TRPA1 and TRPV1 antagonists (HC+JNJ); or vehicle (0.1% DMSO, Veh) for 10 minutes prior to low pH stimulation (pH 5). Antagonism of TRPV1 but not TRPA1 partially inhibited low pH-induced responses in (a) *Guinea pig*, (b) *Mouse and* (c) *Human vagus nerves. A combination of both TRPA1 and TRPV1 antagonists did not inhibit low pH responses any more than TRPV1 antagonism alone. * indicates statistical significance ($p < 0.05$; paired t-test), comparing responses in the same nerve before and after antagonist incubation. Data are presented as mean ± s.e.m. of n = 6 observations for guinea pig and mouse; and n = 2 for human experiments.*

The magnitude of depolarisation induced by capsaicin, acrolein, PGE_2 and BK in the *Asic1*$^{-/-}$ and *Asic3*$^{-/-}$ mice was not altered compared to wild type control, indicating that deletion of these genes did not generally affect the ability of the nerves to respond to agonist stimulation (Figure 6.4A). In contrast, pH 5 in *Asic1*$^{-/-}$ mouse vagus was blunted compared to that observed in wild type tissue ($p < 0.05$); and low pH responses in *Asic3*$^{-/-}$ mouse tissue were augmented in comparison to wild type ($p < 0.05$; Figure 6.4B). Amiloride, a general ASIC inhibitor, partially inhibited low pH responses in guinea pig, wild type mouse and human vagal tissue (Figure 6.5). In contrast, amiloride did not inhibit capsaicin responses in guine-pig or mouse vagus, thereby confirming that there was no off-target effect of this inhibitor on TRPV1 ion channels at the concentration used. In *Asic1*$^{-/-}$ tissue, the TRPV1-selective antagonists capsazepine and JNJ17203212 inhibited low pH responses by 80 ± 11% and 68 ± 12%, respectively ($p < 0.05$). In *Asic3*$^{-/-}$ tissue, incubation with the TRPV1 antagonists inhibited low pH responses by 52 ± 6% and 45 ± 3%, respectively ($p < 0.05$; Fibure 6.6), which is similar to the inhibition observed in wild type mouse tissue (refer to Figure 6.3B). Furthermore, the general ASIC blocker amiloride inhibited low pH responses by 91 ± 10% in *Trpv1*$^{-/-}$ tissue; and 47 ± 4% in *Asic3*$^{-/-}$ tissue ($p < 0.05$); but did not affect low pH stimulation in *Asic1*$^{-/-}$ tissue (3 ± 7%; $p > 0.05$) (Figure 6.6). This is consistent with the above findings implicating a role for ASIC1 but not ASIC3 ion channels in the low pH response.

A.



B.

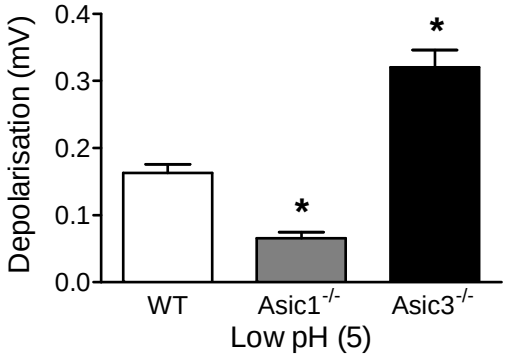


Figure 6.4. Determining a role for ASIC ion channels in low pH-induced isolated vagus nerve responses.

Agonists were applied to wild type (C57Bl/6j), Asic1$^{-/-}$ or Asic3$^{-/-}$ mouse isolated vagus nerves for 2 minutes. (a) *The magnitude of capsaicin (1 µM), acrolein (300 µM), PGE$_2$ (10 µM) or BK (1 µM) responses were not affected by deletion of either the ASIC1 or ASIC3 genes compared to wild type controls.* (b) *Magnitude of depolarisation (mV) induced by low pH (pH 5) stimulation in Asic1$^{-/-}$ tissue was blunted compared to responses in wild type tissue, indicating a role for these ion channels in low pH-induced nerve activation. In contrast, low pH responses in the Asic3$^{-/-}$ mice were enhanced in comparison to wild type. * indicates statistical significance compared to wild type control ($p < 0.05$, Kruskal Wallis one-way ANOVA with Dunn's multiple comparison post-hoc test). Data are presented as mean ± s.e.m. of n = 6-11 observations.*

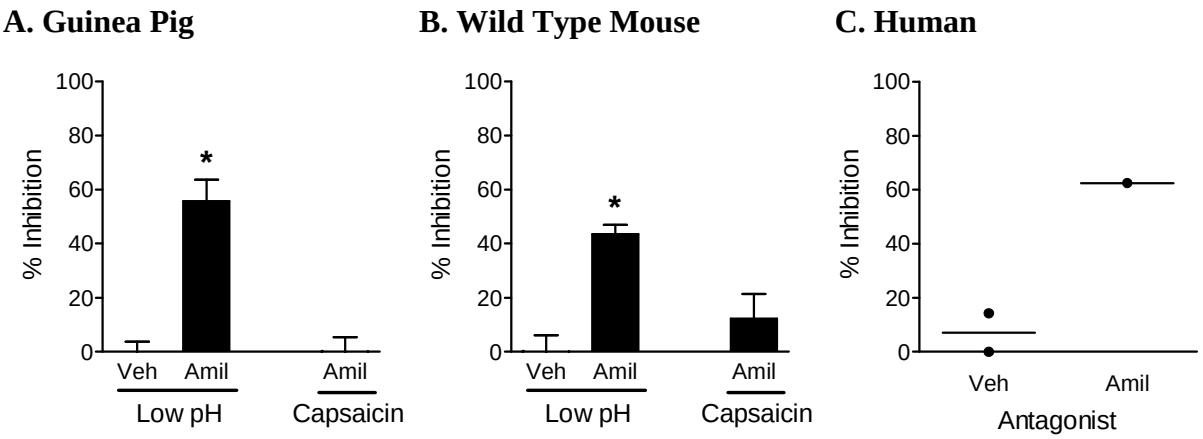


Figure 6.5. Pharmacologically determining a role for ASIC ion channels in low pH-induced isolated vagus nerve responses.

Isolated vagus nerves were incubated with a non-selective ASIC channel inhibitor (100 µM Amiloride; Amil) or vehicle (0.1% DMSO, Veh) for 10 minutes prior to low pH (pH 5) stimulation. ASIC channel antagonism partially inhibited low pH-induced responses in (a) *guinea pig,* (b) *wild type mouse and* (c) *human vagus nerves. * indicates statistical significance (p < 0.05; paired t-test), comparing responses in the same nerve before and after antagonist incubation. Data are presented as mean ± s.e.m. of n = 6 observations for guinea pig and mouse; and n = 1-2 for human experiments.*

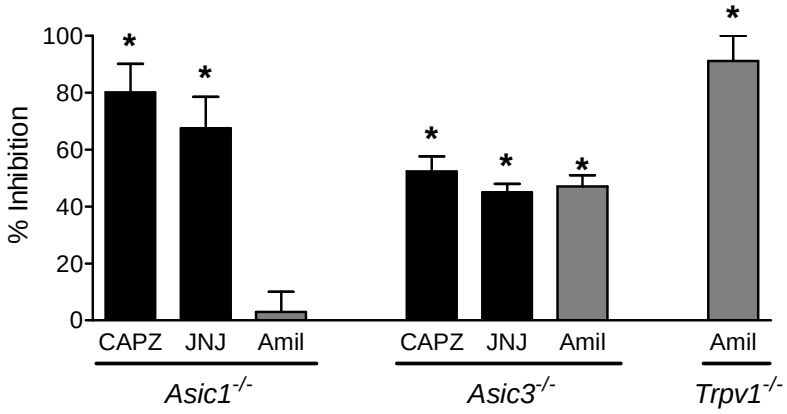


Figure 6.6. Pharmacologically determining a role for the ASIC and TRPV1 ion channels in low pH-induced isolated vagus nerve responses from genetically modified mice.

*Isolated vagus nerves were incubated with a TRPV1-selective antagonist (10 µM capsazepine, CAPZ; 100 µM JNJ17203212, JNJ), or a non-selective ASIC channel inhibitor (100 µM Amiloride, Amil) for 10 minutes prior to low pH (pH 5) stimulation. Incubation with a TRPV1 antagonist on Asic1$^{-/-}$ tissue inhibited low pH responses by approximately 68-80%; and on Asic3$^{-/-}$ tissue inhibited low pH responses by 45-52%. Incubation with a general ASIC inhibitor on Trpv1$^{-/-}$ vagus nerves inhibited low pH responses by approximately 91%. * indicates statistical significance (p < 0.05; paired t-test), comparing responses in the same nerve before and after antagonist incubation. Data are presented as mean ± s.e.m. of n = 6 observations.*

6.3.2 Citric acid-induced guinea pig cough

A concentration-response for citric acid-induced cough in the conscious guinea pig model had been previously established in our lab. Neither vehicle nor 0.1 M citric acid elicited any coughing; whereas 0.2, 0.3 and 0.4 M concentrations elicited robust coughing in most or all animals (Figure 6.7). A sub-maximal concentration of 0.3 M was chosen for use in the antagonist study. Citric acid elicited single loud coughs similar to capsaicin and acrolein stimulation (refer to Figure 3.10).

The ability of TRPA1- (300 mg/kg HC-030031) and TRPV1-selective (100 mg/kg JNJ17203212) antagonists to inhibit citric acid-induced cough was then assessed. When pre-treated with vehicle only, citric acid induced on average 9 ± 2 coughs. Surprisingly, pre-treatment with either TRPA1 or TRPV1 antagonists partially inhibited the number of coughs; citric acid stimulation elicited 2 ± 1 with TRPA1 antagonist pre-treatment, and 2 ± 1 coughs with TRPV1 antagonist pre-treatment ($p < 0.05$). However, i.p. injection with a combination of both TRPA1 and TRPV1 antagonists did not inhibit the cough response any more than either antagonist alone (1 ± 0.5 coughs; Figure 6.8). This is in contrast to the results observed in the *in vitro* isolated vagus nerve model, where TRPA1 antagonism did not inhibit low pH responses.

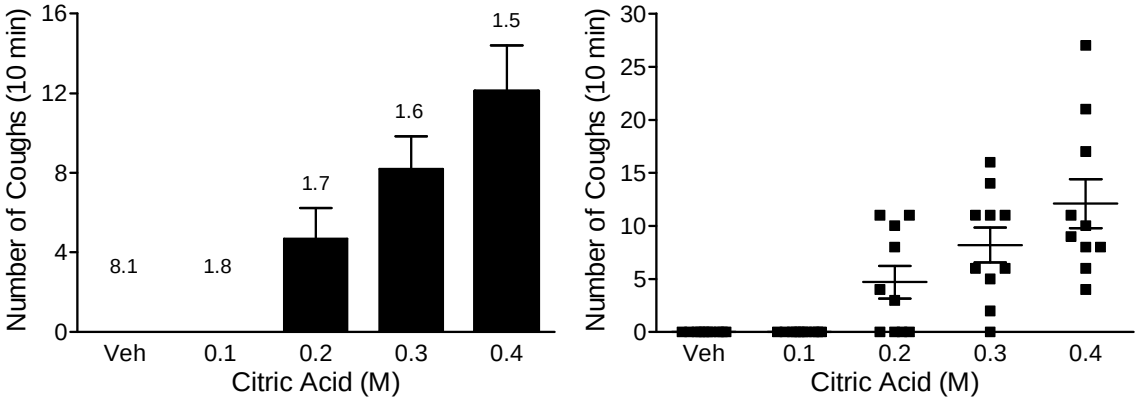


Figure 6.7. Characterising citric acid -induced cough in the conscious guinea pig model.

A concentration response for citric acid-induced cough was established. Citric acid was aerosolised for 10 minutes, and the number of coughs counted during this period. Data are plotted in both bar chart and scatter-graph format as mean ± s.e.m. of n=10 observations. Veh = vehicle (0.9% saline). The pH of each solution is indicated above the corresponding bar on the left-hand bar graph.

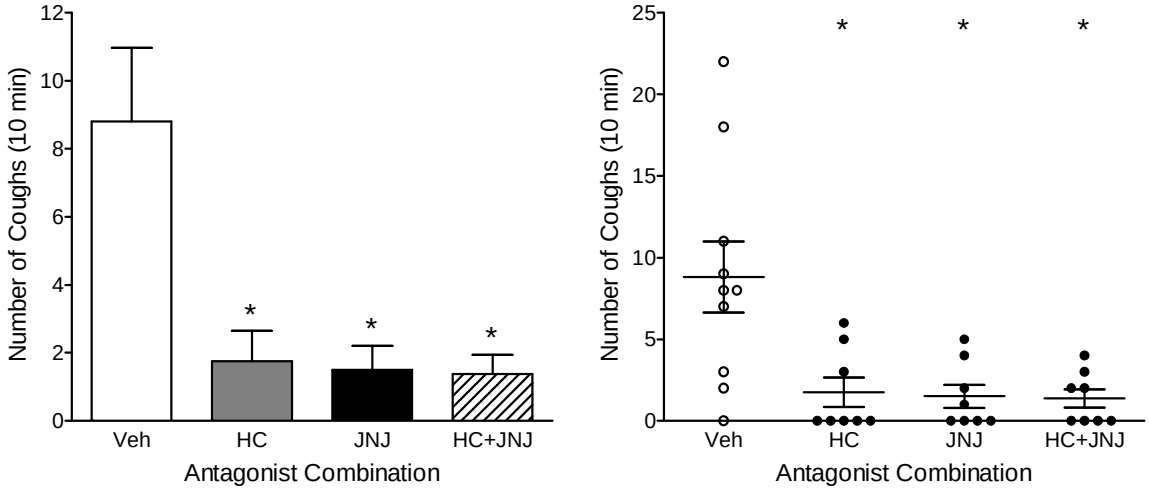


Figure 6.8. Determining a role for TRPA1 and TRPV1 ion channels in citric acid-induced cough.

*One hour prior to 10 minutes stimulation with aerosolised citric acid (0.3 M), guinea pigs were injected i.p with: TRPA1 vehicle plus TRPV1 vehicle (Veh); TRPA1 antagonist (300 mg/kg HC-030031) plus TRPV1 vehicle (HC); TRPV1 antagonist (100 mg/kg JNJ17203212) plus TRPA1 vehicle (JNJ); or TRPA1 antagonist plus TRPV1 antagonist (HC+JNJ). Data are plotted in both bar chart and scatter graph format as mean ± s.e.m. of n=8-10 observations. * indicates statistical significance compared to vehicle control ($p < 0.05$; Kruskal Wallis one-way ANOVA with Dunn's multiple comparison post-hoc test).*

6.4 Discussion

Low pH is an established tussive agent which is commonly used to assess the cough reflex, both in animal research and in the clinic (Karlsson & Fuller, 1999; Morice et al., 2007, 2001). TRPV1 partially mediates the tussive effects of low pH, but it is not currently know what other ion channel(s) are involved. I hypothesised that the TRPA1 ion channel could also be activated by low pH solutions. TRPA1 is a receptor that is activated by a multitude of diverse ligands, and has been demonstrated in this thesis to mediate both PGE_2 and BK-induced tussive responses alongside the TRPV1 ion channel (see chapters 4 & 5). In addition, a role for TRPA1 in detecting intracellular acidity has recently been established (Wang et al., 2011), and it has been implicated in detecting alkaline substances (Dhaka et al., 2009; Fujita et al., 2008). There is also evidence in the literature that ASIC ion channels, particularly the ASIC1 and ASIC3 isoforms, may be involved in low pH responses (Kollarik & Undem, 2002). The aim of this chapter was to investigate the ion channel(s) responsible for low pH-induced cough using the isolated vagus preparation and an *in vivo* model of cough.

A role for TRPV1 in mediating low pH-induced sensory nerve activation *in vitro* was confirmed by pharmacological inhibition with TRPV1-selective antagonists in guinea pig, mouse and human tissue; and assessment of the magnitude of response to low pH in wild type vs. genetically modified *Trpv1*$^{-/-}$ animals. In contrast, antagonism or knockdown of the TRPA1 receptor had no effect on low pH-induced vagus nerve responses in any of these species. This data provided evidence against a role for TRPA1 in low pH-induced vagal sensory afferent stimulation. I therefore went on to investigate whether the ASIC ion channels could be mediating low pH responses. Indeed, pharmacological inhibition of the ASIC channels with a general ASIC antagonist partially inhibited low pH responses in guinea pig and wild type mouse tissue. Because of a lack of selective antagonists which target the respective ASIC sub-types, I also used tissue from genetically modifed *Asic1*$^{-/-}$ and *Asic3*$^{-/-}$ animals to identify which receptor sub-type(s) were mediating the inhibition observed with the general ASIC antagonist. *Asic1*$^{-/-}$ and *Asic3*$^{-/-}$ mice were obtained due to published data suggesting that one of these isoforms was likely to be mediating low pH in the airways. This hypothesis is based on the expression profile of ASIC ion channels in rat DRG neurons; and their observed activation and deactivation kinetics, which are similar to the TRPV1-independent low pH response on single fibres projecting to the airways (Kollarik & Undem, 2002; Kollarik et al., 2007). Using the isolated vagus preparation, I found that low pH

responses in vagal tissue from *Asic1*$^{-/-}$ mice were blunted compared to wild type; whereas responses in *Asic3*$^{-/-}$ mice were augmented. This indicates a role for the ASIC1 ion channels in mediating the low pH tussive response. The enhanced response observed in the *Asic3*$^{-/-}$ mice was unexpected, but is consistent with the literature, in which Chen and colleagues found a reduced time to onset of pain responses and an increase in pain-related behaviours in *Asic3*$^{-/-}$ mice compared to wild type (Chen et al., 2002). In support of a role for the ASIC1 ion channel in low pH-induced sensory nerve activation, TRPV1 antagonists produced 68-80% inhibition of the low pH response in *Asic1*$^{-/-}$ mice, but there was no effect of a general ASIC inhibitor, thus indicating that knockdown of ASIC1 gene accounted for all of this effect. In contrast, TRPV1 antagonists only inhibited the low pH response by 45-52% in *Asic3*$^{-/-}$ mice, which is comparable to the inhibition observed in wild type mice of 45-54%. Furthermore, amiloride produced inhibition of approximately 47% in the *Asic3*$^{-/-}$ tissue, again comparable to the inhibition seen in wild types of around 44%. What I have not investigated in this thesis is the role of ASIC2 ion channels in the response to low pH. Our group has recently obtained permission to bring in some *Asic2*$^{-/-}$ mice, which are being kindly provided by Dr Ladzdunski (Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne, France). Also, it is important to note that there are two isoforms of the ASIC1 channel (ASIC1a and ASIC1b). The knockout mouse colony that I have used in these studies are devoid of functional channels for both of the ASIC1 isoforms and thus further studies will be required to determine whether ASIC1a or ASIC1b (or both) are mediating the effects I have described above.

In contrast to findings in the isolated vagus preparation, pre-treatment with either TRPA1 or TRPV1 antagonists partially inhibited citric acid-induced cough in conscious guinea pigs. However, pre-treatment with a combination of both antagonists did not inhibit cough any more than either antagonist alone, indicating that there is still a role for the ASIC ion channels (likely ASIC1) in mediating citric acid-induced cough. There are a number of possible reasons why the *in vitro* isolated vagus evidence contradicts the *in vivo* cough results. Firstly, in the isolated vagus preparation we are measuring from all vagal afferents, including all the different types of sensory fibre (RAR, SAR, Aδ and C-fibre), as well as airway- and non-airway-projecting fibres. Therefore, any effect of low pH on the TRPA1 ion channel could be masked in this preparation if the effect is small, restricted only to one type of fibre type, or restricted only to the airway-projecting afferents. Furthermore, though we know we are stimulating the vagus nerve with pH 5, we cannot be sure what level of acidity is reached in the lungs when inducing cough with 0.3 M citric acid, which has a solution

acidity of pH 1.6. Therefore, TRPA1 may mediate responses to levels of pH more acidic than pH 5. These are interesting questions which could be answered by using cellular models, whereby we can measure from single cells and distinguish between airway and non-airway afferents. Cellular imaging techniques have only recently been established in our laboratory, therefore in order to investigate these hypotheses I have initially had to characterise a model of vagal ganglia cell imaging, detailed in chapter 7.

In summary, in this chapter I have confirmed a role for the TRPV1 ion channel in mediating sensory nerve activation and cough induced by acidic solutions. Furthermore, the ASIC1 ion channels appear to partially mediate low pH induced sensory nerve activation *in vitro*, but because of the poor selectivity of ASIC channel inhibitors I have not been able to confirm this in the *in vivo* cough model. A role for the TRPA1 ion channel was also investigated, with contradictory evidence indicating an inhibitory effect on citric acid-induced cough *in vivo*, but no effect on low pH-induced sensory nerve activation *in vitro*. More detailed investigation is therefore required to determine whether TRPA1 does indeed play a role in mediating low pH tussive responses.

CHAPTER 7

Characterising a Model of Primary Ganglia Cell Imaging

7.1. Rationale

Cellular cation flux and changes in membrane voltage can be assessed on a population of cells by microscopy, utilising fluorescent dyes. This method could allow us to dissect out the signalling mechanisms of tussive agents on a more basic level, and can be used to look not only at the differences between primary vagal ganglia cells originating from the two different ganglia (nodose and jugular), but also to distinguish between airway and non-airway projecting fibres. This method therefore provides advantages over the isolated vagus preparation, from which we record compound membrane depolarisation from all types of fibres, originating from both vagal ganglia, and projecting not only to the airways but other parts of the body. This *in vitro* preparation could therefore help to investigate the difference in results with regards to the role of TRPA1 in low pH-induced sensory nerve activation and cough observed in chapter 6. For example, it is possible that TRPA1 plays a role in the acid-induced response in airway fibres, but not non-airway fibres; and that this effect is hidden in the natural variability when recording from the axon of the vagus nerve, because both airway and non-airway fibres are present. However, the results from this preparation must still be interpreted with caution, as we are recording from the cell bodies which reside in the vagal ganglia, rather than the nerve endings within the airways. Therefore, the results observed with this preparation may differ from what is actually occurring at the nerve terminals.

We recently developed a multi-dye epifluorescence fast acquisition microscopy technique in our lab. The aims of this chapter were therefore to establish and characterise a model of primary vagal ganglia cell imaging; to confirm the results observed in chapters 4 and 5 for PGE2 and BK-induced sensory nerve activation and cough; and to attempt to clarify the low pH-induced response *in vitro*. The second aim was to stain airway-specific primary ganglia cells using the retrograde tracer DiI, and determine if these cells respond to selected tussive stimuli.

7.2 Methods

7.2.1 Characterisation of the isolated primary vagal ganglia cell imaging model

For a description of the methods for collection and isolation of the primary vagal ganglia cells refer to chapter 2, section 2.5.

7.2.1.1 TRP-selective agonists

Jugular or nodose primary ganglia cells were constantly superfused with 37°C ECS buffer using a house-designed pressurised solution-changing perfusion system allowing complete bath (600 µl volume) replacement in 3s (see chapter 2, Figure 2.5). Potassium chloride solution (50 mM; K50) was applied at the start and end of each experiment for 10s to assess cell viability and allow for normalisation of subsequent agonist signals. Only neurons producing a fast response to K50, which was washable within 5min, and that had diameter of over 20 µm were analysed. Stock solutions of agonists (capsaicin and acrolein) and antagonists (HC-030031 and JNJ17203212) were made up in 100% DMSO and diluted 1/1000 in ECS to make a working solution.

Non-cumulative concentrations of capsaicin (0.1, 0.3, 1, 3, 10 µM), acrolein (0.3, 1, 3, 10, 30, 100 µM) or vehicle (0.1% DMSO) were applied for 20 s in a random order to jugular or nodose cells. After each application of drug, perfusion was switched back to ECS until complete recovery of the baseline calcium level. Intracellular free calcium ($[Ca^{2+}]_i$) imaging was recorded using the Fluo-4 fluorescent dye (see chapter 2, section 2.5 for details), with pictures taken every 1 s from 30s prior to drug application and for 2min afterward, then every 5 s for the rest of the recording. To take into account the multiphasic responses obtained in some cells, calcium flux (total elevation of calcium above resting level over time) was used to measure calcium elevations, which were normalised to calcium flux generated by application of K50 solution. Following characterisation of the CRs, submaximal concentrations of 1 µM capsaicin and 10 µM acrolein were selected for further experiments.

7.2.1.2 TRP-selective antagonists

The ability of TRPA1-selective (HC-030031) and TRPV1-selective (JNJ17203212) antagonists to inhibit acrolein and capsaicin-induced changes in $[Ca^{2+}]_i$ was investigated in jugular cells. CR curves were established for HC-030031 (0.001, 0.01, 0.1, 1 µM) or vehicle (0.1% DMSO) against acrolein; and JNJ17203212 (1, 10, 100 µM) or vehicle (0.1% DMSO) against capsaicin. $[Ca^{2+}]_i$ responses were recorded using Fura-2, with only one concentration of antagonist assessed per plate. Following an initial K50 response, a standard antagonist profile was used (described in chapter 2, section 2.5). Once appropriate concentrations of the antagonists had been determined, the effect of 0.1 µM HC-030031 on capsaicin and 10 µM JNJ17203212 on acrolein stimulated $[Ca^{2+}]_i$ was investigated to establish that there was no off-target effect at the concentration chosen.

7.2.2 Determining a role for TRP channels in primary vagal ganglia cell activation by endogenous mediators

Jugular or nodose cells were constantly superfused with 37°C ECS buffer using an in-house designed system as described above. K50 was applied at the start and at the end of each experiment for 10s to assess cell viability and allow for normalisation of subsequent agonist signals. Only primary ganglia cells producing a fast response to K50, which was washable within 5min, and that had diameter of over 20 µm were analysed. Stock solutions of PGE_2 were made up in 100% ethanol; BK in 100% dH_2O; and HC-030031 and JNJ17203212 in 100% DMSO. Compounds were then diluted 1/1000 in ECS to make a working solution. The pH of ECS buffer was adjusted using HCl to make working solutions of low pH.

A CR curve for changes in $[Ca^{2+}]_i$ was established by applying non-cumulative concentrations of PGE_2 (0.1, 0.3, 1, 3, 10 µM), BK (0.3, 1, 3, 10, 30 µM), low pH (pH 7, 6.5, 6, 5.5, 5), or appropriate vehicle (0.1% ethanol, 0.1% dH_2O, or ECS at pH 7.4, respectively) for 20-60s in a random order to jugular or nodose cells. After each application of drug, perfusion was switched back to ECS until complete recovery of the baseline calcium level. $[Ca^{2+}]_i$ imaging was recorded using Fluo-4, with pictures taken every 1s from 30s prior to drug application and for 2 min afterward, then every 5s for the rest of the recording. To take into account the multiphasic responses obtained in some cells, calcium flux (total elevation of

calcium above resting level over time) was used to measure calcium elevations, which were normalised to calcium flux generated by application of K50 solution.

From the above experiment, sub-maximal concentrations of 1 µM PGE_2, 10 µM BK, and pH 6 were selected for further investigation. Subsequently, the ability of TRPA1-selective (0.1 µM HC-030031), TRPV1-selective (10 µM JNJ17203212) or ASIC (10 µM amiloride) antagonists to inhibit agonist-induced elevation of $[Ca^{2+}]_i$ and changes in membrane voltage in primary jugular cells was investigated. Antagonists were incubated for 60s prior to agonist application. In this set of experiments $[Ca^{2+}]_i$ imaging was recorded using the ratiometric Fluo-2 fluorescent dye and membrane voltage using Di-8-ANEPPS (see chapter 2, section 2.5.2 for details).

7.2.3 Staining of airway primary vagal ganglia cells

Capsaicin (1 µM) and low pH (pH6) were assessed for their ability to activate airway-specific vagal ganglia cells. Guinea pigs were dosed i.n. with a fluorescent dye (DiI; see chapter 2, section 2.5.4 for details) to selectively stain airway cells. Fourteen days later, the animals were sacrificed by i.p. injection of sodium pentobarbitone (200 mg/kg), the vagal ganglia were dissected, and primary jugular cells isolated. The resulting cells were loaded with Fura-2, and changes in $[Ca^{2+}]_i$ assessed as described above. Care was taken to avoid exposure of the ganglia to light during the isolation process, to avoid photo bleaching of the DiI.

7.2.4 Data Analysis

Calcium and membrane voltage recordings were analysed using Image J software (Image Processing and Analysis in Java, National Institute of Health, USA). Magnitude of agonist responses in the presence of antagonist were compared to control responses within the same cell by paired t-test. Significance was set at $p < 0.05$, and all data were plotted as mean ± s.e.m. of *N* (number of animals) and *n* (number of cells) observations.

7.3 Results

Calcium imaging is regularly used as a method to investigate cellular activation. This is a newly established and previously uncharacterised model in our labs, but has the potential to allow more in-depth investigation of how the vagal afferents mediate responses to tussive irritants. Though this thesis deals only with the characterisation of this model, we hope in the future to be able to probe the intracellular signalling pathways downstream of GPCR activation, and to evaluate airway-specific and non-airway cellular responses. This could help to further elucidate the mechanisms which drive the cough response, and aid in determining how these mechanisms are altered in disease states.

7.3.1 Characterisation of the primary vagal ganglia cell imaging preparation

In the initial experiments establishing concentration-responses for the agonists, only $[Ca^{2+}]_i$ was measured using the Fluo-4 fluorescent dye. In subsequent antagonist studies this was changed for the ratiometric Fura-2 calcium dye. Fura-2 is more appropriate for studies conducted over a longer period of time, as photo bleaching of the dye over time does not affect the magnitude of agonist responses when expressed as a ratio of fluorescence. Furthermore, CRs for the agonists were established in both nodose and jugular primary cells; but the effect of antagonists was assessed only in jugular cells.

7.3.1.1 TRP-selective agonists

Concentration-responses for capsaicin and acrolein were established in both nodose and jugular cells. Capsaicin induced a large mono-phasic increase in $[Ca^{2+}]_i$ in primary jugular cells, with an EC_{50} of 1.4 µM and a maximum response (R_M, expressed as % of K50 response) of 63 ± 17% at 10 µM. In contrast, capsaicin did not reliably trigger a significant response in primary nodose cells at any concentration tested (R_M 0.7 ± 0.5% at 10 µM) (Figure 7.1).

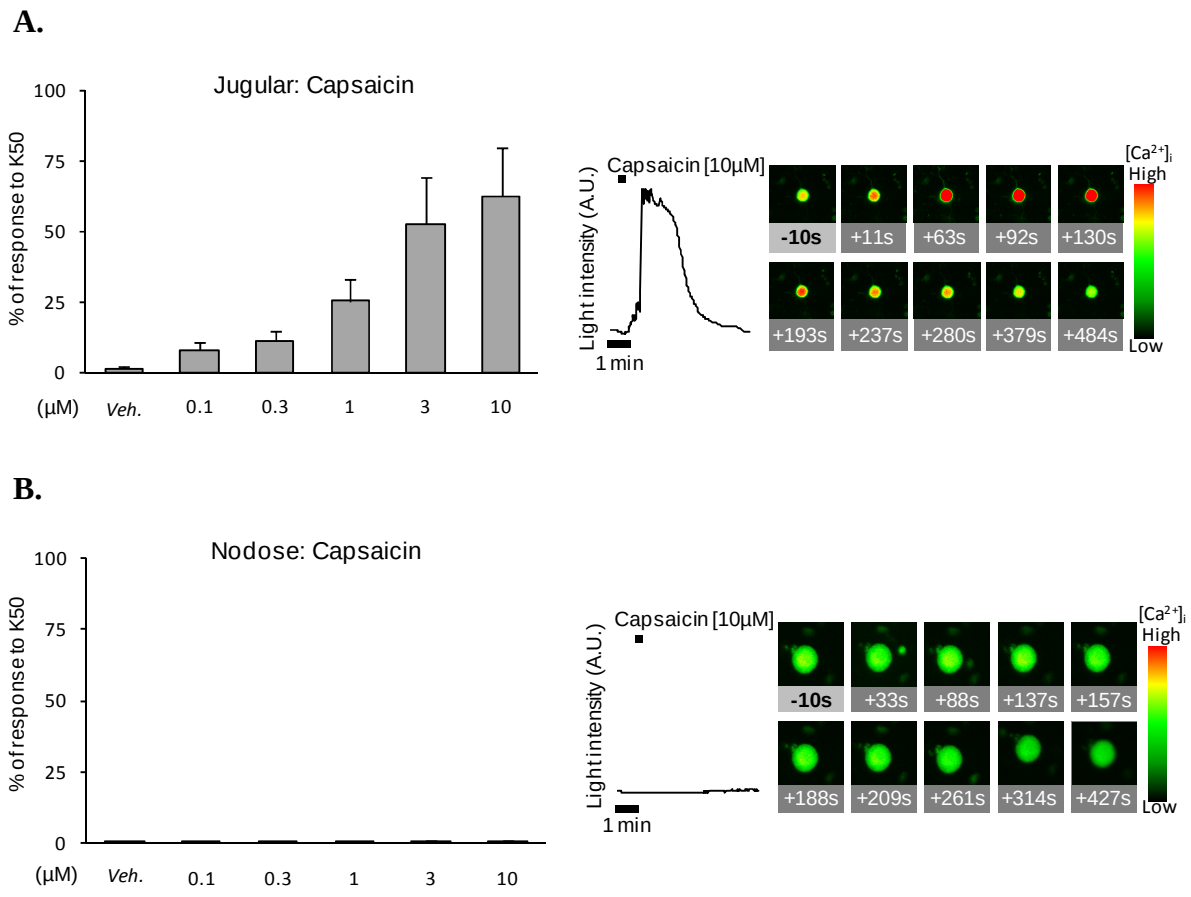


Figure 7.1. Concentration-related activation of primary vagal ganglia cells following exposure to capsaicin.

Capsaicin induced concentration-dependent increases in $[Ca^{2+}]_i$ in (a) jugular cells, but not (b) nodose cells. Histograms on the left show changes in $[Ca^{2+}]_i$ with application of concentrations of capsaicin, normalised and expressed as % response to K50 control. Data are expressed as mean ± s.e.m. of N=4-6, n=9 observations. The trace in the middle panel shows a representative recording of the light intensity over time following exposure to 10 μM capsaicin. Time and duration of capsaicin application are indicated by a black bar above the trace; a 1 min time scale is indicated by the black bar below the trace. The panel on the right displays selected pseudo-coloured fluorescence images taken during recording of the middle panel trace. The time of each snapshot is indicated below the picture, with zero being the start of capsaicin application. A colour code used to indicate light intensity is shown on the right of each set of images, with all light intensities normalised to peak amplitude of the $[Ca^{2+}]_i$ response to K50.

Different types of response were observed for acrolein in the jugular compared to nodose ganglia. Primary jugular cells responded in a multi-phasic pattern, showing periods of repetitive sharp $[Ca^{2+}]_i$ elevations, with an EC_{50} of 6.2 µM and 197 ± 31% R_M at 100 µM. Whereas, acrolein-induced $[Ca^{2+}]_i$ elevations in the nodose ganglia were mono-phasic, with an EC_{50} of 10 µM and 247 ± 94% R_M at 30 µM (Figure 7.2).

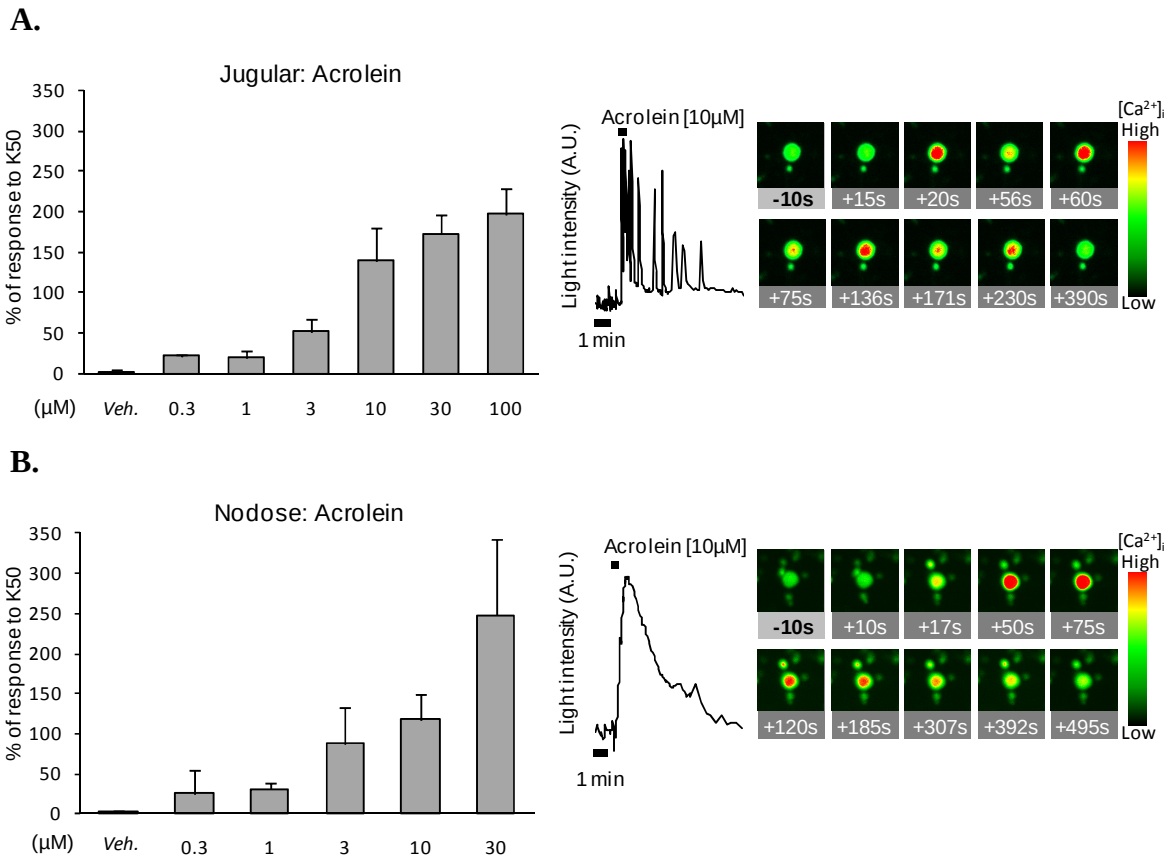


Figure 7.2. Concentration-related activation of primary vagal ganglia cells following exposure to acrolein.

Acrolein induced concentration-dependent increases in $[Ca^{2+}]_i$ in (a) *jugular and* (b) *nodose cells. Histograms on the left show changes in $[Ca^{2+}]_i$ with application of concentrations of acrolein, normalised and expressed as % response to K50 control. Data are expressed as mean ± s.e.m. of N=4, n=6-7 observations. The trace in the middle panel shows a representative recording of the light intensity over time following exposure to 10 µM acrolein. Time and duration of acrolein application are indicated by a black bar above the trace; a 1 min time scale is indicated by the black bar below the trace. The panel on the right displays selected pseudo-coloured fluorescence images taken during recording of the middle panel trace. The time of each snapshot is indicated below the picture, with zero being the start of acrolein application. A colour code used to indicate light intensity is shown on the right of each set of images, with all light intensities normalised to peak amplitude of the $[Ca^{2+}]_i$ response to K50.*

7.3.1.2 TRP-selective antagonists

CRs for the TRPV1-selective antagonist JNJ17203212, and TRPA1-selective antagonist HC-030031 were established in primary jugular cells for their ability to inhibit agonist induced $[Ca^{2+}]_i$ (Figure 3.12). JNJ17203212 concentration-dependently inhibited $[Ca^{2+}]_i$ caused by the TRPV1-selective agonist capsaicin, with a maximal effect of 86 ± 2% at 10 µM. Alternately, HC-030031 concentration-dependently inhibited $[Ca^{2+}]_i$ induced by the TRPA1-selective agonist acrolein, with a maximal effect of 76 ± 8% at 0.1 µM. At the concentration which caused maximal inhibition of its own receptor, 10 µM JNJ17203212 did not inhibit acrolein, and 0.1 µM HC-030031 did not inhibit capsaicin stimulation of jugular cells.

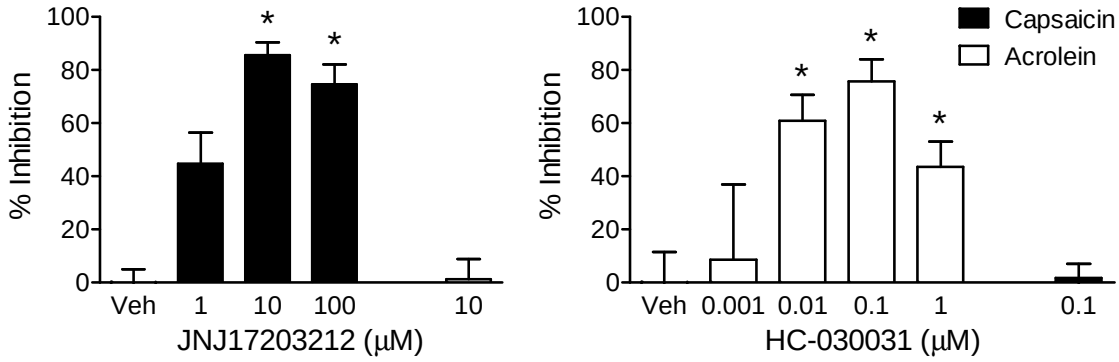


Figure 7.3. Characterising the ability of TRPV1- and TRPA1-selective antagonists to inhibit $[Ca^{2+}]_i$ in guinea pig isolated primary vagal jugular cells.

*Concentration responses were established for TRPV1 (JNJ17203212) and TRPA1 (HC-030031) selective antagonists or vehicle (0.1% DMSO, Veh) against TRPV1 (1 µM capsaicin) and TRPA1 (10 µM acrolein) selective agonists. The concentration which maximally inhibited its own receptor was then tested against the alternate agonist to establish selectivity at the chosen concentration. Data represent mean ± s.e.m of N=3-4, n = 4-19 observations. * indicates statistical significance (p < 0.05; paired t-test), comparing responses in the same cell before and after antagonist incubation.*

7.3.2 Determining a role for TRP channels in primary vagal ganglia cell activation by endogenous mediators

In this set of studies the Fura-2 calcium dye was used in combination with Di-8-ANEPPS to assess cell membrane voltage changes, in jugular primary cells only.

7.3.2.1 PGE_2

PGE2 increased $[Ca^{2+}]_i$ in primary jugular cells with an R_M of 37 ± 14% at 10 µM, and an EC_{50} of 0.6 µM; whereas, in primary nodose cells R_M was 12 ± 3% at 10 µM with an EC_{50} of 1.3 µM (Figure 7.4). Changes in calcium flux with PGE_2 stimulation were variable, generally showing multi-phasic responses in both jugular and nodose cells.

Mirroring observations in the isolated vagus nerve, either TRPA1 (0.1 µM HC-030031) or TRPV1 (10 µM JNJ17203212) antagonism partially inhibited PGE_2-induced increases in $[Ca^{2+}]_i$ (55 ± 4% and 40 ± 9%, respectively) and membrane voltage changes (47 ± 3% and 38 ± 7%, respectively; $p < 0.05$) in guinea pig primary cells isolated from the jugular ganglia (Figure 7.5). Furthermore, when used in combination, both TRPA1 and TRPV1 antagonism inhibited $[Ca^{2+}]_i$ elevation by 88 ± 3%; and depolarisation of the cell membrane by 78 ± 10% ($p < 0.05$). In contrast, vehicle incubation had no effect on either $[Ca^{2+}]_i$ (-1 ± 11%) or membrane voltage changes (8 ± 6%; $p > 0.05$).

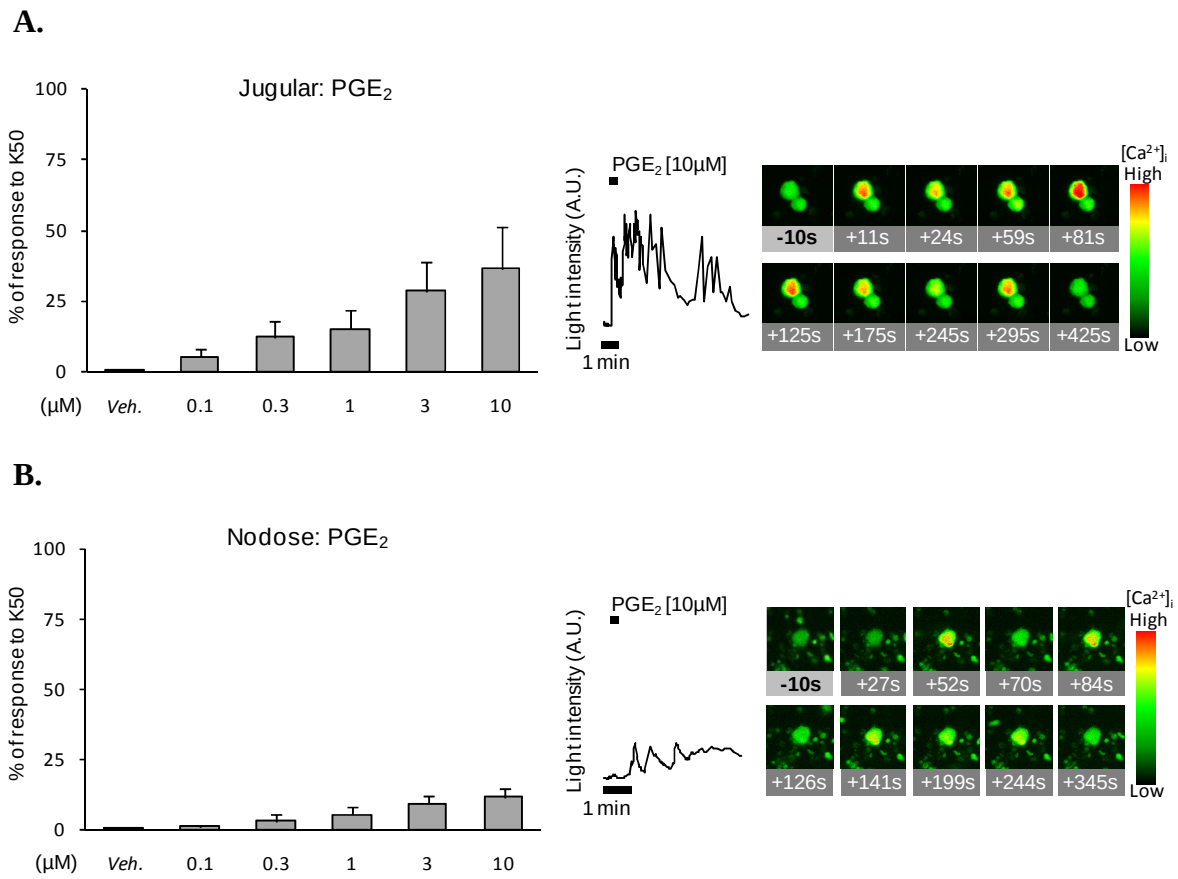


Figure 7.4. Concentration-related activation of primary vagal ganglia cells following exposure to PGE_2.

PGE_2 induced concentration-dependent increases in $[Ca^{2+}]_i$ in (a) *jugular and* (b) *nodose primary cells. Histograms on the left show changes in $[Ca^{2+}]_i$ with application of concentrations of PGE_2, normalised and expressed as % response to K50 control. Data are expressed as mean ± s.e.m. of N=4, n=6 observations. The trace in the middle panel shows a representative recording of the light intensity over time following exposure to 10 μM PGE_2. Time and duration of PGE_2 application are indicated by a black bar above the trace; a 1 min time scale is indicated by the black bar below the trace. The panel on the right displays selected pseudo-coloured fluorescence images taken during recording of the middle panel trace. The time of each snapshot is indicated below the picture, with zero being the start of PGE_2 application. A colour code used to indicate light intensity is shown on the right of each set of images, with all light intensities normalised to peak amplitude of the $[Ca^{2+}]_i$ response to K50.*

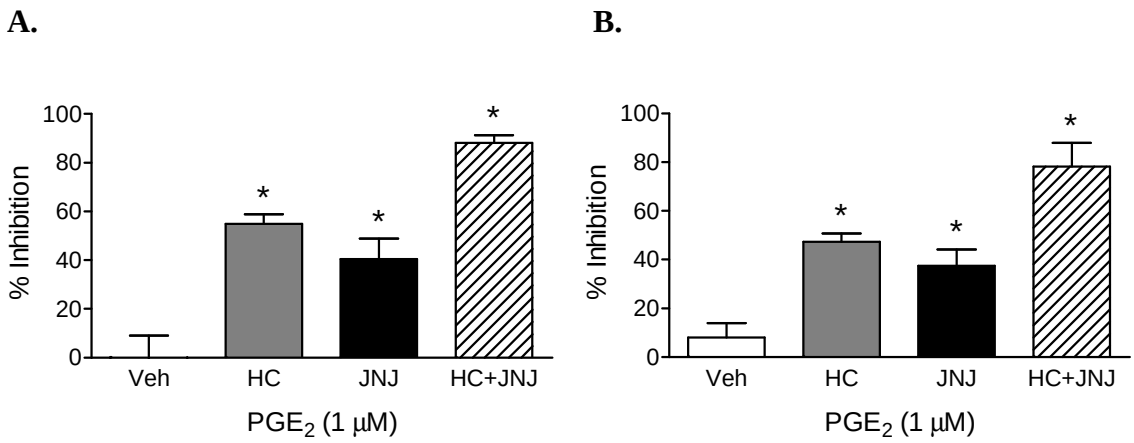


Figure 7.5. Pharmacologically determining a role for TRPA1 and TRPV1 ion channels in PGE_2 activation of isolated primary jugular cells.

*Isolated vagal jugular cells were incubated with a selective TRPA1 antagonist (0.1 µM HC-030031, HC); a selective TRPV1 antagonist (10 µM JNJ17203212, JNJ); a combination of both TRPA1 and TRPV1 antagonists (HC+JNJ); or vehicle (0.1% DMSO, Veh) for 60 seconds prior to PGE_2 (1 µM) stimulation. Antagonism of either TRPA1 or TRPV1 partially inhibited PGE_2-induced (a) $[Ca^{2+}]_i$ and (b) membrane voltage responses in guinea pig primary jugular cells, whereas a combination of both TRPA1 and TRPV1 antagonist virtually abolished PGE_2 stimulation. Vehicle incubation did not have a significant effect on either $[Ca^{2+}]_i$ or membrane voltage. * indicates statistical significance ($p < 0.05$; paired t-test), comparing responses in the same cell before and after antagonist incubation. Data are presented as mean ± s.e.m. of N = 3-4, n = 10-16 observations.*

7.3.2.2 Bradykinin

BK increased $[Ca^{2+}]_i$ in primary jugular cells with an R_M of 29 ± 13% at 10 µM, and an EC_{50} of 1.3 µM; whereas, in primary nodose cells R_M was only 8 ± 3% at 10 µM with an EC_{50} of 7.4 µM (Figure 7.6). Calcium responses to BK stimulation were bi-phasic, showing an initial sharp elevation with short duration, followed by a more prolonged elevation in $[Ca^{2+}]_i$.

Inhibition of either the TRPA1 (0.1 µM HC-030031) or TRPV1 (10 µM JNJ17203212) ion channels partially reduced BK-induced increases in $[Ca^{2+}]_i$ (45 ± 5% and 46 ± 7%, respectively) and membrane voltage changes (39 ± 4% and 42 ± 6, respectively; $p < 0.05$) in guinea pig primary vagal ganglia cells isolated from the jugular ganglia (Figures 7.7). Furthermore, when used in combination, both TRPA1 and TRPV1 antagonism inhibited

$[Ca^{2+}]_i$ elevation by 80 ± 12%; and depolarisation of the cell membrane by 77 ± 9% ($p < 0.05$). In contrast, vehicle incubation had no effect on either BK-induced $[Ca^{2+}]_i$ (-4 ± 8%) or membrane voltage changes (-2 ± 8%; $p > 0.05$). This data supports the results observed in the isolated vagus preparation.

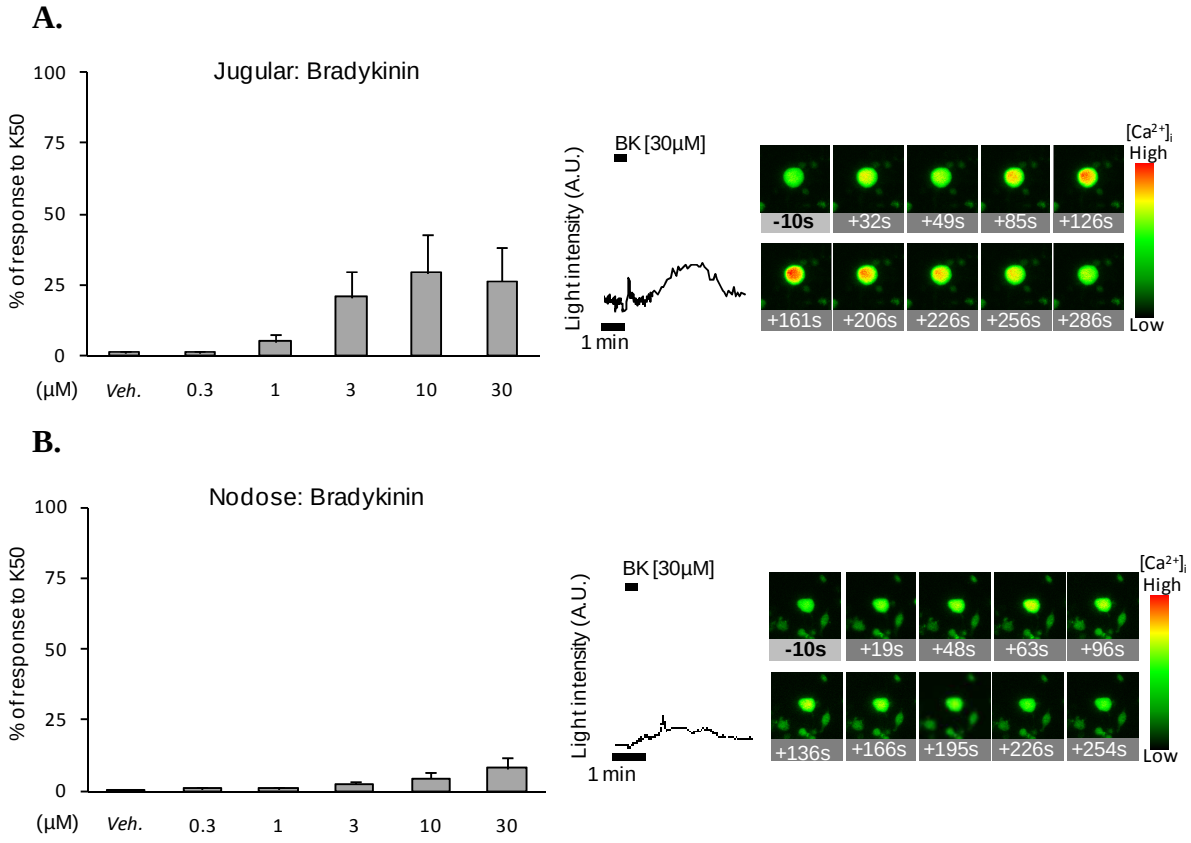


Figure 7.6. Concentration-related activation of primary vagal ganglia cells following exposure to BK.

BK induced concentration-dependent increases in $[Ca^{2+}]_i$ in (a) *jugular and* (b) *nodose cells. Histograms on the left show changes in $[Ca^{2+}]_i$ with application of concentrations of BK, normalised and expressed as % response to K50 control. Data are expressed as mean ± s.e.m. of N=3, n=4 observations. The trace in the middle panel shows a representative recording of the light intensity over time following exposure to 30 µM BK. Time and duration of BK application are indicated by a black bar above the trace; a 1 min time scale is indicated by the black bar below the trace. The panel on the right displays selected pseudo-coloured fluorescence images taken during recording of the middle panel trace. The time of each snapshot is indicated below the picture, with zero being the start of BK application. A colour code used to indicate light intensity is shown on the right of each set of images, with all light intensities normalised to peak amplitude of the $[Ca^{2+}]_i$ response to K50.*

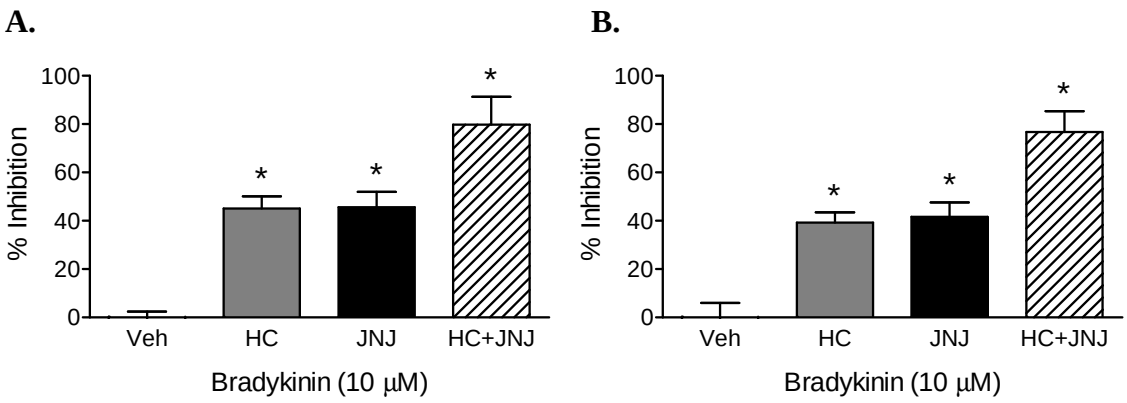


Figure 7.7. Pharmacologically determining a role for TRPA1 and TRPV1 ion channels in BK-stimulated activation of isolated primary jugular cells.

Isolated vagal jugular cells were incubated with a selective TRPA1 antagonist (0.1 µM HC-030031, HC); a selective TRPV1 antagonist (10 µM JNJ17203212, JNJ); a combination of both TRPA1 and TRPV1 antagonists (HC+JNJ); or vehicle (0.1% DMSO, Veh) for 60 seconds prior to BK (10 µM) stimulation. Antagonism of either TRPA1 or TRPV1 partially inhibited BK-induced (a) *$[Ca^{2+}]_i$ and* (b) *membrane voltage responses in guinea pig primary jugular cells, whereas a combination of both TRPA1 and TRPV1 antagonist virtually abolished BK stimulation. Vehicle incubation did not have a significant effect on either $[Ca^{2+}]_i$ or membrane voltage. * indicates statistical significance ($p < 0.05$; paired t-test), comparing responses in the same cell before and after antagonist incubation. Data are presented as mean ± s.e.m. of N = 3, n = 13-19 observations.*

7.3.2.3 Low pH

Low pH increased $[Ca^{2+}]_i$ in primary jugular cells with an R_M of 82 ± 20% at pH 5, and an EC_{50} of pH 6.1; whereas, in primary nodose cells R_M was 17 ± 3% at pH 5, with an EC_{50} of pH 5.7 (Figure 7.8). Calcium responses to low pH were transient and mono-phasic, showing a sharp increase in $[Ca^{2+}]_i$, which quickly came back to baseline. An acidity of pH 6, exhibiting submaximal but robust responses in jugular cells, was chosen for further experiments.

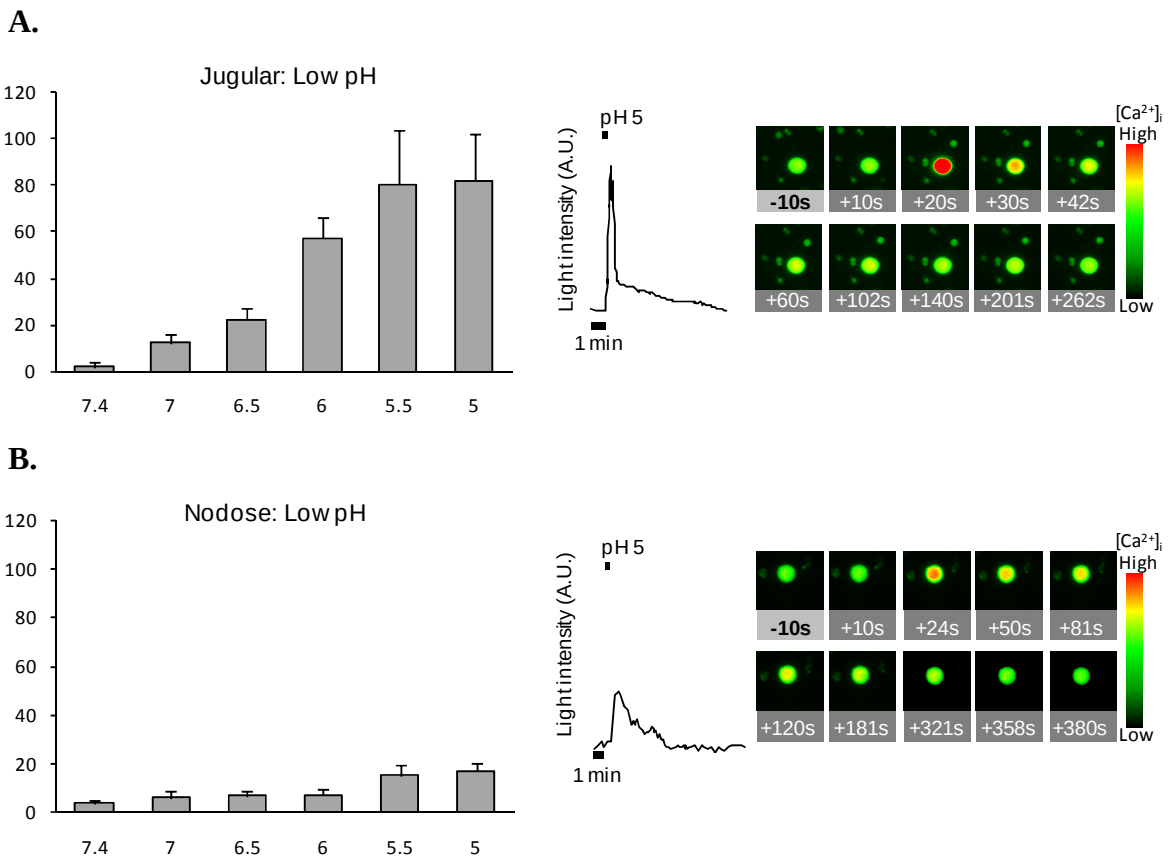


Figure 7.8. Concentration-related activation of primary vagal ganglia cells following exposure to low pH.

Low pH induced concentration-dependent increases in $[Ca^{2+}]_i$ *in* (a) *jugular and* (b) *nodose primary cells. Histograms on the left show changes in* $[Ca^{2+}]_i$ *with application of concentrations of low pH, normalised and expressed as % response to K50 control. Data are expressed as mean ± s.e.m. of N=3-4, n=12-13 observations. The trace in the middle panel shows a representative recording of the light intensity over time following exposure to pH 6. Time and duration of low pH application are indicated by a black bar above the trace; a 1 min time scale is indicated by the black bar below the trace. The panel on the right displays selected pseudo-coloured fluorescence images taken during recording of the middle panel trace. The time of each snapshot is indicated below the picture, with zero being the start of low pH application. A colour code used to indicate light intensity is shown on the right of each set of images, with all light intensities normalised to peak amplitude of the* $[Ca^{2+}]_i$ *response to K50.*

Incubation with vehicle (0.1% DMSO) did not have any effect on $[Ca^{2+}]_i$ or membrane voltage with subsequent low pH stimulation. In agreement with results observed in the vagus nerve, antagonism of the TRV1 channel (10 µM JNJ17203212) inhibited low pH-induced increases in $[Ca^{2+}]_i$ and changes in membrane voltage by approximately half (52 ± 14% and 46 ± 16%, respectively; $p < 0.05$) (Figure 7.9). Furthermore, inhibition of ASIC channels (10 µM amiloride) blocked low pH $[Ca^{2+}]_i$ and membrane voltage responses by 36 ± 15% and 29 ± 10%, respectively; and inhibition of both ASIC and TRPV1 channels blocked low pH responses by 94 ± 2% and 74 ± 7%, respectively ($p < 0.05$). Amiloride (10 µM) was also tested against capsaicin and acrolein responses, and was shown to have no effect on low pH-induced changes in either $[Ca^{2+}]_i$ (2 ± 7% and -4 ± 9%, respectively; $p > 0,05$) or membrane voltage (9 ± 5% and 0.2 ± 7%, respectively; $p > 0.05$), confirming that this inhibitor was not having an off-target effect at the concentration chosen for investigation. TRPA1 inhibition (0.1 µM HC-030031) also had no overall effect on $[Ca^{2+}]_i$ or membrane voltage changes (13 ± 11% and -1 ± 11%, respectively; $p > 0.05$).

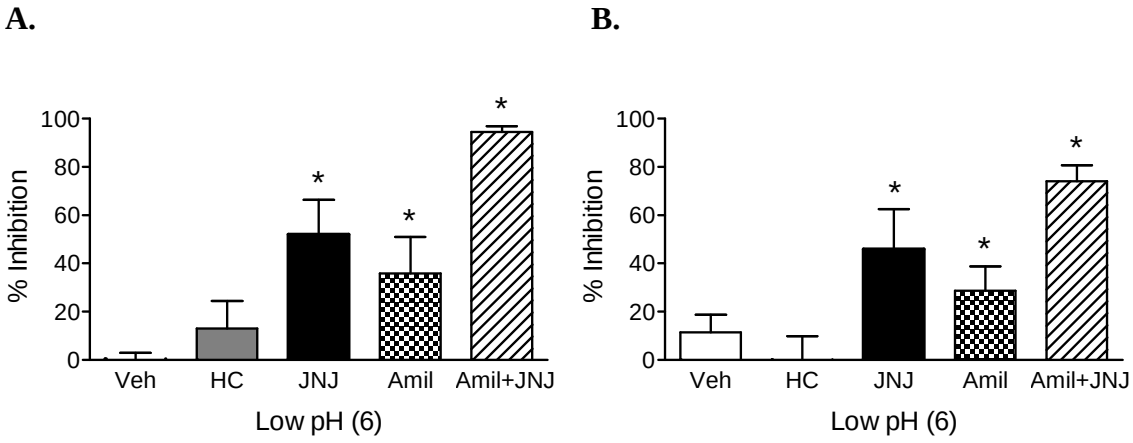


Figure 7.9. Pharmacologically determining a role for TRPA1, TRPV1 and ASIC ion channels in low pH-stimulated activation of isolated primary jugular cells.

Isolated vagal jugular cells were incubated with a selective TRPA1 antagonist (0.1 µM HC-030031, HC); a selective TRPV1 antagonist (10 µM JNJ17203212, JNJ); a non-selective ASIC antagonist (10 µM Amiloride, Amil); or vehicle (0.1% DMSO, Veh) for 60 seconds prior to low pH (pH 6) stimulation. Antagonism of TRPV1 or ASIC ion channels partially inhibited low pH-induced (a) *$[Ca^{2+}]_i$ and* (b) *membrane voltage responses in guinea pig primary jugular cells, whereas TRPA1 and vehicle had no significant effect. * indicates statistical significance ($p < 0.05$; paired t-test), comparing responses in the same cell before and after antagonist incubation. Data are presented as mean ± s.e.m. of N = 3-4, n = 9-15 observations.*

During analysis of these results, some interesting observations were made. Specifically, inhibition of the TRPV1 ion channel appeared to indicate two mutually exclusive sets of primary jugular cells that respond to low pH: those sensitive to TRPV1 inhibition, and those non-sensitive. These results were mirrored with the general ASIC inhibitor amiloride, again indicating two mutually exclusive sets of jugular cells: those sensitive to ASIC inhibition and those non-sensitive. This data is presented in Figure 7.10A, whereby incubation with JNJ17203212 or amiloride either completely abolished low pH-induced $[Ca^{2+}]_i$, or had no effect. Overall, this caused approximately 50% inhibition of the response (see bar graphs in Figure 7.9), which is what would have been evident in the compound depolarisation response on the isolated vagus nerve. Conversely, the effect of TRPA1 inhibition was much more variable, with a spread between approximately 0-60% inhibition of the low pH $[Ca^{2+}]_i$ response (Figure 7.10A). These results are in contrast to those observed for PGE_2 and BK, whereby (except for some outliers) inhibition of TRPA1 or TRPV1 caused inhibition of $[Ca^{2+}]_i$ responses which were clustered around 50% (Figure 7.10B & 7.10C).

7.3.3 Activation of airway-stained primary jugular ganglia cells

Jugular cells stained with the retrograde tracer DiI were successfully isolated. It was subsequently confirmed that stimulation with both capsaicin and low pH (Figure 7.11) cause a transient increase in $[Ca^{2+}]_i$ in DiI-stained cells.

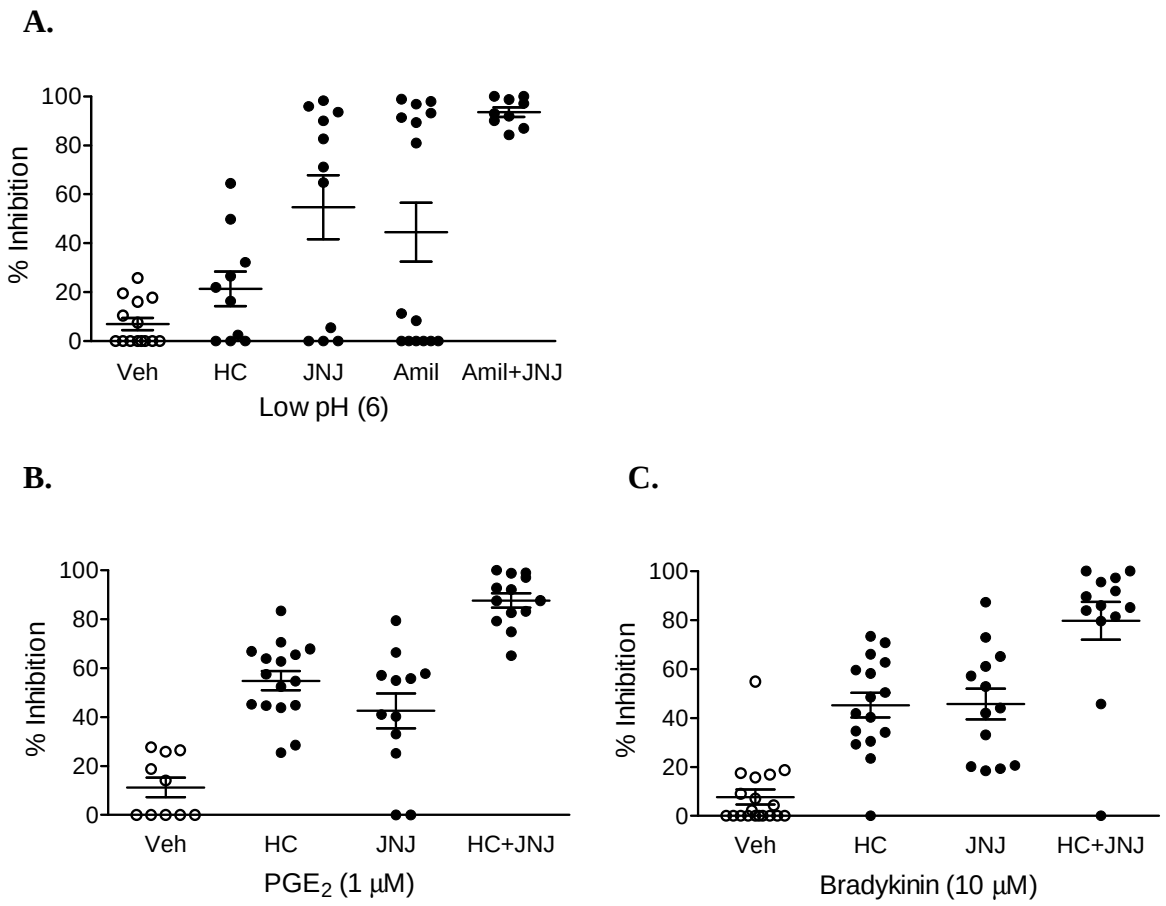
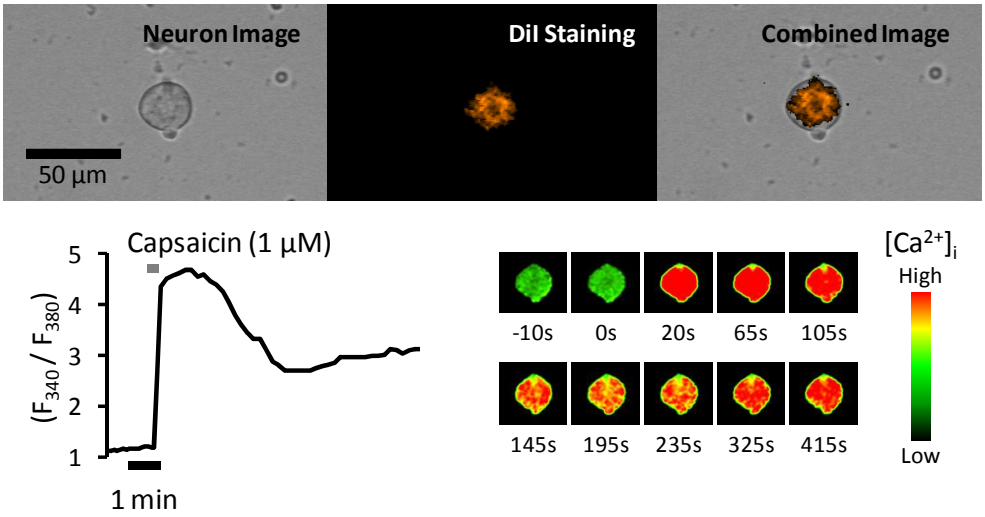


Figure 7.10. Effects of TRPA1, TRPV1 and ASIC inhibitors on $[Ca^{2+}]_i$ increases stimulated by endogenous irritants in primary jugular cells.

Isolated vagal jugular cells were incubated with a selective TRPA1 antagonist (0.1 µM HC-030031, HC); a selective TRPV1 antagonist (10 µM JNJ17203212, JNJ); a non-selective ASIC antagonist (10 µM Amiloride, Amil); a combination of TRPA1 and TRPV1 antagonists (HC+JNJ);a combination of ASIC and TRPV1 antagonists (Amil+JNJ); or vehicle (0.1% DMSO, Veh) for 60 seconds prior to (a) *low pH (pH 6),* (b) *PGE_2 (1 µM) or* (c) *BK (10 µM) agonist stimulation. This expands on earlier observations by looking at the spread of the $[Ca^{2+}]_i$ data. For clarity, in the absence of inhibition (where the agonist response during antagonist incubation was equal to or greater than control), responses have been normalised to '0% inhibition'.* (a) *Further examination of the low pH data reveals two distinct populations of TRPV1-sensitive and TRPV1-non sensitive, as well as ASIC-sensitive and ASIC-non sensitive primary cells; exhibited by either complete block or no effect of the low pH response with the TRPV1 or ASIC inhibitors. Conversely, inhibition of the TRPA1 ion channel produced a wide range of effects, from 0-60% inhibition of the low pH response. This is in contrast to* (b) *PGE_2 and* (c) *BK, where inhibiton of TRPA1 or TRPV1 ion channels consistently inhibited the agonist responses by around 50% each. Data are presented as mean ± s.e.m of N = 3-4, n = 9-19 observations.*

A.



B.

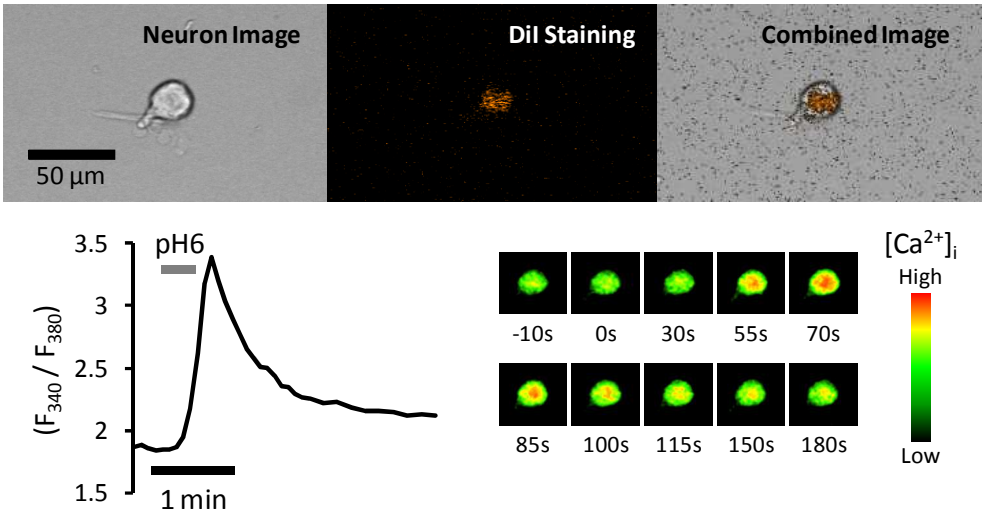


Figure 7.11. Capsaicin and low pH stimulate airway-labelled primary jugular ganglia cells.

Stimulation with (a) *capsaicin (1 µM) or* (b) *low pH (pH 6) caused a transient increase in* $[Ca^{2+}]_i$ *in jugular cells labelled with the retrograde tracer DiI. The top left panel shows a bright field image of the selected cell; the middle panel shows an image capture of the cell fluorescence when excited at λ=520-550 nm; and the panel on the top right is a combined image confirming that the fluorescence is restricted to the selected cell. The bottom left trace shows a representative recording of Fura-2 light intensity ratio over time following exposure to capsaicin. Time and duration of agonist application is indicated by a grey bar above the trace; and a 1 min time scale is indicated by the black bar below the trace. The panel on the bottom right displays selected pseudo-coloured fluorescence images taken during recording of the trace, with time of each snapshot indicated below the picture (zero being the start of agonist application). A colour code used to indicate light intensity is displayed on the right of the set of images, with all light intensities normalised to peak amplitude of* $[Ca^{2+}]_i$ *response to K50.*

7.4 Discussion

We have recently established a primary vagal ganglia cell imaging preparation in our labs. This model will allow us to investigate the signalling mechanisms involved in the tussive response in more depth than what is possible with the isolated vagus preparation. In particular we are interested in probing the intracellular signalling pathways that are involved in activating TRPA1 and TRPV1 ion channels downstream of GPCR activation following PGE_2 and BK stimulation; and this preparation could help to clarify how low pH is being mediated by the TRPV1, ASIC and possibly TRPA1 ion channels. However, before we could start these studies, I needed to characterise and validate the model. The focus of this chapter was, therefore, to replicate the experiments performed in previous chapters; to corroborate my current findings that both TRPA1 and TRPV1 mediate PGE_2 and BK-induced responses; and to further investigate the involvement of TRPA1, TRPV1 and ASIC ion channels in low pH-induced primary jugular ganglia cell activation.

The selective agonist acrolein produced concentration-related increases in $[Ca^{2+}]_i$ in both nodose and jugular primary cells; whereas capsaicin reliably produced concentration-related increases in $[Ca^{2+}]_i$ in most jugular but few nodose cells. Expression of TRPV1 (Kwong et al., 2008), and a response to capsaicin stimulation (Undem et al., 2004) has been previously observed in guinea pig C-fibres originating from the nodose ganglia and projecting to the airways, but only a small percentage of the whole population of nodose neurons consist of C-fibres. Because my data is representative of all cells producing a response to K50, the CR of the few nodose cells that did respond to capsaicin is lost in the overall data analysis. It was decided to move ahead with antagonist studies in primary jugular cells only, as both acrolein and capsaicin produced robust responses in these cells. A concentration-response for the selective antagonists JNJ17203212 and HC-030031 were then established against their respective agonist; and the concentration which exhibited the highest inhibition of its own receptor was tested against the alternate agonist to demonstrate that there were no off-target effects.

Once I had established appropriate concentrations of antagonist to use, I proceeded to characterise the effects of PGE_2, BK and low pH on primary vagal cells. All three of these compounds caused concentration-related increases in $[Ca^{2+}]_i$ in both nodose and jugular cells. Furthermore, TRPA1 and TRPV1-selective antagonists partially inhibited PGE_2 and BK-

induced $[Ca^{2+}]_i$ and membrane voltage changes in primary jugular cells; and a combination of both antagonists showed greater inhibition than either antagonist alone. These results agree with those presented in the previous chapters using the isolated vagus preparation and an *in vivo* guinea pig model of cough. This indicates that the results from the imaging preparation closely correspond to the effects in previously validated models of cough, and can therefore be used to investigate the cough reflex.

Investigating the low pH response using this model produced some interesting results. That is, although overall effects of TRPV1 or ASIC channel inhibition showed partial inhibition of the $[Ca^{2+}]_i$ and membrane voltage response, there appeared to be two mutually exclusive sets of cells: those sensitive to TRPV1 or ASIC channel inhibition, and those non-sensitive. This is in contrast to PGE_2 and BK, in which either TRPA1 or TRPV1 antagonists inhibited $[Ca^{2+}]_i$ and membrane voltage by approximately half each; and indicates that TRPV1 and ASIC ion channels may not be co-expressed on the same nerve fibres. This warrants further investigation, and will be discussed in the Future Studies section in chapter 8. Conversely, the ability of a TRPA1 antagonist to inhibit low pH responses varied greatly. One explanation for this could be if (like TRPV1 and ASIC ion channels) there are TRPA1 antagonist-sensitive and non-sensitive cells. In this instance, you would expect some natural variability in the amount of inhibition observed in TRPA1 antagonist-sensitive cells, as well as a set of cells in which there would be no inhibitory effect at all (the TRPA1 antagonist-non-sensitive cells). This may also help to explain the lack of an effect of TRPA1 inhibition seen in the isolated vagus nerve preparation, where we are measuring compound depolarisation from all types of nerve fibre, which could hide the small effect of TRPA1 inhibition within the natural variability in response. The above hypothesis also fits with the *in vivo* cough data, as we have already observed that 50% inhibition of afferent nerve responses with TRPA1 or TRPV1 antagonists leads to greater than 50% inhibition of PGE_2 and BK-induced cough. Therefore, a small effect of TRPA1 inhibition at the level of the nerve fibres could lead to a significant inhibition of the cough reflex induced by citric acid *in vivo*.

The final set of experiments looked at the ability of capsaicin or low pH to activate airway-specific primary jugular cells, which were stained with the retrograde tracer DiI. I was successful in identifying stained and non-stained cells, indicating airway-projecting and non airway-projecting nerve fibres, respectively. Once a stained cell was identified, the cell was subsequently stimulated with either capsaicin or low pH using the normal protocol, and

changes in $[Ca^{2+}]_i$ were measured. Indeed, both capsaicin and low pH caused calcium influx in stained primary cells. It is possible that TRPA1 mediates low pH effects in the airway nerve fibres, but not non-airway fibres. Therefore, recording from airway-only cells may help to clarify whether TRPA1 plays a role in low pH-induced sensory nerve activation leading to cough. These experiments will be discussed in the Future Studies section of chapter 8.

In summary, in this chapter I have characterised a model of primary vagal ganglia cell imaging. The results for primary vagal cell activation mirrored those of previous chapters, indicating an equal role for the TRPA1 and TRPV1 ion channels in mediating the effects of PGE_2 and BK; and a role for TRPV1 and ASIC ion channels in mediating the effects of low pH. The analysis also presented novel and interesting data, indicating that TRPV1 and ASIC ion channels may be expressed on different sets of afferent nerve fibres. Furthermore, the spread of the data with the TRPA1 antagonist suggests that this ion channel may also play a small role in some afferents. Finally, I established the ability of both capsaicin and low pH to activate airway-specific cells, which could further clarify a role for TRPA1 in the low pH response with further investigation, as it is possible that this ion channel is involved in mediating the response to acidic solutions in airway but not non-airway projecting nerve fibres. Therefore, the primary vagal ganglia cell imaging preparation will provide us with the opportunity for more in-depth analysis of the mechanisms driving the cough reflex.

CHAPTER 8

Discussion and Future Studies

8.1. Summary and discussion

Cough is a common symptomatic complaint. Though the majority of people suffer from acute cough associated with upper respiratory tract infections, which functions to rid the airways of inhaled irritants and mucus; approximately 7% of the population suffer from chronic, non-productive cough that is associated with inflammatory diseases (e.g. asthma, COPD, cancer), but can also be idiopathic (Ford et al., 2006; Irwin et al., 1998; Morice et al., 2007). Of increasing concern is that the global incidence of chronic respiratory diseases is on the rise (Barnes, 2010a, 2010b; World Health Organisation, 2007, 2011a, 2011b), and as such the prevalence of chronic cough is also likely to increase. Furthermore, up to 40% of parents with children aged 0-17 months seek medical help associated with coughing, a trend which has been recently highlighted as a concerning issue (Hay et al., 2005; Sands et al., 2011). This is particularly worrying because of the recent evidence that cough and cold remedies can cause adverse events in children under 11 years of age (American Academy of Pediatrics, 1997; Centre for Disease Control, 2007; Gunn et al., 2001; Vassilev et al., 2009), which prompted the US Food and Drug Administration to recommend against the use of over-the-counter therapies to treat children under 2 years of age due to potentially life-threatening side-effects (US Food and Drug Administration, 2008).

The majority of existing anti-tussive treatments are easily available as self medication programmes, purchased over-the-counter. However, many of these treatments show little efficacy and are associated with adverse side effects that can impede daily activity (Karlsson & Fuller, 1999; Vassilev et al., 2009). Indeed, the ‘gold-standard’ in cough therapy are opioid-based drugs, which cause sedation and nausea. The development of more efficacious and targeted compounds that selectively inhibit the cough reflex and display a better safety profile are therefore urgently required. Furthermore, the ideal therapy would not inhibit the functional cough reflex associated with healthy outcomes such as removing mucus and other irritants from the airways; but would target only the enhanced, non-functional cough. Currently, the mechanisms driving the cough reflex are poorly understood, and need to be ascertained before better, targeted drug therapies can be developed.

A number of the TRP family of ion channels are known to be expressed in the peripheral nervous system, and can be activated by a variety of irritants (Caspani & Heppenstall, 2009). Furthermore, the TRP channels have been linked to various roles in sensory perception, and

are associated with the pathogenesis of a range of diseases (Caterina et al., 1997; Nilius, 2007). As such, a great deal of research has recently focused on the TRPs as pharmacological targets. In particular, the TRPV1 ion channel has a well-established role in mediating cough induced by the tussive irritants capsaicin and low pH in both animals and humans; and it has been found that patients suffering from chronic cough exhibit an increase in TRPV1 expression in the lungs (Groneberg et al., 2004). This has established the TRPV1 ion channel as a promising target for anti-tussive therapy. Unfortunately, TRPV1 is widely expressed throughout the body, and is involved in temperature homeostasis. As such, current TRPV1 antagonists are associated with development of hyperthermia, which is a confounding factor that may hinder the development of these compounds as cough therapies (Gavva et al., 2008; Lehto et al., 2008). Furthermore, TRPV1 is not activated by many irritants known to initiate cough. This evidence implicates the involvement of one or more other receptors in mediating the cough reflex.

TRPA1 binds a diverse number of irritant compounds that are associated with respiratory irritation, including those present in the environment, foodstuffs, and mediators released endogenously in the body. This highlighted the TRPA1 ion channel as another potential mediator of the cough reflex. The initial aim of this thesis was therefore to determine whether activation of the TRPA1 ion channel does indeed cause cough. To approach this question, I characterised models of sensory nerve activation and cough using both TRPA1 and TRPV1 selective agonists and antagonists. *In vitro* assays are often utilised for pharmacological proof-of-concept studies as they are high through-put, require fewer animals, and are more cost-effective than *in vivo* models. I was able to establish that TRPA1 and TRPV1 agonists stimulate sensory afferent nerves in guinea pig, mouse and human vagal tissue, thereby demonstrating that this effect was conserved across species. Furthermore, this activation could be blocked by TRPA1- and TRPV1-selective antagonists. However, results obtained *in vitro* do not always translate *in vivo*, as these models pose more complex pharmacokinetic and pharmacodynamic issues. I therefore went on to confirm that TRPA1 and TRPV1 agonists induce coughing in conscious guinea pigs, and again that this can be blocked with selective inhibitors. Indeed, since beginning this research, it has been confirmed that TRPA1 agonists cause cough in humans (Birrell et al., 2009). This has implications for the development of chronic cough in disease, as isocyanates (which have been implicated in the late asthmatic response) are now also known to bind to TRPA1 (Bautista et al., 2006; Finotto et al., 1991; Vandenplas et al., 1993). A role for this family of compounds in cough is

supported by recent data showing that allyl-isothiocyanate causes cough in guinea pigs (Andrè et al., 2009).

Inflammatory airways disease is associated with an enhanced release of PGE_2 and BK, and these two irritants are thought to be involved in the hypersensitisation to tussive stimuli leading to chronic cough (Choudry et al., 1989; Fox et al., 1996). Furthermore, both PGE_2 and BK can cause cough in humans and animals when inhaled. These two mediators bind to their associated GPCRs on cell membranes, leading to the release of intracellular secondary messengers. For PGE_2 this has been shown to be the EP_3 receptor (Maher et al., 2009); and I established in this thesis that for BK in guinea pigs and humans it is the B_2 receptor, but that both B_1 and B_2 play a role in the mouse. In order to induce cough, activation of the GPCR subsequently needs to cause opening of membrane-bound ion channels in order to lead to a net change in cell membrane potential and generation of action potentials (see Figures 2.2 & 2.3, chapter 2). Having established a role for the TRPA1 and TRPV1 ion channels in the cough reflex, I then wanted to investigate whether they were involved in the tussive response to endogenous mediators associated with inflammatory disease. Using the *in vitro* isolated vagus nerve preparation, and the *in vivo* guinea pig cough model that I had characterised previously, I was able to establish that TRPA1 and TRPV1 partially mediate the response to both PGE_2 and BK downstream of GPCR activation. These results indicate that both TRPA1 and TRPV1 could be important in the pathogenesis of chronic cough associated with airways inflammation.

Finally, I wanted to investigate the tussive response to low pH. It has been observed that patients who suffer from chronic cough exhibit a decrease in lung pH compared to healthy volunteers (Hunt et al., 2000; Kostikas et al., 2002). Similar to PGE_2 and BK, low pH is also known to both sensitise the cough reflex to stimulation by other agonists, and to induce coughing itself when inhaled as an aerosol. TRPV1 has a well-established role in partially mediating the tussive effects of low pH (Kollarik & Undem, 2002; Lalloo et al., 1995). Though a role for the ASIC ion channels in the cough response to low pH has been suggested (Kollarik & Undem, 2002), this has not been explicitly investigated. Furthermore, TRPA1 has been shown to play a role in sensing alkaline solutions and intracellular acidic pH (Dhaka et al., 2009; Fujita et al., 2008; Wang et al., 2011), and thus I hypothesised that this ion channel may also play a role in sensing acidity within the lungs. Again using the isolated vagus nerve preparation and guinea pig cough model, I clearly demonstrated a role for

TRPV1 in partially mediating the cough response to low pH. This confirms previous observations. Furthermore, using a general ASIC channel inhibitor, and mice devoid of functional ASIC1 and ASIC3 ion channels, I also demonstrated a role for ASIC1 in low pH activation of vagal sensory afferents. Because of the poor selectivity of the available ASIC inhibitors, I did not use an ASIC inhibitor in the *in vivo* cough model. Conversely, a role for TRPA1 in mediating the tussive effects of low pH was less clear. In the *in vitro* vagus nerve model there did not appear to be any effect of inhibiting the TRPA1 ion channel on low pH responses in any of the species tested (guinea pig, mouse and human). In contrast, the TRPA1-selective antagonist HC-030031 did inhibit citric acid-induced coughing in the conscious guinea pig cough model, to a similar extent of that seen with the TRPV1 antagonist JNJ17203212. Although, when a combination of both TRPA1 and TRPV1 antagonists were used there was no further inhibition of the cough response, indicating that ASIC channels may still play a role in low pH-induced cough *in vivo*. Figure 8.1 diagramatically summarises the findings of this thesis, showing the proposed pathways for stimulation of cough with PGE_2, BK and low pH.

A model of primary vagal ganglia cell imaging could provide us with the opportunity to investigate how agonists and antagonists mediate the cough reflex in more detail. This preparation allows the ability to record from single primary ganglia cells, isolated from the nodose or jugular ganglia, which can also be identified as coming from the airways via retrograde labelling. This method therefore provides advantages over the isolated vagus preparation, from which we are recording the compound response of all types of nerve fibres, originating from both vagal ganglia, and terminating in the airway as well as other visceral organs. In particular, we hope to use this preparation to determine the intracellular signalling pathways downstream of EP_3 and B_2 GPCR activation which lead to opening of the TRPA1 and TRPV1 ion channels; and to clarify the effects of TRPA1 inhibition on low pH-induced responses in an *in vitro* model. Because this is a newly established method within our laboratory, I have focussed on characterising the imaging preparation as a reliable *in vitro* model of cough. To do this, I began by characterising the effects of TRPA1 and TRPV1 selective agonists and antagonists in the primary vagal cells. I then verified that the TRPA1 and TRPV1 ion channels partially mediated the effects of PGE_2 and BK. These results mirrored those observed in both the isolated vagus nerve preparation, and *in vivo* guinea pig cough model, confirming that imaging of ganglia cells can be used as a model to investigate the cough reflex.

Having characterised the imaging model, I went on to investigate low pH-induced activation of vagal ganglia cells. Overall results mirrored those observed in the isolated vagus nerve. That is, low pH responses were partially inhibited by either TRPV1 or ASIC channel inhibition, but not TRPA1 inhibition. However with closer analysis, I also observed that there appeared to be distinct sets of cells that were either sensitive or non-sensitive to TRPV1 or ASIC channel inhibition. Additional experiments are required, but this could indicate that TRPV1 and ASIC channels are not co-expressed on primary vagal ganglia cells. Furthermore, there was a variable effect of TRPA1 inhibition on low pH responses in jugular cells, from approximately 0-60% inhibition of the calcium response. This is much greater than the natural variation seen with other experiments in this preparation. Therefore, this could again indicate distinct sets of cells that are either sensitive or non-sensitive to TRPA1 inhibition; but also that the inhibitory effect of TRPA1 on primary vagal cells is only partial (rather than complete inhibition observed with either neurons sensitive to TRPV1 or ASIC antagonists). One explanation for these results is that TRPA1 plays a role in sensing low pH in airway-projecting cells, but not non-airway cells. We are able to investigate this hypothesis using the imaging model by retrogradelly staining airway cells using the fluorescent dye DiI. The final experiment in my thesis therefore investigated the ability of capsaicin and low pH to stimulate DiI-stained cells. I established that both of these agonists do indeed activate airway cells, and I can therefore move ahead with further studies investigating how TRPV1, ASICs and TRPA1 mediate low pH-induced activation of airway-specific cells. These studies will be discussed in section 8.3.2.

8.2 Limitations of the thesis

Some of the limitations of the experimental techniques used in this thesis have been discussed previously. Briefly, the isolated vagus nerve preparation provides a relatively high-throughput *in vitro* method that has been shown to parallel cough responses seen in the conscious guinea pig model *in vivo*, and also human cough responses in the clinic. Though mice do not possess a cough response, and therefore cannot be used for *in vivo* research, the afferent arm of the reflex still appears to be intact, and responses in this species parallel those seen in guinea pigs and humans. Moreover, the trunk of the vagus nerve carries all types of afferent nerve fibres, as well as parasympathetic nerves, and fibres innervating other organ systems such as the heart and gastrointestinal tract. In addition, the effect on the isolated

nerve trunk does not necessarily represent what is happening at the nerve terminals within the airway. Despite these limitations, one major advantage of the isolated vagus preparation is that our group has access to human tissue from donor and recipient transplant patients, which are surplus to clinical requirement. Therefore, we are able to replicate our findings using human tissue, and demonstrate that our animal models do indeed translate to humans. But, due to the scarcity of donor tissue, the n-number of experiments using human vagus nerves in this thesis are low (n = 1-8); furthermore we cannot control for the time lapse between retrieval of the tissue to arrival at the laboratory, and the tissue will become less viable with time.

The primary vagal ganglia cell imaging model provides advantages over the isolated vagus preparation as an *in vitro* model, but there are still a number of limitations with this method. Firstly, we cannot be sure what cellular changes take place during the isolation process. For example, upregulation or downregulation of receptors and ion channels or other phenotypical changes may occur. Moreover, though we can record from single cells, we still cannot distinguish between the type of nerve fibre (e.g. Aδ or C-fibre). An advantage of the system is the ability to retrogradelly label airway cells using the fluorescent tracer DiI. However, we are dosing this dye intranasally, which means that some of the dye may be swallowed and could stain cells projecting from the oesophagus. Also, at present we do not have access to human vagal ganglia and therefore cannot replicate our findings in a human model.

8.3 Conclusions

Identification of the TRPA1 ion channel as a key tussive mediator represents a key step forward in our understanding of the mechanisms which drive the cough reflex. Furthermore, the finding that TRPA1 and TRPV1 mediate the effects of the endogenous irritants PGE_2, BK and possibly low pH is novel and exciting. This data suggests that TRPA1 antagonists, possibly in combination with TRPV1 antagonists, should be considered as a promising therapeutic target for cough.

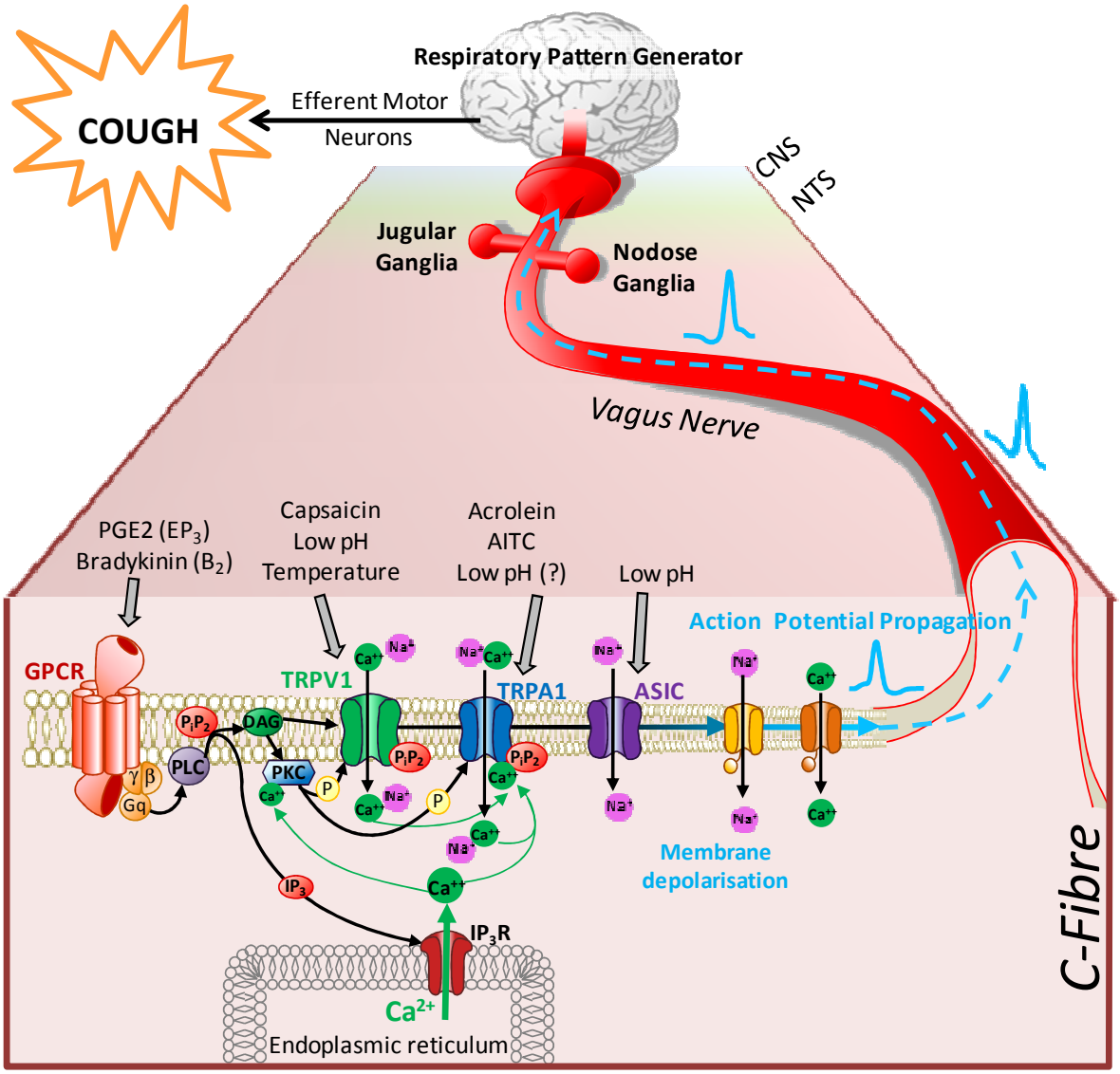


Figure 8.1. Summary: Activation of the cough reflex by TRPA1, TRPV1 and ASIC ion channels.

TRPA1, TRPV1 or ASIC ion channels can be directly gated by chemical irritants; changes in temperature; and changes in pH. Alternately, endogenous mediators such as PGE_2 or BK can cause TRP channel activation by binding to their GPCR, and initiating subsequent intracellular signalling cascades. One proposed intracellular cascade involves activation of PLC, which leads to hydrolysis of PIP_2, a complex that normally binds to and inhibits TRPA1 and TRPV1; therefore, removal of this inhibition could act to sensitise these ion channels. Hydrolysis of PIP_2 also leads to the production of DAG (which activates PKCs) and IP_3. DAG is proposed to directly activate TRPV1, and PKC phosphorylates both TRPA1 and TRPV1 ion channels. IP_3 binds to its receptor on the endoplasmic reticulum, causing release of intracellular calcium stores and further activation of TRPA1 and PKCs. Stimulation of these ion channels causes cation influx, which depolarises the cell membrane. If depolarisation reaches the activation threshold, voltage gated Na_V and Ca_V ion channels also open in

cascade, and action potentials are generated and propogated along the nerve fibre through the vagus nerve to where it synapses in the NTS. Information is then passed to the respiratory pattern generator in the CNS, causing activation of efferent motor neurons, and ultimately leading to cough. Abbreviations: CNS = central nervous system; NTS = nucleus tractus solitarius; AITC = allyl isothiocyanate; GPCR = G protein coupled receptor; PIP_2 = phosphatidylinositol 4,5-biphosphate; IP_3 = inositol triphosphate; DAG = diacylglycerol; PLC = phospholipase C; PKC = phosphokinase C; P = phosphorylation.

8.4 Future Studies

The aim of this thesis was to identify the ion channels involved in mediating tussive effects of PGE_2, BK and low pH. Having identified a role for TRPA1 and TRPV1 in the cough response to PGE_2 and BK, this section identifies further work aimed at elucidating the signalling pathways downstream of GPCR coupling that lead to ion channel opening. In addition, I will discuss how I plan to proceed in determining a role for TRPA1 in mediating low pH responses in the airways. Finally, I will outline the future direction of this research investigating the involvement of TRPA1 and TRPV1 receptors in the enhanced cough associated with inflammatory disease.

8.4.1 Determining the signalling pathways leading to stimulation of TRPA1 and TRPV1 downstream of GPCR activation by PGE_2 or BK

It has been previously established in our labs that PGE_2 causes cough by binding to the EP_3 GPCR (Maher et al., 2009); and I established in this thesis that in guinea pigs and humans, BK induces cough via activation of the B_2 GPCR. Intracellular secondary messengers are subsequently released, which ultimately lead to opening of the TRPA1 and TRPV1 ion channels. Inhibitors of the potential secondary messenger mechanisms (e.g. phosphokinase A, phosphokinase C or diacylglycerol antagonists) could be utilised in the imaging model in an attempt to block PGE_2 or BK-induced activation. Subsequently, selective activators of these pathways (e.g. 1-oleoyl-2-acetyl-sn-glycerol, a cell membrane-permeable analogue of diacylglycerol) in combination with TRPA1 and TRPV1-selective antagonists could be used to confirm that the identified pathway does indeed cause activation of these ion channels.

8.4.2 Investigating a role for TRPA1 in the low pH-induced sensory afferent response in vitro

8.4.2.1 Determining the effect of other TRPA1-selective antagonists

A potential explanation for the lack of effect of the TRPA1 antagonist on low pH-induced responses in the *in vitro* vagus nerve and isolated primary ganglia models is that acidic solutions interfere with the ability of HC-030031 to inhibit the TRPA1 ion channel. Therefore, it would be interesting to investigate the ability of other TRPA1-selective antagonists (e.g. AP18) to inhibit low pH responses in these models.

8.4.2.2 Airway-specific sensory primary vagal ganglia cells

I have established that low pH solutions cause activation of airway-specific primary vagal ganglia cells stained using DiI. As discussed previously, it is possible that TRPA1 is mediating an effect on airway nerves, but not non-airway nerves, and that this effect is being lost in the natural variability of the *in vitro* preparations utilised. Therefore, using the selective TRPA1 inhibitor HC-030031 I plan to investigate whether TRPA1 plays a role in mediating the low pH response in airway stained cells *in vitro*. In measuring responses only from the airway-associated cells this could remove some of the variability observed when testing on all (airway and non-airway) cells. Furthermore, I plan to isolate primary vagal cells from mouse ganglia, which will allow the use of genetically modified animals to verify the antagonist studies. This could also allow me to confirm my findings in the isolated vagus model which indicate a role for ASIC1 but not ASIC3 ion channels in low pH-induced sensory nerve activation.

8.4.2.3 Investigating a role for TRPA1 in mediating low pH-induced sensory nerve activation using more acidic solutions

An alternative hypothesis as to why we are seeing an inhibitory effect of TRPA1 antagonists on the low pH cough response *in vivo*, but not in the *in vitro* preparations, is that the nerve endings within the airway are being exposed to a higher level of acidity with inhalation of citric acid than what we are using *in vitro* (pH 5 for isolated vagus, and pH 6 for isolated primary cells). To test this theory I plan to use isolated vagus nerves from wild type and genetically modified *Trpa1*$^{-/-}$, *Trpv1*$^{-/-}$, *Asic1*$^{-/-}$, and *Asic3*$^{-/-}$ animals, and perform

concentration responses for pH to establish what range of acidity each of these ion channels detects. I will be using the isolated vagus preparation as the axon of the vagus nerve is more robust than the isolated primary vagal ganglia cells, for which a pH < 5.5 kills the cells. I have run a pilot trial for this on wild type vagus nerves, and established that the tissue is still viable even after stimulation with pH 3 (Figure 8.2). If this hypothesis is correct, I should see a blunted pH response in *Trpa1*$^{-/-}$ mouse vagus compared to wild type for pH < 5.

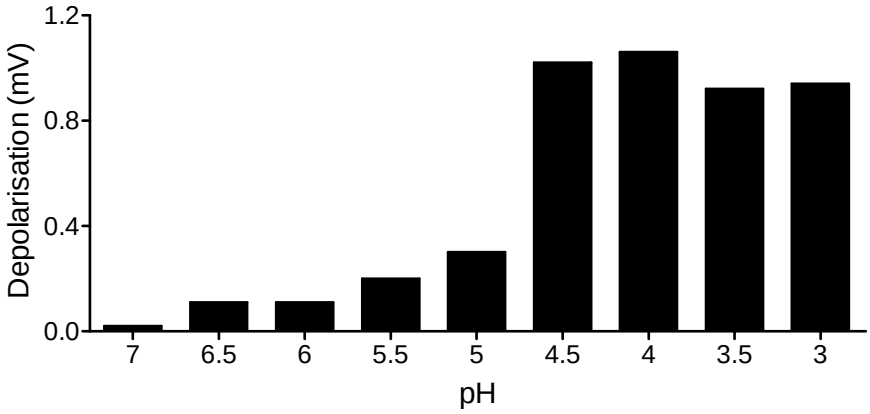


Figure 8.2. Concentration-related increases in mouse vagus nerve depolarisation with decreasing pH.

Isolated wild type (C57Bl/6j) mouse vagus was exposed to non-cumulative concentrations of pH for 2 minutes, followed by wash-out to retain baseline. Decreasing pH levels caused concentration-related increases in sensory afferent depolarisation. Data are presented as the peak depolarisation (in millivolts) of n = 1 observation.

8.4.3 Investigating a role for ASIC ion channels in citric-acid induced cough

In this thesis I investigated the ability of TRPA1 and TRPV1 selective antagonists to inhibit citric acid-induced cough *in vivo*. Having also established a role for the ASIC ion channels in the sensory afferent response to low pH *in vitro*, it would be interesting to follow this up in our model of conscious guinea pig cough. The general ASIC inhibitor amiloride was not used to inhibit citric acid cough in this thesis because this compound displays poor potency, and has inhibitory effects on other receptors of the epithelial sodium channel family (ENaC; Dubé et al., 2005). A recently described non-amiloride inhibitor of ASIC ion channels, A-317567, shows better potency than amiloride in *in vivo* models of inflammatory hyperalgesia and

post-operative pain, and has no apparent effects on renal ENaCs (Dubé et al., 2005). This compound could therefore provide a better pharmacological tool for *in vivo* research investigating the role ASIC family of ion channels in cough.

8.4.4 Investigating a role for TRPA1 and TRPV1 in the enhanced cough response using models of respiratory disease

In this thesis, a role for TRPA1 and TRPV1 in mediating the effects of PGE_2, BK and low pH have been investigated under 'normal' conditions. This is necessary in order to understand how the acute cough reflex is being controlled, in order to then determine what changes occur during disease. It would subsequently be interesting to establish whether these ion channels play a role in modification of the cough reflex in disease states. There is enhanced release of PGE_2 and BK, and a decrease in pH in the airways of patients suffering from inflammatory airways diseases (e.g. asthma and COPD), and these irritants have been associated with sensitisation of the cough reflex (Choudry et al., 1989; Fox et al., 1996; Karlsson & Fuller, 1999; Morice et al., 2007). Therefore, it is possible that cough hypersensitivity could be controlled with therapies that inhibit the TRPA1 and TRPV1 receptors. Our lab has established animal models of asthma (induced by ovalbumin or LPS sensitisation) and cigarette smoke exposure. In the cigarette smoke model we have already successfully observed enhanced sensory nerve and cough responses to the TRPV1 agonist capsaicin, which suggests that this is an ideal model in which to investigate excessive cough. Using these models, we could determine the ability of TRPA1 and TRPV1 antagonists to inhibit the excessive cough responses using the *in vitro* and *in vivo* models characterised in this thesis.

8.4.5 Measuring RNA and protein expression in airway-labelled primary vagal ganglia cells

An increase in TRPV1 expression has been found in the lungs of patients suffering from chronic cough (Groneberg et al., 2004), providing another potential mechanism for the enhanced cough response to TRPV1 agonists in inflammatory airway disease. Therefore, it would be interesting to investigate RNA and protein expression of the TRP channels using RT-PCR and western blot techniques. This would allow us to compare the level of expression of these receptors in the healthy state with that in our disease models, discussed above.

Appendix

Chemicals and reagents

The table below outlines all the chemical reagents and compounds used in this thesis. The vehicle or diluents have been described where appropriate.

Drug / reagent	**Source**	**Vehicle or diluents (where applicable)**
Acrolein	Sigma-Aldrich	0.1% DMSO in Krebs solution (*in vitro*) 0.9% saline (*in vivo*)
Agarose	Promega	2% in 1xTBE
AITC (allyl-isothiocyanate)	Sigma-Aldrich	0.1% DMSO in Krebs solution
Amiloride	Sigma-Aldrich	0.1% DMSO in Krebs solution
Boric Acid	Sigma-Aldrich	-
Bradykinin	Sigma-Aldrich	0.1% dH_2O (*in vitro*) 0.9% saline (*in vivo*)
$CaCl_2$	VWR	-
Capsaicin	Sigma-Aldrich	0.1% DMSO in Krebs solution (*in vitro*) 1% ethanol plus 1% Tween 80 in 0.9% saline (*in vivo*)
Capsazepine	Sigma-Aldrich	0.1% DMSO in Krebs solution
Cinnamaldehyde	Sigma-Aldrich	0.1% DMSO in Krebs solution
Citric Acid	Sigma-Aldrich	0.9% saline
Collagenase	Worthington	Ca^{2+}-free, Mg^{2+}-free Hank's balanced salt solution
Di-8-ANEPPS (4-[2-[6-(dioctylamino)-2-naphthalenyl]ethenyl]-1-(3-sulfopropyl)-pyridinium)	Invitrogen	0.4% DMSO in extracellular solution

Drug / reagent	**Source**	**Vehicle or diluents (where applicable)**
DiI (DiIC18(3), 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate)	Invitrogen	2% ethanol in 0.9% saline
Dispase II	Roche	Ca^{2+}-free, Mg^{2+}-free Hank's balanced salt solution
EDTA	Promega	-
Ethanol	VWR	-
F12	Invitrogen	-
FBS (foetal bovine serum)	Sigma-Aldrich	-
Fluo-4 AM	Invitrogen	Extracellular solution
Glucose	VWR	-
HBSS	Invitrogen	-
HC-030031	ChemBridge	0.1% DMSO in Krebs solution (*in vitro*) 0.5% methyl cellulose in 0.9% saline (*in vivo*)
HEPES	Sigma-Aldrich	-
Hyperladder IV	Bioline Ltd	-
Indomethacin	Sigma-Aldrich	0.1% DMSO in Krebs solution
Isopropanol	Sigma-Aldrich	-
JNJ17203212	GlaxoSmithKlein	0.1% DMSO in Krebs solution (*in vitro*) 15% solutol in 5% dextrose solution (*in vivo*)
KCl	VWR	Extracellular solution
KH_2PO_4	VWR	-
L15	Sigma-Aldrich	-
Laminin	Sigma-Aldrich	dH_2O
Lys-[Des-Arg^9]Bradykinin	Tocris	0.1% DMSO in Krebs solution
Methyl cellulose	Sigma-Aldrich	-

Drug / reagent	**Source**	**Vehicle or diluents (where applicable)**
$MgSO_4$	VWR	-
NaCl	VWR	-
$NaH_2PO_4.2H_2O$	VWR	-
$NaHCO_3$	VWR	-
Nuclease-free water	Promega	-
Nucleon resin	Tepnel Life Sciences	-
Papain	Sigma-Aldrich	Ca^{2+}-free, Mg^{2+}-free Hank's balanced salt solution
Penicillin/streptomycin	Sigma-Aldrich	-
Percoll	Sigma-Aldrich	-
Petroleum jelly	Vaseline	-
PGE_2	Sigma Aldrich	0.1% ethanol in Krebs solution (*in vitro*) 0.1 M phosphate buffer (*in vivo*)
Proteinase K	Tepnel Life Sciences	Nuclease-free H_2O
R715	Tocris	0.1% DMSO in Krebs solution
Reagent M1	Tepnel Life Sciences	-
Reagent M2	Tepnel Life Sciences	-
Resiniferatoxin	LC Labs	0.1% DMSO in Krebs solution
0.9% saline (sterile)	Fresenius Kabi	-
Safeview	NBS Biologicals Ltd	-
Sodium pentobarbitone	Merial Animal Health	-
Trizma Base	Sigma-Aldrich	-
Tween 80	Sigma-Aldrich	-
WIN 64338	Tocris	0.1% DMSO in Krebs solution

Reference List

Advenier, C., Girard, V., Naline, E., Vilain, P., & Emonds-Alt, X. (1993). Antitussive effect of SR 48968, a non-peptide tachykinin NK_2 receptor antagonist. *European Journal of Pharmacology, 250*(1), 169-171.

Ahluwalia, J., Urban, L., Capogna, M., Bevan, S., & Nagy, I. (2000). Cannabinoid 1 receptors are expressed in nociceptive primary sensory neurons. *Neuroscience, 100*(4), 685-688.

Alvarez de la Rosa, D., Zhang, P., Shao, D., White, F., & Canessa, C. (2002). Functional implications of the localization and activity of acid-sensitive channels in rat peripheral nervous system. *Proceedings of the National Academy of Sciences of the United States of America, 99*(4), 2326-31.

American Academy of Pediatrics (1997). Use of codeine- and dextromethorphan-containing cough remedies in children. *Pediatrics, 99*(6), 918-920.

Anavi-Goffer, S., & Coutts, A. (2003). Cellular distribution of vanilloid VR1 receptor immunoreactivity in the guinea-pig myenteric plexus. *European Journal of Pharmacology, 458*(1-2), 61-71.

Andersson, D., Gentry, C., Moss, S., & Bevan, S. (2008). Transient receptor potential A1 is a sensory receptor for multiple products of oxidative stress. *The Journal of Neuroscience, 28*(10), 2485-94.

Andrè, E., Campi, B., Materazzi, S., Trevisani, M., Amadesi, S., Massi, D., et al. (2008). Cigarette smoke-induced neurogenic inflammation is mediated by α,β-unsaturated aldehydes and the TRPA1 receptor in rodents. *The Journal of Clinical Investigation, 118*(7), 2574-82.

Andrè, E., Gatti, R., Trevisani, M., Preti, D., Baraldi, P. G., Patacchini, R., & Geppetti, P. (2009). Transient receptor potential ankyrin receptor 1 is a novel target for pro-tussive agents. *British Journal of Pharmacology, 158*(6), 1621-8.

Avelino, A., Cruz, C., Nagy, I., & Cruz, F. (2002). Vanilloid receptor 1 expression in the rat urinary tract. *Neuroscience*, *109*(4), 787-98.

Bandell, M., Story, G., Hwang, S., Viswanath, V., Eid, S., Petrus, M., et al. (2004). Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. *Neuron*, *41*(6), 849-57.

Bang, S., & Hwang, S. (2009). Polymodal ligand sensitivity of TRPA1 and its modes of interactions. *The Journal of General Physiology*, *133*(3), 257-62.

Barnes, P. (2010a). New therapies for chronic obstructive pulmonary disease. *Medical Principles and Practice*, *19*(5), 330-8.

Barnes, P. (2010b). New therapies for asthma: is there any progress? *Trends in Pharmacological Sciences*, *31*(7), 335-43.

Bautista, D., Jordt, S., Nikai, T., Tsuruda, P., Read, A., Poblete, J., et al. (2006). TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell*, *124*(6), 1269-82.

Bautista, D., Movahed, P., Hinman, A., Axelsson, H., Sterner, O., Högestätt, E., et al. (2005). Pungent products from garlic activate the sensory ion channel TRPA1. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(34), 12248-52.

Belvisi, M. (2003). Airway sensory innervation as a target for novel therapies: an outdated concept? *Current Opinion in Pharmacology*, *3*(3), 239-243.

Belvisi, M., & Bolser, D. (2002). Summary: Animal models for cough. *Pulmonary Pharmacology & Therapeutics*, *15*(3), 249-50.

Belvisi, M., & Geppetti, P. (2004). Cough 7: Current and future drugs for the treatment of chronic cough. *Thorax*, *59*(5), 438-440.

Belvisi, M., & Hele, D. (2006). Cough: Citric acid and nerves. *Drug Discovery Today: Disease Models*, *3*(3), 237-241.

Belvisi, M., Miura, M., Stretton, D., & Barnes, P. (1992). Capsazepine as a selective antagonist of capsaicin-induced activation of C-fibres in guinea-pig bronchi. *European Journal of Pharmacology*, *215*(2-3), 341-4.

Belvisi, M., Patel, H., Freund-Michel, V., Hele, D., Crispino, N., & Birrell, M. (2008). Inhibitory activity of the novel CB_2 receptor agonist, GW833972A, on guinea-pig and human sensory nerve function in the airways. *British Journal of Pharmacology*, *155*(4), 547-57.

Bessac, B., Sivula, M., von Hehn, C., Escalera, J., Cohn, L., & Jordt, S. (2008). TRPA1 is a major oxidant sensor in murine airway sensory neurons. *The Journal of Clinical Investigation*. doi:10.1172/JCI34192.tating

Bhattacharya, A., Scott, B., Nasser, N., Ao, H., Maher, M., Dubin, A., et al. (2007). Pharmacology and antitussive efficacy of 4-(3-Trifluoromethyl-pyridin-2-yl)-peperazine-1-carboxylic Acid (5-Trifluoromethyl-pyridin-2-yl)-amide (JNJ17203212), a Transient Receptor Potential Vanilloid 1 antagonist in guinea pigs. *The Journal of Pharmacology and Experimental Therapeutics*, *323*(2), 665-674.

Birder, L., Kanai, A., de Groat, W., Kiss, S., Nealen, M., Burke, N., et al. (2001). Vanilloid receptor expression suggests a sensory role for urinary bladder epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(23), 13396-401.

Birrell, M., Belvisi, M., Grace, M., Sadofsky, L., Faruqi, S., Hele, D., et al. (2009). TRPA1 agonists evoke coughing in guinea pig and human volunteers. *American Journal of Respiratory and Critical Care Medicine*, *180*(11), 1042-7.

Bolser, D., Aziz, S., DeGennaro, F., Kreutner, W., Egan, R., Siegel, M., & Chapman, R. (1993). Antitussive effects of GABA-B agonists in the cat and guinea-pig. *British Journal of Pharmacology*, *110*(1), 491-5.

Bolser, D., DeGennaro, F., O'Reilly, S., Chapman, R., Kreutner, W., Egan, R., & Hey, J. (1994). Peripheral and central sites of action of GABA-B agonists to inhibit the cough reflex in the cat and guinea pig. *British Journal of Pharmacology*, *113*(4), 1344-8.

Bolser, D., DeGennaro, F., O'Reilly, S., McLeod, R., & Hey, J. (1997). Central antitussive activity of the NK_1 and NK_2 tachykinin receptor antagonists, CP-99,994 and SR 48968, in the guinea-pig and cat. *British Journal of Pharmacology*, *121*(2), 165-70.

Bos, C., Richel, D., Ritsema, T., Peppelenbosch, M., & Versteeg, H. (2004). Prostanoids and prostanoid receptors in signal transduction. *The International Journal of Biochemistry & Cell Biology*, *36*(7), 1187-205.

Bose, B., Cater, J., & Clark, R. (1987). A once daily theophylline preparation in prevention of nocturnal symptoms in childhood asthma. *European Journal of Pediatrics*, *146*, 524-527.

Buckley, J., Birrell, M., Maher, S., Nials, A., Clarke, D., & Belvisi, M. (2011). EP_4 receptor as a new target for bronchodilator therapy. *Thorax*. doi:10.1136/thx.2010.158568

Buckley, N., McCoy, K., Mezey, E., Bonner, T., Zimmer, A., Felder, C., & Glass, M. (2000). Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB_2 receptor. *European Journal of Pharmacology*, *396*(2-3), 141-9.

Calixto, J., Cabrini, D., Ferreira, J., & Campos, M. (2000). Kinins in pain and inflammation. *Pain*, *87*(1), 1-5.

Campbell, M., Eliraz, A., Johansson, G., Tornling, G., Nihlén, U., Bengtsson, T., & Rabe, K. (2005). Formoterol for maintenance and as-needed treatment of chronic obstructive pulmonary disease. *Respiratory Medicine*, *99*(12), 1511-20.

Canning, B. (2002). Interactions between vagal afferent nerve subtypes mediating cough. *Pulmonary Pharmacology & Therapeutics*, *15*, 187-192.

Canning, B. (2007). Encoding of the cough reflex. *Pulmonary Pharmacology & Therapeutics*, *20*(4), 396-401.

Canning, B. (2009). Central regulation of the cough reflex: Therapeutic implications. *Pulmonary Pharmacology & Therapeutics*, *22*(2), 75-81.

Canning, B., & Mori, N. (2011). Encoding of the cough reflex in anesthetized guinea pigs. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *300*(2), R369-77.

Canning, B., Mazzone, S., Meeker, S., Mori, N, Reynolds, S., & Undem, B. (2004). Identification of the tracheal and laryngeal afferent neurones mediating cough in anaesthetized guinea-pigs. *The Journal of Physiology*, *557*(Pt 2), 543-58.

Canning, B., Mori, N., & Mazzone, S. (2006). Vagal afferent nerves regulating the cough reflex. *Respiratory Physiology & Neurobiology*, *152*(3), 223-42.

Carr, M. (2004). Plasticity of vagal afferent fibres mediating cough. *Pulmonary Pharmacology & Therapeutics*, *17*(6), 447-51.

Carr, M., Hunter, D., Jacoby, D., & Undem, B. (2002). Expression of tachykinins in nonnociceptive vagal afferent neurons during respiratory viral infection in guinea pigs. *American Journal of Respiratory and Critical Care Medicine*, *165*, 1071-5.

Carr, M., Kollarik, M., Meeker, S., & Undem, B. (2003). A role for TRPV1 in bradykinin-induced excitation of vagal airway afferent nerve terminals. *The Journal of Pharmacology and Experimental Therapeutics*, *304*(3), 1275-1279.

Carruthers, S. (1986). Severe coughing during captopril and enalapril therapy. *Canadian Medical Association Journal*, *135*, 217-218.

Caspani, O., & Heppenstall, P. (2009). TRPA1 and cold transduction: An unresolved issue? *The Journal of General Physiology*, *133*(3), 245-9.

Caterina, M., Leffler, A., Malmberg, A., Martin, W., Trafton, J., Petersen-Zeitz, K., et al. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science*, *288*, 306-313.

Caterina, M., Schumacher, M., Tominaga, M., Rosen, T., Levine, J., & Julius, D. (1997). The capsaicin receptor: A heat-activated ion channel in the pain pathway. *Nature*, *389*, 816-24.

Cavanaugh, E., Simkin, D., & Kim, D. (2008). Activation of transient receptor potential A1 channels by mustard oil, tetrahydrocannabinol and Ca^{2+} reveals different functional channel states. *Neuroscience, 154*(4), 1467-76.

Cazzola, M., Matera, M., Liccardi, G., De Prisco, F., D'Amato, G., & Rossi, F. (1993). Theophylline in the inhibition of angiotensin-converting enzyme inhibitor-induced cough. *Respiration, 60*(4), 212-5.

Centre for Disease Control. (2007). Infant deaths associated with cough and cold medications - two states, 2005. *Morbidity and Mortality Weekly Report, 56*(1), 1-4.

Chang, A., Phelan, P., Carlin, J., Sawyer, S., & Robertson, C. (1998). A randomised, placebo controlled trial of inhaled salbutamol and beclomethasone for recurrent cough. *Archives of Disease in Childhood, 79*(1), 6-11.

Chen, C., Zimmer, A., Sun, W., Hall, J., & Brownstein, M. (2002). A role for ASIC3 in the modulation of high-intensity pain stimuli. *Proceedings of the National Academy of Sciences of the United States of America, 99*(13), 8992-7.

Chen, J., Zhang, X., Kort, M., Huth, J., Sun, C., Miesbauer, L., et al. (2008). Molecular determinants of species-specific activation or blockade of TRPA1 channels. *The Journal of Neuroscience, 28*(19), 5063-71.

Chong, C., Chen, C., Ma, H., Wu, Y., Chen, Y., & Wang, T. (2005). Comparison of lidocaine and bronchodilator inhalation treatments for cough suppression in patients with chronic obstructive pulmonary disease. *Emergency Medicine Journal, 22*(6), 429-32.

Chou, Y., Scarupa, M., Mori, N, & Canning, B. (2008). Differential effects of airway afferent nerve subtypes on cough and respiration in anesthetized guinea pigs. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology, 295*(5), R1572-84.

Choudry, N., Fuller, R., & Pride, N. (1989). Sensitivity of the human cough reflex: Effect of inflammatory mediators prostaglandin E_2, bradykinin, and histamine. *American Review of Respiratory Disease, 140*, 137-41.

Clapham, D. (2003). TRP channels as cellular sensors. *Nature, 426*, 517-24.

Claudino, R., Kassuya, C., Ferreira, J., & Calixto, J. (2006). Pharmacological and molecular characterization of the mechanisms involved in prostaglandin E_2-induced mouse paw edema. *The Journal of Pharmacology and Experimental Therapeutics*, *318*(2), 611-618.

Coleridge, H., Coleridge, J., Ginzel, K., Baker, D., Banzett, R., & Morrison, M. (1976). Stimulation of "irritant" receptors and afferent C-fibres in the lungs by prostaglandins. *Nature, 264*, 451-3.

Coleridge, J., & Coleridge, H. (1984). Afferent vagal C fibre innervation of the lungs and airways and its functional significance. *Reviews of Physiology, Biochemistry and Pharmacology, 99*, 1-110.

Costello, J., Dunlop, L., & Gardiner, P. (1985). Characteristics of prostaglandin induced cough in man. *British Journal of Clinical Pharmacology*, *20*(4), 355-9.

Davis, J., Gray, J., Gunthorpe, M., Hatcher, J., Davey, P., Overend, P., et al. (2000). Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Letters to Nature, 405*, 183-7.

Denda, M., Fuziwara, S., Inoue, K., Denda, S., Akamatsu, H., Tomitaka, A, & Matsunaga, K. (2001). Immunoreactivity of VR1 on epidermal keratinocyte of human skin. *Biochemical and Biophysical Research Communications*, *285*(5), 1250-2.

Dhaka, A., Uzzell, V., Dubin, A., Mathur, J., Petrus, M., Bandell, M., & Patapoutian, A. (2009). TRPV1 is activated by both acidic and basic pH. *The Journal of Neuroscience, 29*(1), 153-8.

Dicpinigaitis, P., & Dobkin, J. (1997). Antitussive effect of the GABA-agonist baclofen. *Chest, 111*(4), 996-999.

Diogenes, A., Akopian, A., & Hargreaves, K. (2007). NGF up-regulates TRPA1: implications for orofacial pain. *Journal of Dental Research, 86*(6), 550-555.

Doherty, M., Mister, R., Pearson, M., & Calverley, P. (2000). Capsaicin responsiveness and cough in asthma and chronic obstructive pulmonary disease. *Thorax, 55*(8), 643-9.

Dubé, G., Lehto, S., Breese, N., Baker, S., Wang, X., Matulenko, M., et al. (2005). Electrophysiological and in vivo characterization of A-317567, a novel blocker of acid sensing ion channels. *Pain, 117*(1-2), 88-96.

Eid, S., Crown, E., Moore, E., Liang, H., Choong, K., Dima, S., et al. (2008). HC-030031, a TRPA1 selective antagonist, attenuates inflammatory- and neuropathy-induced mechanical hypersensitivity. *Molecular Pain, 4*.

Eilers, H., Cattaruzza, F., Nassini, R., Materazzi, S., Andre, E., Chu, C., et al. (2010). Pungent general anesthetics activate transient receptor potential-A1 to produce hyperalgesia and neurogenic bronchoconstriction. *Anesthesiology, 112*(6), 1452-63.

Ellis, K., & Fozard, J. (2002). Species differences in bradykinin receptor-mediated responses of the airways. *Autonomic & Autacoid Pharmacology, 22*(1), 3-16.

Ellul-Micallef, R. (1983). Effect of terbutaline sulphate in chronic "allergic" cough. *British Medical Journal, 287*, 940-3.

Fahy, J., Wong, H., Geppetti, P., Reis, J., Harris, S., Maclean, D., et al. (1995). Effect of an NK_1 receptor antagonist (CP-99,994) on hypertonic saline-induced bronchoconstriction and cough in male asthmatic subjects. *American Journal of Respiratory and Critical Care Medicine, 152*(3), 879-84.

Fairfax, A., Clarke, R., Chatterjee, S., Connolly, C., Higenbottam, T., Holgate, S., et al. (1990). Controlled-release theophylline in the treatment of nocturnal asthma. *Journal of International Medical Research, 18*(4), 273-81.

Finotto, S., Fabbri, L., Rado, V., Mapp, C., & Maestrelli, P. (1991). Increase in numbers of CD8 positive lymphocytes and eosinophils in peripheral blood of subjects with late asthmatic reactions induced by toluene diisocyanate. *British Journal of Industrial Medicine, 48*(2), 116-21.

Fong, J., Sandhu, G., Ellaway, P., Davey, N., Strutton, P., Murphy, K., & Guz, A. (2004). What do we know about how humans cough? *Pulmonary Pharmacology & Therapeutics, 17*(6), 431-4.

Fontana, G., Pantaleo, T., Lavorini, F., Mutolo, D., Polli, G., & Pistolesi, M. (1999). Coughing in laryngectomized patients. *American Journal of Respiratory and Critical Care Medicine*, *160*(5 Pt 1), 1578-84.

Ford, A., Forman, D., Moayyedi, P., & Morice, A. (2006). Cough in the community: a cross sectional survey and the relationship to gastrointestinal symptoms. *Thorax*, *61*(11), 975-9.

Fox, A., Barnes, P., Venkatesan, P., & Belvisi, M. (1997). Activation of large conductance potassium channels inhibits the afferent and efferent function of airway sensory nerves in the guinea pig. *The Journal of Clinical Investigation*, *99*(3), 513-9.

Fox, A., Lalloo, U., Belvisi, M., Bernareggi, M., Chung, K., & Barnes, P. (1996). Bradykinin-evoked sensitization of airway sensory nerves: A mechanism for ACE-inhibitor cough. *Nature Medicine*, *2*(7), 814-7.

Freund-Michel, V., Birrell, M., Giembycz, M., Hele, D., Haj-Yahia, S., & Belvisi, M. (2010). β_2-agonists block tussive responses in guinea pigs via an atypical cAMP-dependent pathway. *The European Respiratory Journal*, *35*(3), 647-54.

Fujita, F., Uchida, K., Moriyama, T., Shima, A., Shibasaki, K., Inada, H., et al. (2008). Intracellular alkalization causes pain sensation through activation of TRPA1 in mice. *The Journal of Clinical Investigation*, *118*(12), 4049-4057.

Fuller, R., & Choudry, N. (1987). Increased cough reflex associated with angiotensin converting enzyme inhibitor cough. *British Medical Journal*, *295*, 1025-1026.

Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., et al. (1995). Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *European Journal of Biochemistry*, *232*(1), 54-61.

Gallos, G., & Flood, P. (2010). Wasabi and a volatile anesthetic. *Anesthesiology*, *112*(6), 1309-10.

Garrity, P. (2011). Weakly acidic, but strongly irritating: TRPA1 and the activation of nociceptors by cytoplasmic acidification. *The Journal of General Physiology*, *137*(6), 489-91.

Gauvreau, G., Watson, R., & O'Byrne, P. (1999). Protective effects of inhaled PGE_2 on allergen-induced airway responses and airway inflammation. *American Journal of Respiratory and Critical Care Medicine*, *159*(1), 31-6.

Gavva, N., Treanor, J., Garami, A., Fang, L., Surapaneni, S., Akrami, A., et al. (2008). Pharmacological blockade of the vanilloid receptor TRPV1 elicits marked hyperthermia in humans. *Pain*, *136*(1-2), 202-10.

Girard, V., Naline, E., Vilain, P., Emonds-Alt, X., & Advenier, C. (1995). Effect of the two tachykinin antagonists, SR 48968 and SR 140333, on cough induced by citric acid in the unanaesthetized guinea-pig. *European Respiratory Journal*, *8*(7), 1110-1114.

Grace, M., Birrell, M., Dubuis, E., & Belvisi, M. (2011). Tobacco smoke induced cough: Mechanisms driving acute and chronic cough pathology. In A. M. Moldoveanu (Ed.), *Advanced Topics in Environmental Health and Air Pollution Case Studies* (2nd ed., pp. 97-120). In Tech.

Griffin, G., Fernando, S., Ross, R., McKay, N., Ashford, M., Shire, D., et al. (1997). Evidence for the presence of CB_2-like cannabinoid receptors on peripheral nerve terminals. *European Journal of Pharmacology*, *339*(1), 53-61.

Grilo, A., Sáez-Rosas, M., Santos-Morano, J., Sánchez, E., Moreno-Rey, C., Real, L., et al. (2011). Identification of genetic factors associated with susceptibility to angiotensin-converting enzyme inhibitors-induced cough. *Pharmacogenetics and Genomics*, *21*(1), 10-17.

Groneberg, D., Niimi, A., Dinh, Q., Cosio, B., Hew, M., Fischer, A., & Chung, K. (2004). Increased expression of transient receptor potential vanilloid-1 in airway nerves of chronic cough. *American Journal of Respiratory and Critical Care Medicine*, *170*(12), 1276-80.

Gu, Q., & Lee, L. (2006). Characterization of acid signaling in rat vagal pulmonary sensory neurons. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *291*(1), L58-65.

Gunn, V., Taha, S., Liebelt, E., & Serwint, J. (2001). Toxicity of over-the-counter cough and cold medications. *Pediatrics*, *108*(3).

Gunthorpe, M., & Chizh, B. (2009). Clinical development of TRPV1 antagonists: Targeting a pivotal point in the pain pathway. *Drug Discovery Today*, *14*(1-2), 56-67.

Harding, S. (2006). Gastroesophageal reflux and chronic cough. *GI Motility Online*. doi:doi:10.1038/gimo77

Hardy, M., Lawrence, C., Standen, N., & Rodrigo, G. (2006). Can optical recordings of membrane potential be used to screen for drug-induced action potential prolongation in single cardiac myocytes? *Journal of Pharmacological and Toxicological Methods*, *54*(2), 173-82.

Hay, A., Heron, J., & Ness, A. (2005). The prevalence of symptoms and consultations in pre-school children in the Avon Longitudinal Study of Parents and Children (ALSPAC): A prospective cohort study. *Family Practice*, *22*(4), 367-74.

Herkenham, M., Lynn, A., Johnson, M., Melvin, L., de Costa, B., & Rice, K. (1991). Characterization and localization of cannabinoid receptors in rat brain: A quantitative in vitro autoradiographic study. *The Journal of Neuroscience*, *11*(2), 563-83.

Herxheimer, H., & Stresemann, E. (1961). The effect of bradykinin in aerosol in guinea-pigs and man. *Proceedings of the Physiological Society* (pp. 38-9). doi:10.1097/00000441-196106000-00014

Hesselager, M., Timmermann, D., & Ahring, P. (2004). pH dependency and desensitization kinetics of heterologously expressed combinations of acid-sensing ion channel subunits. *The Journal of Biological Chemistry*, *279*(12), 11006-15.

Higenbottam, T. (2002). Chronic cough and the cough reflex in common lung diseases. *Pulmonary Pharmacology & Therapeutics*, *15*(3), 241-7.

Hinman, A., Chuang, H., Bautista, D., & Julius, D. (2006). TRP channel activation by reversible covalent modification. *Proceedings of the National Academy of Sciences of the United States of America, 103*(51), 19564-8.

Ho, C., Gu, Q., Hong, J., & Lee, L. (2000). Prostaglandin E_2 enhances chemical and mechanical sensitivities of pulmonary C fibers in the rat. *American Journal of Respiratory and Critical Care Medicine*, *162*(2 Pt 1), 528-33.

Hunt, J., Fang, K., Malik, R., Snyder, A., Malhotra, N., Platts-Mills, T., & Gaston, B. (2000). Endogenous airway acidification: Implications for asthma pathophysiology. *American Journal of Respiratory and Critical Care Medicine*, *161*, 694-699.

Hwang, S., & Oh, U. (2002). Hot channels in airways: pharmacology of the vanilloid receptor. *Current Opinion in Pharmacology, 2*, 235-242.

Hwang, S., Cho, H., Kwak, J., Lee, S., Kang, C., Jung, J., et al. (2000). Direct activation of capsaicin receptors by products of lipoxygenases: Endogenous capsaicin-like substances. *Proceedings of the National Academy of Sciences*, *97*(11), 6155-6160.

Ichikawa, H., & Sugimoto, T. (2003). The co-expression of VR1 and VRL-1 in the rat vagal sensory ganglia. *Brain Research*, *980*, 293-296.

Ichikawa, H., & Sugimoto, T. (2004). The co-expression of $P2X_3$ receptor with VR1 and VRL-1 in the rat trigeminal ganglion. *Brain Research, 998*(1), 130-135.

Irwin, R., Boulet, L., Cloutier, M., Fuller, R., Gold, P., Hoffstein, V., et al. (1998). Managing cough as a defense mechanism and as a symptom. *Chest, 114*(2 supplement), 133S-181S.

Jaquemar, D., Schenker, T., & Trueb, B. (1999). An ankyrin-like protein with transmembrane domains is specifically lost after oncogenic transformation of human fibroblasts. *The Journal of Biological Chemistry*, *274*(11), 7325-7333.

Ji, R., Samad, T., Jin, S., Schmoll, R., & Woolf, C. (2002). p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron*, *36*(1), 57-68.

Jia, Y., McLeod, R., Wang, X., Parra, L., Egan, R., & Hey, J. (2002). Anandamide induces cough in conscious guinea-pigs through VR1 receptors. *British Journal of Pharmacology*, *137*(6), 831-6.

Joos, G., Germonpré, P., & Pauwels, R. (2000). Role of tachykinins in asthma. *Allergy*, *55*(4), 321-37.

Jordt, S., Tominaga, M., & Julius, D. (2000). Acid potentiation of the capsaicin receptor determined by a key extracellular site. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(14), 8134-9.

Jordt, S., Bautista, D., Chuang, H., McKerny, D., Zygmunt, P., Hogestatt, E., et al. (2004). Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Letters to Nature, 427*(15), 260-5.

Kagaya, M., Lamb, J., Robbins, J., Page, C., & Spina, D. (2002). Characterization of the anandamide induced depolarization of guinea-pig isolated vagus nerve. *British Journal of Pharmacology*, *137*(1), 39-48.

Karashima, Y., Talavera, K., Everaerts, W., Janssens, A., Kwan, K., Vennekens, R., et al. (2009). TRPA1 acts as a cold sensor in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(4), 1273-8.

Karlsson, J., & Fuller, R. (1999). Pharmacological regulation of the cough reflex - from experimental models to antitussive effects in man. *Pulmonary Pharmacology & Therapeutics*, *12*(4), 215-28.

Katsumata, U., Kiyohisa, S., Ukiie, Y., Sasaki, H., & Takishima, T. (1991). Bradykinin-induced cough reflex markedly increases in patients with cough associated with captopril and enalapril. *Tohoku Journal of Experimental Medicine, 164*, 103-109.

Kaufman, M., Coleridge, H., Coleridge, J., & Baker, D. (1980). Bradykinin stimulates afferent vagal C-fibers in intrapulmonary airways of dogs. *Journal of Applied Physiology: Respiratory, Environmental and Exercise Physiology, 48*(3), 511-7.

Kawakami, Y., Uchiyama, K., Irie, T., & Murao, M. (1973). Evaluation of aerosols of prostaglandins E_1 and E_2 as bronchodilators. *European Journal of Clinical Pharmacology*, *6*(2), 127-32.

Kay, L., Yeo, W., & Peachell, P. (2006). Prostaglandin E_2 activates EP_2 receptors to inhibit human lung mast cell degranulation. *British Journal of Pharmacology*, *147*(7), 707-13.

Kollarik, M., & Undem, B. (2002). Mechanisms of acid-induced activation of airway afferent nerve fibres in guinea-pig. *The Journal of Physiology*, *543*(2), 591-600.

Kollarik, M., & Undem, B. (2004). Activation of bronchopulmonary vagal afferent nerves with bradykinin, acid and vanilloid receptor agonists in wild-type and TRPV1$^{-/-}$ mice. *The Journal of Physiology*, *555*(Pt 1), 115-23.

Kollarik, M., Ru, F., & Undem, B. (2007). Acid-sensitive vagal sensory pathways and cough. *Pulmonary Pharmacology & Therapeutics*, *20*(4), 402-11.

Kostikas, K., Papatheodorou, G., Ganas, K., Psathakis, K., Panagou, P., & Loukides, S. (2002). pH in expired breath condensate of patients with inflammatory airway diseases. *American Journal of Respiratory and Critical Care Medicine*, *165*(10), 1364-1370.

Kuzhikandathil, E., Wang, H., Szabo, T., Morozova, N., Blumberg, P., & Oxford, G. (2001). Functional analysis of capsaicin receptor (vanilloid receptor subtype 1) multimerization and agonist responsiveness using a dominant negative mutation. *The Journal of Neuroscience*, *21*(22), 8697-706.

Kwan, K., Allchorne, A., Vollrath, M., Christensen, A., Zhang, D., Woolf, C., & Corey, D. (2006). TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron*, *50*(2), 277-89.

Kwong, K., & Lee, L. (2002). PGE_2 sensitizes cultured pulmonary vagal sensory neurons to chemical and electrical stimuli. *Journal of Applied Physiology*, *93*(4), 1419-28.

Kwong, K., Carr, M., Gibbard, A., Savage, T., Singh, K., Jing, J., et al. (2008). Voltage-gated sodium channels in nociceptive versus non-nociceptive nodose vagal sensory neurons innervating guinea pig lungs. *The Journal of Physiology*, *586*(5), 1321-36.

Lalloo, U., Fox, A., Belvisi, M., Chung, K., & Barnes, P. (1995). Capsazepine inhibits cough induced by capsaicin and citric acid but not by hypertonic saline in guinea pigs. *Journal of Applied Physiology*, *79*(4), 1082-7.

Laude, E., Higgins, K., & Morice, A. (1993). A comparative study of the effects of citric acid, capsaicin and resiniferatoxin on the cough challenge in guinea-pig and man. *Pulmonary Pharmacology*, *6*, 171-175.

Lee, L., Kwong, K., Lin, Y., & Gu, Q. (2002). Hypersensitivity of bronchopulmonary C-fibers induced by airway mucosal inflammation: cellular mechanisms. *Pulmonary Pharmacology & Therapeutics*, *15*(3), 199-204.

Lee, M., & Undem, B. (2008). Basic mechanisms of cough: current understanding and remaining questions. *Lung*, *186 Suppl*, S10-6.

Lee, M., Undem, B., Brown, C., & Carr, M. (2006). Effect of nociceptin in acid-evoked cough and airway sensory nerve activation in guinea pigs. *American Journal of Respiratory and Critical Care Medicine*, *173*(3), 271-5.

Lehto, S., Tamir, R., Deng, H., Klionsky, L., Kuang, R., Le, A., et al. (2008). Antihyperalgesic effects of (R,E)-N-(2-Hydroxy-2,3-dihydro-1H-inden-4-yl)-3-(2-(piperidin-1-yl)-4-(trifluoromethyl)phenyl)-acrylamide (AMG8562), a novel transient receptor potential vanilloid type 1 modulator that does not cause hyperthermia in rats. *The Journal of Pharmacology and Experimental Therapeutics*, *326*(1), 218-229.

Lewis, C., Ambrose, C., Banner, K., Battram, C., Butler, K., Giddings, J., et al. (2007). Animal models of cough: literature review and presentation of a novel cigarette smoke-enhanced cough model in the guinea-pig. *Pulmonary Pharmacology & Therapeutics*, *20*(4), 325-33.

Lowry, R., Higenbottam, T., Johnson, T., & Godden, D. (1987). Inhibition of artificially induced cough in man by bronchodilators. *British Journal of Clinical Pharmacology*, *24*(4), 503-10.

Macpherson, L., Dubin, A., Evans, M., Marr, F., Schultz, P., Cravatt, B., & Patapoutian, A. (2007). Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. *Nature, 445*, 541-5.

Maher, S., Birrell, M., & Belvisi, M. (2009). Prostaglandin E_2 mediates cough via the EP_3 receptor: implications for future disease therapy. *American Journal of Respiratory and Critical Care Medicine, 180*(10), 923-8.

Martin, J., Suzuki, M., Maghni, K., Pantano, R., Ramos-Barbón, D., Ihaku, D., et al. (2002). The immunomodulatory actions of prostaglandin E_2 on allergic airway responses in the rat. *Journal of Immunology, 169*(7), 3963-9.

Materazzi, S., Nassini, R., Andrè, E., Campi, B., Amadesi, S., Trevisani, M., et al. (2008). Cox-dependent fatty acid metabolites cause pain through activation of the irritant receptor TRPA1. *Proceedings of the National Academy of Sciences of the United States of America, 105*(33), 12045-50.

Mazzone, S. (2004). Sensory regulation of the cough reflex. *Pulmonary Pharmacology & Therapeutics, 17*(6), 361-8.

Mazzone, S., Mori, N, & Canning, B. (2005). Synergistic interactions between airway afferent nerve subtypes regulating the cough reflex in guinea-pigs. *The Journal of Physiology, 569*(Pt 2), 559-73.

McCormick, A., Fleming, D., & Charlton, J. (1995). Morbidity Statistics from General Practice: Fourth national study 1991-1992. *Office of Population Censuses and Surveys, Series MB5*(3).

McCoy, J., Wicks, J., & Audoly, L. (2002). The role of prostaglandin E_2 receptors in the pathogenesis of rheumatoid arthritis. *Journal of Clinical Investigation, 110*(5), 651-8.

McLeod, R., Bolser, D., Jia, Y., Parra, L., Mutter, J., Wang, X., et al. (2002). Antitussive effect of nociceptin/orphanin FQ in experimental cough models. *Pulmonary Pharmacology & Therapeutics, 15*(3), 213-6.

McLeod, R., Fernandez, X., Correll, C., Phelps, T., Jia, Y., Wang, X., & Hey, J. (2006). TRPV1 antagonists attenuate antigen-provoked cough in ovalbumin sensitized guinea pigs. *Cough*, *2*(10). doi:10.1186/1745-9974-2-10

McLeod, R., Tulshian, D., Bolser, D., Varty, G., Baptista, M., Fernandez, X., et al. (2010). Pharmacological profile of the NOP agonist and cough suppressing agent SCH 486757 (8-[Bis(2-Chlorophenyl)Methyl]-3-(2-Pyrimidinyl)-8-Azabicyclo[3.2.1]Octan-3-Ol) in preclinical models. *European Journal of Pharmacology*, *630*, 112-20.

McNally, E. (1987). Cough due to captopril. *The Western Journal of Medicine*, *146*(2), 226-228.

McNamara, C., Mandel-Brehm, J., Bautista, D., Siemens, J., Deranian, K., Zhao, M., et al. (2007). TRPA1 mediates formalin-induced pain. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(33), 13525-30.

Melillo, E., Woolley, K., Manning, P., Watson, R., & O'Byrner, P. (1994). Effect of inhaled PGE_2 on exercise-induced bronchoconstriction in asthmatic subjects. *American Journal of Respiratory and Critical Care Medicine*, *149*(5), 1138-41.

Mezey, E., Tóth, Z., Cortright, D., Arzubi, M., Krause, J., Elde, R., et al. (2000). Distribution of mRNA for vanilloid receptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(7), 3655-60.

Montell, C., & Rubin, G. (1989). Molecular characterization of the Drosophila trp locus: a putative integral membrane protein required for phototransduction. *Neuron*, *2*(4), 1313-23.

Montuschi, P., Kharitonov, S., Ciabattoni, G., & Barnes, P. (2003). Exhaled leukotrienes and prostaglandins in COPD. *Thorax*, *58*(7), 585-8.

Morice, A., Fontana, G., Belvisi, M., Birring, S., Chung, K., Dicpinigaitis, P., et al. (2007). ERS guidelines on the assessment of cough. *The European Respiratory Journal*, *29*(6), 1256-76.

Morice, A., Kastelik, J., & Thompson, R. (2001). Cough challenge in the assessment of cough reflex. *British Journal of Clinical Pharmacology*, *52*(4), 365-75.

Moriyama, T., Higashi, T., Togashi, K., Iida, T., Segi, E., Sugimoto, Y., et al. (2005). Sensitization of TRPV1 by EP_1 and IP reveals peripheral nociceptive mechanism of prostaglandins. *Molecular Pain*, *1*(3). doi:10.1186/1744-8069-1-3

Mulrennan, S., Wright, C., Thompson, R., Goustas, P., & Morice, A. (2004). Effect of salbutamol on smoking related cough. *Pulmonary Pharmacology & Therapeutics*, *17*(3), 127-31.

Munro, S., Thomas, K., & Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature*, *365*, 61-5.

Myers, A., Kajekar, R., & Undem, B. (2002). Allergic inflammation-induced neuropeptide production in rapidly adapting afferent nerves in guinea pig airways. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *282*(4), L775-81.

Nagata, K., Duggan, A., Kumar, G., & García-Añoveros, J. (2005). Nociceptor and hair cell transducer properties of TRPA1, a channel for pain and hearing. *The Journal of Neuroscience*, *25*(16), 4052-61.

Nakajima, T., Nishimura, Y., Nishiuma, T., Kotani, Y., Nakata, H., & Yokoyama, M. (2006). Cough sensitivity in pure cough variant asthma elicited using continuous capsaicin inhalation. *Allergology International*, *55*(2), 149-55.

Nasra, J., & Belvisi, M. (2009). Modulation of sensory nerve function and the cough reflex: understanding disease pathogenesis. *Pharmacology & Therapeutics*, *124*(3), 354-75.

Nassenstein, C., Kwong, K., Taylor-Clark, T., Kollarik, M., Macglashan, D., Braun, A., & Undem, B. (2008). Expression and function of the ion channel TRPA1 in vagal afferent nerves innervating mouse lungs. *The Journal of Physiology*, *586*(6), 1595-604.

Nassini, R., Materazzi, S., De Siena, G., De Cesaris, F., & Geppetti, P. (2010). Transient receptor potential channels as novel drug targets in respiratory diseases. *Current Opinion in Investigational Drugs*, *11*(5), 535-42.

Nemoto, T., Aoki, H., Ike, A., Yamada, K., Kondo, T., Kobayashi, S., & Inagawa, T. (1976). Serum prostaglandin levels in asthmatic patients. *The Journal of Allergy and Clinical Immunology*, *57*(2), 89-94.

Newton, R., Eddleston, J., Haddad, E., Hawisa, S., Mak, J., Lim, S., et al. (2002). Regulation of kinin receptors in airway epithelial cells by inflammatory cytokines and dexamethasone. *European Journal of Pharmacology*, *441*(3), 193-202.

Niimi, A., & Chung, K. (2004). Airway inflammation and remodelling changes in patients with chronic cough: do they tell us about the cause of cough? *Pulmonary Pharmacology & Therapeutics*, *17*(6), 441-6.

Nilius, B. (2007). TRP channels in disease. *Biochimica et Biophysica Acta*, *1772*(8), 805-12. doi:10.1016/j.bbadis.2007.02.002

Obata, K., Katsura, H., Mizushima, T., Yamanaka, H., Kobayashi, K., Dai, Y., et al. (2005). TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. *The Journal of Clinical Investigation*, *115*(9), 2393-2401.

O'Connell, F., Thomas, V., Studham, J., Pride, N., & Fuller, R. (1996). Capsaicin cough sensitivity increases during upper respiratory infection. *Respiratory Medicine*, *90*(5), 279-86.

Paredes, R. M., Etzler, J., Watts, L., & Lechleiter, J. (2009). Chemical calcium indicators. *Methods*, *46*(3), 143-151.

Patapoutian, A., Tate, S., & Woolf, C. (2009). Transient receptor potential channels: targeting pain at the source. *Nature Reviews. Drug Discovery*, *8*(1), 55-68.

Patel, H., Barnes, P., Takahashi, T., Tadjkarimi, S., Yacoub, M., & Belvisi, M. (1995). Evidence for prejunctional muscarinic autoreceptors in human and guinea pig trachea. *American Journal of Respiratory and Critical Care Medicine*, *152*(3), 872-8.

Patel, H., Birrell, M., Crispino, N., Hele, D., Venkatesan, P., Barnes, P., et al. (2003). Inhibition of guinea-pig and human sensory nerve activity and the cough reflex in

guinea-pigs by cannabinoid (CB_2) receptor activation. *British Journal of Pharmacology*, *140*(2), 261-8.

Pavord, I., Wong, C., Williams, J., & Tattersfield, A. (1993). Effect of inhaled prostaglandin E_2 on allergen-induced asthma. *American Review of Respiratory Disease*, *148*(1), 87-90.

Pechlivanova, D., & Georgiev, V. (2005). Effects of single and long-term theophylline treatment on the threshold of mechanical nociception: Contribution of adenosine A1 and α_2-adrenoceptors. *Methods and Findings in Experimental and Clinical Pharmacology*, *27*(9), 659.

Pecova, R., Zucha, J., Pec, M., Neuschlova, M., Hanzel, P., & Tatar, M. (2008). Cough reflex sensitivity testing in seasonal allergic rhinitis patients and healthy volunteers. *The Journal of Physiology and Pharmacology*, *59*(Suppl 6), 557-564.

Petrus, M., Peier, A., Bandell, M., Hwang, S., Huynh, T., Olney, N., et al. (2007). A role of TRPA1 in mechanical hyperalgesia is revealed by pharmacological inhibition. *Molecular Pain*, *3*(40).

Plevkova, J., Varechova, S., Brozmanov, M., & Tatar, M. (2006). Testing of cough reflex sensitivity in children suffering from allergic rhinitis and common cold. *Journal of Physiology and Pharmacology*, *57*(Supp 4), 289-296.

Poonyachoti, S., Kulkarni-Narla, A., & Brown, D. (2002). Chemical coding of neurons expressing delta- and kappa-opioid receptor and type I vanilloid receptor immunoreactivities in the porcine ileum. *Cell and Tissue Research*, *307*(1), 23-33.

Pounsford, J., Birch, M., & Saunders, K. (1985). Effect of bronchodilators on the cough response to inhaled citric acid in normal and asthmatic subjects. *Thorax*, *40*(9), 662-7.

Profita, M., Sala, A., Bonanno, A., Riccobono, L., Siena, L., Melis, M., et al. (2003). Increased prostaglandin E_2 concentrations and cyclooxygenase-2 expression in asthmatic subjects with sputum eosinophilia. *Journal of Allergy and Clinical Immunology*, *112*(4), 709-716.

Rao, S., Mudipalli, R., Remes-Troche, J., Utech, C., & Zimmerman, B. (2007). Theophylline improves esophageal chest pain - a randomized, placebo-controlled study. *The American Journal of Gastroenterology*, *102*(5), 930-8.

Rech, J., Eckert, W., Maher, M., Banke, T., Bhattacharya, A., & Wickenden, A. (2010). Recent advances in the biology and medicinal chemistry of TRPA1. *Future Medicinal Chemistry*, *2*(5), 843-58.

Regoli, D., Nsa Allogho, S., Rizzi, A., & Gobeil, F. (1998). Bradykinin receptors and their antagonists. *European Journal of Pharmacology*, *348*(1), 1-10.

Reynolds, S., Mackenzie, A., Spina, D., & Page, C. (2004). The pharmacology of cough. *Trends in Pharmacological Sciences*, *25*(11), 569-76.

Rodgers, H., Pang, L., Holland, E., Corbett, L., Range, S., & Knox, A. (2002). Bradykinin increases IL-8 generation in airway epithelial cells via COX-2-derived prostanoids. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *283*(3), L612-8.

Sands, R., Shanmugavadivel, D., Stephenson, T., & Wood, D. (2011). Medical problems presenting to paediatric emergency departments: 10 years on. *Emergency Medicine Journal*.

Scherrer, D., Daeffler, L., Trifilieff, A., & Gies, J. (1995). Effects of WIN 64338, a nonpeptide bradykinin B_2 receptor antagonist, on guinea-pig trachea. *British Journal of Pharmacology*, *115*(7), 1127-8.

Schroeder, K., & Fahey, T. (2002). Systematic review of randomised controlled trials of over the counter cough medicines for acute cough in adults. *British Medical Journal*, *324*, 1-6.

Shin, J., Cho, H., Hwang, S., Jung, J., Shin, C., Lee, S., et al. (2002). Bradykinin-12-lipoxygenase-VR1 signaling pathway for inflammatory hyperalgesia. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(15), 10150-5.

Simon, S., & Liedtke, W. (2008). How irritating: The role of TRPA1 in sensing cigarette smoke and aerogenic oxidants in the airways. *The Journal of Clinical Investigation*, *118*(7), 2383-2386.

Singh, S., & Deshpande, S. (2009). Nociceptive vascular reflexes evoked by scorpion venom modulate cardiorespiratory parameters involving vanilloid receptor 1 in anaesthetised rats. *Neuroscience Letters*, *451*(3), 194-8.

Smith, C., Adamson, D., Choudry, N., & Fuller, R. (1991). The effect of altering airway tone on the sensitivity of the cough reflex in normal volunteers. *European Respiratory Journal*, *4*(9), 1078-9.

Story, G., Peier, A., Reeve, A., Eid, S., Mosbacher, J., Hricik, T., et al. (2003). ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell*, *112*(6), 819-29.

Takahashi, T., Belvisi, M., Patel, H., Ward, J., Tadjkarimi, S., Yacoub, M., & Barnes, P. (1994). Effect of Ba 679 BR, a novel long-acting anticholinergic agent, on cholinergic neurotransmission in guinea pig and human airways. *American Journal of Respiratory and Critical Care Medicine*, *150*(6), 1640-5.

Tatar, M., Sant'Ambrogio, G., & Sant'Ambrogio, F. (1994). Laryngeal and tracheobronchial cough in anesthetized dogs. *Journal of Applied Physiology*, *76*(6), 2672-9.

Taylor-Clark, T., McAlexander, M., Nassenstein, C., Sheardown, S., Wilson, S., Thornton, J., et al. (2008a). Relative contributions of TRPA1 and TRPV1 channels in the activation of vagal bronchopulmonary C-fibres by the endogenous autacoid 4-oxononenal. *The Journal of Physiology*, *586*(14), 3447-59.

Taylor-Clark, T., Nassenstein, C., McAlexander, M., & Undem, B. (2009). TRPA1: a potential target for anti-tussive therapy. *Pulmonary Pharmacology & Therapeutics*, *22*(2), 71-4.

Taylor-Clark, T., Undem, B., MacGlashan, D., Ghatta, S., Carr, M., & McAlexander, M. (2008b). Prostaglandin-induced activation of nociceptive neurons via direct interaction

with transient receptor potential A1 (TRPA1). *Molecular Pharmacology*, *73*(2), 274-281.

Taylor-Clark, T., & Undem, B. (2006). Transduction mechanisms in airway sensory nerves. *Journal of Applied Physiology*, *101*(3), 950-9.

Tominaga, M., & Tominaga, T. (2005). Structure and function of TRPV1. *Pflügers Archiv: European Journal of Physiology*, *451*(1), 143-50.

Tominaga, M., Caterina, M., Malmberg, A., Rosen, T., Gilbert, H., Skinner, K., et al (1998). The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron*, *21*(3), 531-43.

Tominaga, M., Wada, M., & Masu, M. (2001). Potentiation of capsaicin receptor activity by metabotropic ATP receptors as a possible mechanism for ATP-evoked pain and hyperalgesia. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(12), 6951-6.

Trevisani, M., Milan, A., Gatti, R., Zanasi, A., Harrison, S., Fontana, G., et al. (2004). Antitussive activity of iodo-resiniferatoxin in guinea pigs. *Thorax*, *59*(9), 769-72.

Trevisani, M., Siemens, J., Materazzi, S., Bautista, D., Nassini, R., Campi, B., et al. (2007). 4-Hydroxynonenal, an endogenous aldehyde, causes pain and neurogenic inflammation through activation of the irritant receptor TRPA1. *Proceedings of the National Academy of Sciences*, *104*(33), 13519-13524.

US Food and Drug Administration. (2008). FDA Advisory: FDA recommends that over-the-counter (OTC) cough and cold products not be used for infants and children under 2 years of age. Retrieved from www.hhs.gov.uk.

Undem, B., Chuaychoo, B., Lee, M., Weinreich, D., Myers, A., & Kollarik, M. (2004). Subtypes of vagal afferent C-fibres in guinea-pig lungs. *The Journal of Physiology*, *556*(Pt 3), 905-17.

Usmani, O., Belvisi, M., Patel, H., Crispino, N., Birrell, M., Korbonits, M., et al. (2005). Theobromine inhibits sensory nerve activation and cough. *The FASEB Journal*, *19*(2), 231-3.

Valenzano, K., Grant, E., Wu, G., Hachicha, M., Schmid, L., Tafesse, L., et al. (2003). N-(4-Tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carbox-amide (BTCT), an novel orally effective vanilloid receptor 1 antagonist with analgesic properties: I . in vitro characterization and pharmacokinetic properties. *The Journal of Pharmacology and Experimental Therapeutics*, *306*, 377-386.

Vandenplas, O., Malo, J., Saetta, M., Mapp, C., & Fabbri, L. (1993). Occupational asthma and extrinsic alveolitis due to isocyanates: Current status and perspectives. *British Journal of Industrial Medicine*, *50*, 213-228.

Vassilev, Z., Chu, A.., Ruck, B., Adams, E., & Marcus, S. (2009). Adverse reactions to over-the-counter cough and cold products among children: the cases managed out of hospitals. *Journal of Clinical Pharmacy and Therapeutics*, *34*(3), 313-8.

Vianna, R., & Calixto, J. (1998). Characterization of the receptor and the mechanisms underlying the inflammatory response induced by des-Arg9-BK in mouse pleurisy. *British Journal of Pharmacology*, *123*(2), 281-91.

Walker, K., Urban, L., Medhurst, S., Patel, S., Panesar, M., Fox, A., & McIntyre. (2003). The VR1 antagonist capsazepine reverses mechanical hyperalgesia in models of inflammatory and neuropathic pain. *The Journal of Pharmacology and Experimental Therapeutics*, *304*(1), 56-62.

Walters, E., Bevan, C., Parrish, R., Davies, B., & Smith, A. (1982). Time-dependent effect of prostaglandin E_2 inhalation on airway responses to bronchoconstrictor agents in normal subjects. *Thorax*, *37*(6), 438-42.

Wang, D. (2005). The vanilloid receptor and hypertension. *Acta pharmacologica Sinica*, *26*(3), 286-94.

Wang, D., & Li, J. (1999). Antihypertensive mechanisms underlying a novel salt-sensitive hypertensive model induced by sensory denervation. *Hypertension*, *33*(1 Pt 2), 499-503.

Wang, S., Dai, Y., Fukuoka, T., Yamanaka, H., Kobayashi, K., Obata, K., et al. (2008). Phospholipase C and protein kinase A mediate bradykinin sensitization of TRPA1: A molecular mechanism of inflammatory pain. *Brain*, *131*(Pt 5), 1241-51.

Wang, Y., Chang, R., Allgood, S., Silver, W., & Liman, E. (2011). A TRPA1-dependent mechanism for the pungent sensation of weak acids. *Journal of General Physiology*, *137*(6), 493-505.

Ward, J., Barnes, P., Tadjkarimi, S., Yacoub, M., & Belvisi, M. (1995). Evidence for the involvement of cGMP in neural bronchodilator responses in human trachea. *The Journal of Physiology*, *483*(2), 525-36.

Ward, S., Bayguinov, J., Won, K., Grundy, D., & Berthoud, H. (2003). Distribution of the vanilloid receptor (VR1) in the gastrointestinal tract. *The Journal of Comparative Neurology*, *465*(1), 121-35.

Widdicombe, J. (1995). Neurophysiology of the cough reflex. *European Respiratory Journal*, *8*(7), 1193-1202.

Widdicombe, J. (1998). Afferent receptors in the airways and cough. *Respiration Physiology*, *114*(1), 5-15.

Widdicombe, J. (2002). Neuroregulation of cough: Implications for drug therapy. *Current Opinion in Pharmacology*, *2*(3), 256-63.

Wilgus, T., Parrett, M., Ross, M., Tober, K., Robertson, F., & Oberyszyn, T. (2002). Inhibition of ultraviolet light B-induced cutaneous inflammation by a specific cyclooxygenase-2 inhibitor. *Advances in Experimental Medicine and Biology*, *507*, 85-92.

World Health Organisation. (2007). *Global surveillance, prevention and control of chronic respiratory disease: A comprehensive approach*. *Environmental Research* (Vol. 4). Geneva: WHO Press. doi:10.1016/0013-9351(71)90024-7

World Health Organisation. (2011a). Asthma. *Factsheet No. 307*. Retrieved from http://www.who.int/mediacentre/factsheets/fs307/en/index.html

World Health Organisation. (2011b). Chronic Obstructive Pulmonary Disease. *Fact Sheet No. 315*. Retrieved from http://www.who.int/mediacentre/factsheets/fs315/en/index.html

Zhang, W., Bhola, N., Kalyankrishna, S., Gooding, W., Hunt, J., Seethala, R., et al. (2008). Kinin B2 receptor mediates induction of cyclooxygenase-2 and is overexpressed in head and neck squamous cell carcinomas. *Molecular Cancer Research*, *6*(12), 1946-56.

Zurborg, S., Yurgionas, B., Jira, J., Caspani, O., & Heppenstall, P. (2007). Direct activation of the ion channel TRPA1 by Ca^{2+}. *Nature Neuroscience*, *10*(3), 277-9.

Zygmunt, P., Petersson, J., Andersson, D., Chuang, H., Sørgård, M., Di Marzo, V., et al. (1999). Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature*, *400*, 452-7.