An investigation into the efficacy and mechanisms of action of novel therapeutics for chronic cough

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Abstract

Whilst cough is a defensive reflex, in respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) the cough reflex may become exaggerated and unproductive, leading to presentation of chronic cough as a troublesome symptom. Chronic cough is the most common reason that patients seek ambulatory care in the UK, and cough medications are one of the largest segments of the global over-the-counter drugs market, yet there are no cough medications (prescription or OTC) available that are both efficacious AND safe, indicating a large unmet clinical need.

β-adrenergic receptor (β-AR) agonists, methylxanthines (theophylline) and fatty acid amide hydrogenase inhibitors (FAAHi) are peripherally acting drugs with a proven safety profile in humans for other uses, but are not currently recognised as anti-tussive medications. This thesis aimed to examine whether these drugs possess anti-tussive activity, and if so, the mechanisms by which they inhibit cough.

These drugs were all effective at blocking the *in vivo* cough response to tussive stimuli in naïve guinea pigs, and in addition β-AR agonists and theophylline inhibited tussive stimulus evoked cough in guinea pigs previously exposed to cigarette-smoke, which display a clinically relevant enhanced cough phenotype. *In vitro* and *in vivo* assays of guinea pig vagus nerve/neuron activation were used to show that these compounds inhibit depolarisation of airway-innervating peripheral sensory nerves. Pharmacological and genetic tools were used to investigate the receptors and signalling pathways activated by these drugs. Whilst β-AR agonists, theophylline and FAAHi had different mechanisms of action, a common component was that all three classes of compound act on various potassium channels, thereby reducing the excitability of sensory neurons.

This work suggests that drugs that inhibit peripheral sensory nerve activity have the potential to be efficacious as anti-tussives. Specifically the compounds tested have improved clinical side-effect profiles over currently used anti-tussives, and therefore have potential as therapies for chronic cough.

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"You see, I consider that a man's brain originally is like a little empty attic, and you have to stock it with such furniture as you choose. A fool takes in all the lumber of every sort that he comes across, so that the knowledge which might be useful to him gets crowded out, or at best is jumbled up with a lot of other things so that he has a difficulty in laying his hands upon it. Now the skillful workman is very careful indeed as to what he takes into his brain-attic. He will have nothing but the tools which may help him in doing his work, but of these he has a large assortment, and all in most perfect order. It is a mistake to think that that little room has elastic walls and can distend to any extent. Depend upon it there comes a time when for every addition of knowledge you forget something that you knew before. It is of the highest importance, therefore, not to have useless facts elbowing out the useful ones."

"No data yet. It is a capital mistake to theorise before you have all the evidence. It biases the judgment."

- Sherlock Holmes

From 'A Study in Scarlet', by Sir Arthur Conan Doyle

Declaration of originality

The work contained in this thesis is my own work, except where otherwise indicated and referenced appropriately.

Patch clamp experiments to determine the effect of theophylline were performed by Dr Eric Dubuis. Single fibre experiments (chapters 3, 4, 5) were performed by Dr John Adcock, with my assistance. The experiments to determine the effect of PF04862853 on CA cough and FAA plasma levels (chapter 5) were performed by researchers at Pfizer, UK.

Copyright Declaration

Table of Contents

Table of Contents

List of Figures

List of Tables

List of Abbreviations

$[Ca^{2+}]_i$	Intracellular calcium concentration
ACCP	American College of Chest Physicians
AEA	Anadamide or Arachidonoylethanoamide
AP	Action potential
ASM	Airway smooth muscle
ATP	Adenosine Trisphosphate
AUC	Area under the curve
AUC	Area under the curve
β-AR	β-adrenergic receptor
B_2	Bradykinin receptor type 2
BAL	Bronchoalveolar lavage
BK	Bradykinin
BK_{Ca}	Large-conductance calcium activated potassium channel
C_2	Concentration required to evoke 2 coughs
C_5	Concentration required to evoke 5 coughs
CA	Citric acid
cAMP	cyclic Adenosine Monophosphate
CB	Cannabinoid receptor
CB_1	Cannabinoid receptor type 1
CB_2	Cannabinoid receptor type 2
CCD	Charge-coupled device (digital camera image sensor)
CCIQ	Chronic cough impact questionnaire
cGMP	cyclic Guanosine monophosphate
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CQLQ	Cough-specific quality of life questionnaire
CS	Cigarette smoke
DiI	$DiIC_{18}$ (3 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbo-cyanine perchlorate)
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DRG	Dorsal root ganglia/ganglion
ECS	Extracellular solution
ECS	Extracellular solution
EDTA	Ethylenediaminetetraacetic acid
EP_1	Prostanoid receptor sub-type E1
EP_2	Prostanoid receptor sub-type E1
EP_3	Prostanoid receptor sub-type E1
EP_4	Prostanoid receptor sub-type E1
FAA	Fatty acid amide
FAAH	Fatty acid amide hydrolase
FAAHi	Fatty acid amide hydrolase inhibitor
FBS	Foetal bovine serum
FEV_1	Forced Expiratory Volume in 1s
GABA-B	Gamma-aminobutyric acid receptor type B
GERD	Gastrooesophageal reflux disease
GOLD	Global initiative for chronic Obstructive Lung Disease
GPCR	G-protein coupled receptor
$G_{\alpha i}$	Guanosine nucleotide-binding protein class alpha sub-type i
$G_{\alpha S}$	Guanosine nucleotide-binding protein class alpha sub-type s
HBSS	Hanks balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
IC_{50}	Half maximal inhibitory concentration
ICS	Intracellular solution
IIAM	International Institute for the Advancement of Medical science
IK_{Ca}	Intermediate-conductance calcium activated potassium channel
IL-1β	Interleukin type 1β
IL-8	Interleukin type 8
K_{50}	50mM potassium solution
K_{ATP}	ATP-sensitive potassium channel
KH	Krebs- Henseleit solution
KO	Knock out (of a specific gene)
LABA	Long-acting β-adrenergic receptor agonist
LC-MS	Liquid chromatography mass spectrometry
LCQ	Leicester cough questionnaire
LEA	Linoleoylethanolamide
LT	Leukotriene
mRNA	Messenger ribonucleic acid
NK	Neurokinin
NTS	*Nucleus Tractus Solitarii* (nucleus of the solitary tract)
OEA	Oleoylethanolamide
OTC	Over the counter (non-prescription drug)
PAS	Papain-activation solution
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PEA	Palmitoylethanolamide
PG	Prostaglandin
PGE_2	Prostaglandin Type E2
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein Kinase type A
PKG	Protein Kinase type G
PNDS	Post-nasal drip syndrome
PPAR	Peroxisome proliferator-activated receptor
PRG	Pontine respiratory group
q-PCR	quantitative Polymerase Chain Reaction
RAR	Rapidly adapting receptor
ROS	Reactive Oxygen species
SABA	Short-acting β-adrenergic receptor agonist
SAR	Slowly adapting receptors
SEM	Standard error of the mean
SK_{Ca}	Small-conductance calcium activated potassium channel
SP	Substance P
TBE	Buffer solution containing Tris base, borate and EDTA
TNF-α	Tumour necrosis factor type α
TRPA1	Transient receptor potential channel type Ankyrin 1
TRPM8	Transient receptor potential channel type Melastatin 1
TRPV1	Transient receptor potential channel type Vanniloid 1
TSP	Tobacco smoke particulate
VGPC	Voltage-gated potassium channel
VGSC	Voltage-gated sodium channel
WPI	World Precision Instruments (company)

1 Introduction

1.1 The physiology of the cough reflex

Cough is a phenomenon that all humans will be familiar with, through personal experience during their lifetime. As with most everyday and unconscious bodily functions that are normally taken for granted, a cough is actually a complex and precisely coordinated manoeuvre. The physical portion of cough begins with a sudden contraction of multiple thoracic muscles, causing compression of the lungs (Leith *et al.*, 2011). During the initial compression of the lungs, the glottis remains closed, trapping air within the airways. The volume of the airways therefore decreases, whilst the amount of air (gas) within that volume remains the same, resulting in an increase in intra-luminal air pressure (Ross *et al.*, 1955). Subsequent opening of the glottis allows release of this pressure, which occurs as a rapid and explosive expulsion of (volume) air from the airways at speeds of up to 30mph (Ross *et al.*, 1955). The expulsive release of this pressurised air at high speeds causes the characteristic sound that humans can readily discern as a cough through lifelong experience (Morice *et al.*, 2007b; Smith, 2010).

This complicated manoeuvre has evolved as a defense in order to forcefully expel, via the explosive release of air, materials that may occlude the airways and impair breathing or cause complete asphyxiation (Leith *et al.*, 2011). Such 'materials' can include excess endogenous matter (e.g. sputum) as well as foreign bodies or irritants (e.g. inhaled foodstuffs or noxious gases). Whilst the physical portion of cough begins with muscle contraction, defensive (or 'involuntary') cough really begins with detection of an appropriate stimulus in the airways. Thus defensive cough is best described as a reflex arc, possessing three components; sensing of a 'cough stimulus' by airway afferent nerves (peripheral sensation), processing of afferent input by central nervous system (CNS processing), & innervation of thoracic muscles by efferent neurons (motor innervation) (*Fig. 1.1*).

1.1.1 Peripheral Sensation

Anatomy of airway sensory neurons

The defensive cough reflex begins with a tussive stimulus acting on the termini of afferent nerves which protrude into various levels of the airway smooth muscle and epithelium. The majority of the afferent (or sensory) nerves that innervate the airways have cell bodies that are housed either within the jugular (superior vagal) or nodose (inferior vagal) ganglia, which are located slightly caudally to the jugular foramen. Airway sensory nerves
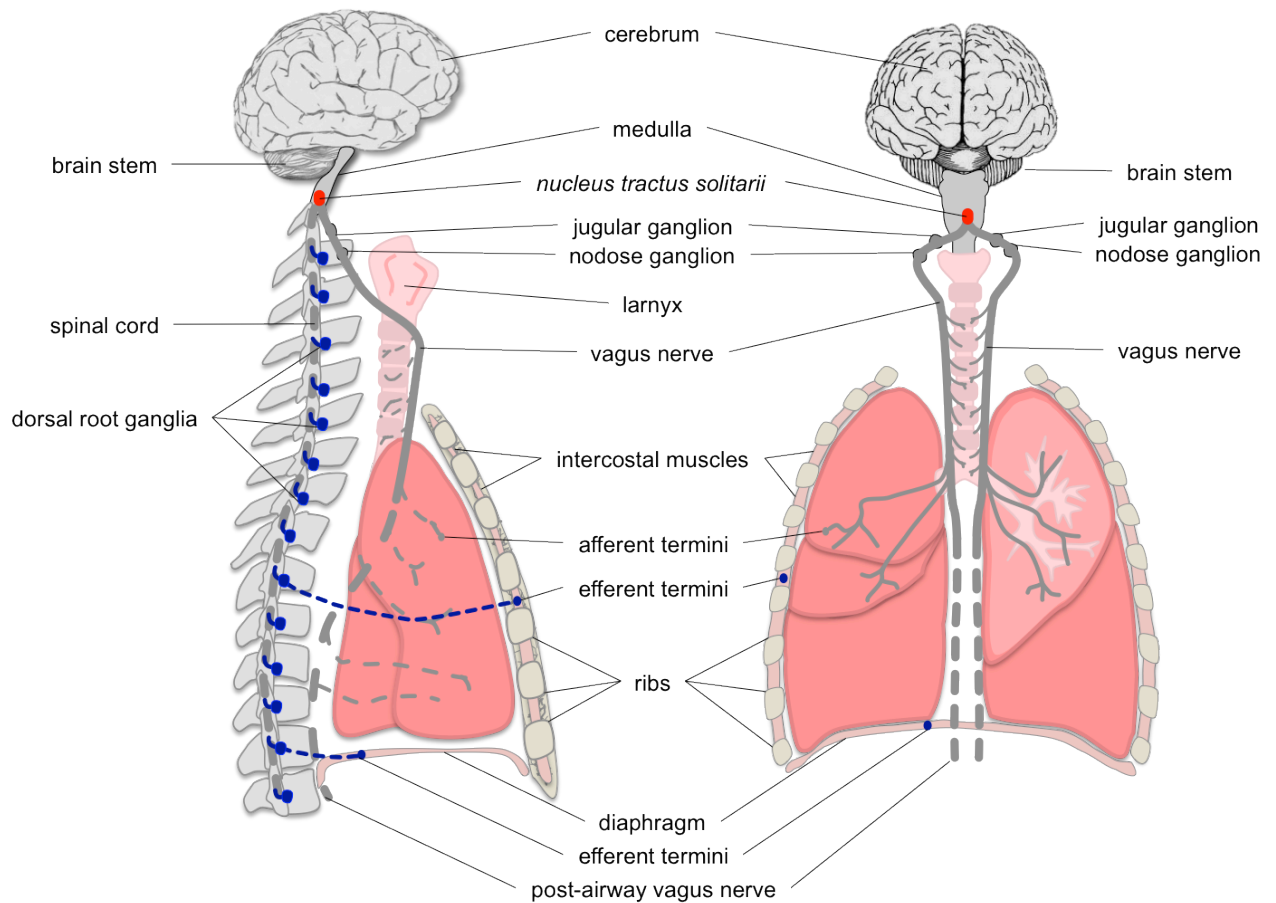
Figure 1.1 Diagram of the cough reflex arc
Illustration of the relative locations of peripheral sensory nerves and soma (dark grey), nucleus tractus solitarii (red) and efferent nerves (blue) to central nervous system, lungs, trachea, diaphragm, and intercostal muscles. N.B. in the interests of clarity only a few afferent and efferent nerve endings are depicted.

are pseudo-unipolar in their morphology; the spheroidal cell body extends a single short process, which is then bifurcated into peripherally and centrally projecting axonal processes (Undem & Weinreich, 2005) (*Fig.1.2*). Once the axonal processes of these nerves leave the ganglia they form part of the vagus nerve. It should be noted that the vagus nerve is a paired nerve – that is to say that an individual possesses two vagus nerves, including two sets of nodose and jugular ganglia, positioned symmetrically along the anterior-posterior axis (*Fig. 1.1*). The vagus nerve is the longest cranial nerve (number 10), containing the axonal processes of motor and sensory nerves innervating many mid-line organs and other tissues, as well as those innervating the airways (Standring, 2008).

central protrusion into NTS

central nerve terminus

axon within vagus nerve

neuron nucleus

cell body (soma)

soma within vagal ganglia

Node of Ranvier
Schwann cell
Schwann cell nucleus

myelinated axons only

peripheral protrusion into airways

peripheral nerve terminus
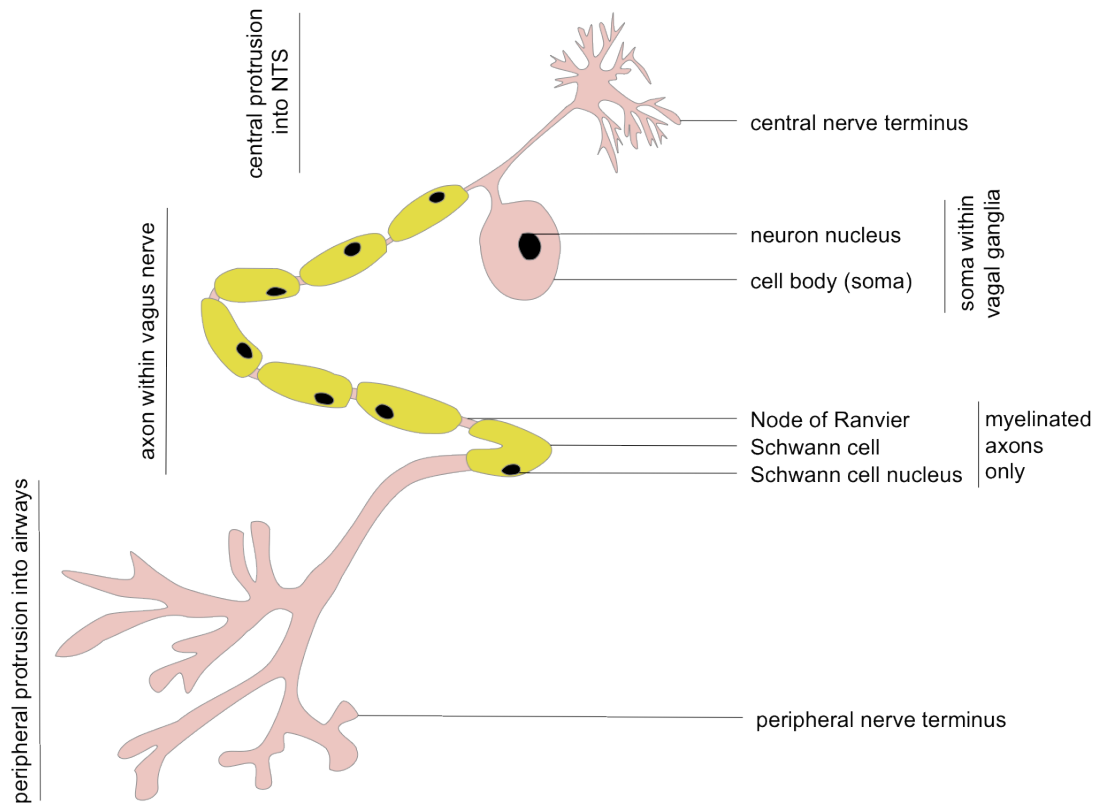
Figure 1.2 Anatomy of airway afferent vagal neuron
Illustration of the anatomy and morphology of an airway-terminating afferent neuron. The portions of the nerve located within the airways, within the vagus nerve, within the vagal (jugular or nodose) ganglia and the nucleus tractus solitarii (NTS) are indicated. N.B. Schwann cells (which collectively form the myelin sheath) and nodes of Ranvier are present only on myelinated neurons.

Caudally to the jugular and nodose ganglia, the vagus nerve runs parallel to the trachea, inside the carotid sheath, along with the carotid artery and jugular vein. Posterior to the trachea, the vagus nerve continues down into the abdomen. Along its length, nerve fibres branch off laterally from the vagus nerve to innervate nearby organs and tissues, including the trachea, bronchi and lungs. Rostral to the jugular and nodose ganglia, the vagus nerve enters the skull though the jugular foramen, and terminates in the solitary tract (*tractus solitarius*). The solitary tract is a compact bundle of axonal processes (including vagal neurons), and is surrounded by the nucleus of the solitary tract (*nucleus tractus solitarii*, or NTS), which itself is an embedded posterolateral portion of the medulla oblongata. The sensory and motor neurons in the solitary tract synapse (interface) with the neurons of the NTS. This synapse is the point at which electrically coded signals conducted by airway-terminating sensory neurons are passed from the peripheral nervous system to the central nervous system.

Transducing tussive stimuli into electrical signals

In the case of airway-terminating afferent neurons, electrical signals are generated by the activation of receptors on the caudal termini protruding into the airway tissues, at the caudally opposite end of the axonal processes to the NTS. Stimuli that activate airway-terminating sensory nerves can be chemical (e.g. inhaled noxious gas) or physical/mechanical (e.g. inhaled foodstuff) in nature, with different stimuli acting on populations of afferent nerve endings. In order to transduce the various types of stimuli into electrical impulses, many different types of specialised receptor are present on the termini of airway sensory nerves. These nerves therefore can be broadly categorised into mechano-sensitive and chemo-sensitive neurons, although there is some overlap between these two categories. As such, airway afferent nerves are classified by not just their responsiveness to different types of stimulus, but also conduction velocity, location of termini within the airway lumen, anatomy, and also developmental origin of neuronal soma (discussed in greater detail in *Introduction 1.2*).

Generator and Action Potentials

Activation of the relevant receptor(s) by an appropriate stimulus leads to a localised membrane depolarisation at the nerve terminus, known as a generator potential. The generator potential is an analogue signal, as the magnitude of depolarisation is positively correlated with the 'strength' of the stimulus – i.e. the greater the number of receptors and potency of receptor activation, the greater the generator potential. If the generator potential reaches a critical threshold, an action potential (AP) is initiated. The magnitude of an AP is fixed, and not related to the 'strength' of the stimulus (i.e. number of receptors activated), and therefore the conversion of the local generator potential to an AP can be considered the conversion of an analogue into a digital signal. The critical threshold for the generation of an AP is the gating voltage for voltage-gated sodium channels (VGSCs) in the plasma membrane (*Fig. 1.3*). When the voltage of the generator potential increases sufficiently, the pore domain of VGSCs opens, enabling positively charged sodium ions (Na^+) to move into the cell along their electrochemical gradient, causing greater depolarisation of the membrane. This further activates proximal VGSCs, which results in a spreading wave of localised depolarisation. The depolarisation spreads along the axonal membrane of the neuron as additional VGSCs open in response to the depolarisation. Whilst the opening of VGSCs allows the influx of sodium ions, opening of the channel causes it to self-inactivate shortly after opening, via a mechanism known as "ball and chain" inactivation, which has three main stages (*as illustrated in Figure 1.3*).
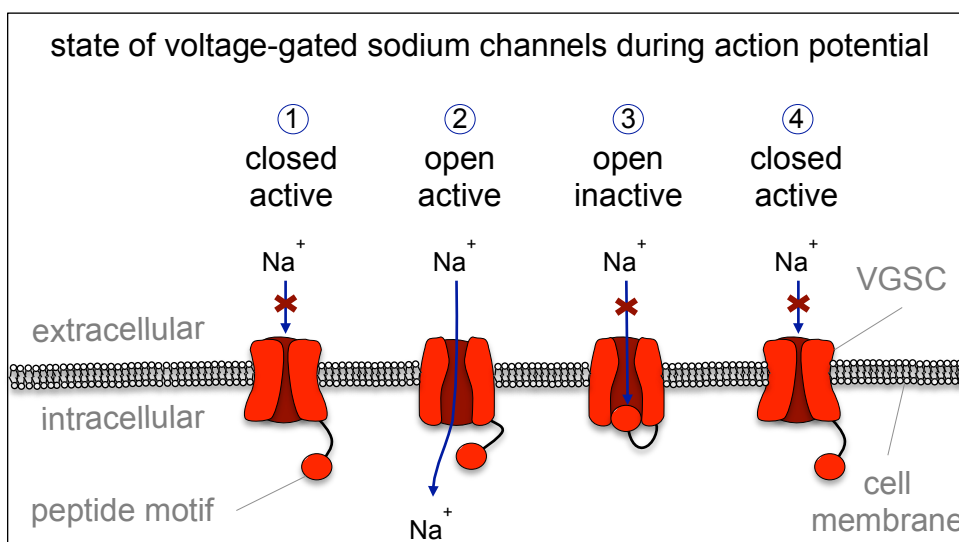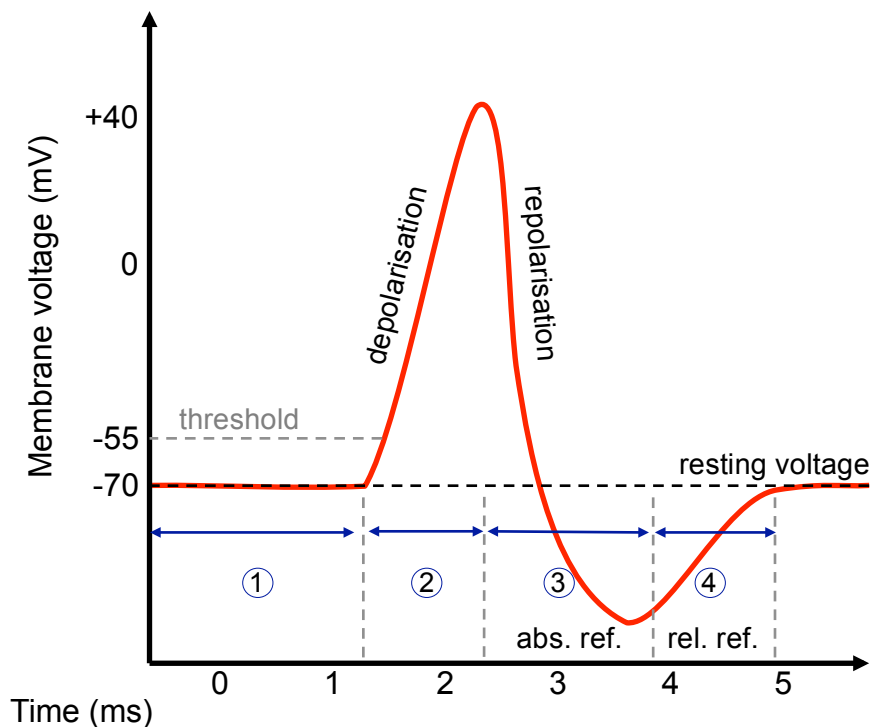
Figure 1.3 Action potential wave & voltage-gated sodium channel states
[A] Local axon membrane voltage over time, during an action potential. Numbers in circles relate to panel B. [B] Idealised cartoon of VGSC channel opening and mechanism of "ball-and-chain" inactivation. At resting potential (1), the central pore of the VGSC is closed and impermeable to sodium ions (Na^+), and a peptide motif (ball) resides in the cytoplasmic compartment as part of the cytoplasmic peptide tail (chain) of the VGSC protein. As the channel pore opens (2) due to increased voltage, sodium ions move along their electrochemical gradient into the neuron, and the peptide motif begins to move to a docking domain in the internal pore that is exposed in the open VGSC configuration. Binding of the peptide motif (3) blocks the opened pore, preventing further Na^+. Restoration of the membrane potential (4) by potassium ion efflux (not shown) causes closing of the pore domain, undocking of the peptide motif, and restoration of the VGSC to a resting state.

In essence, opening of the channels by an increase in voltage allows the influx of sodium ions for a short period before the channel then becomes physically inactivated in a voltage-insensitive manner. The result is that the influx of sodium ions through VGSCs in their open and activated period causes a local depolarisation great enough to induce nearby additional VGSCs to open before the original VGSCs are inactivated. A consequence of the fast inactivation of the VGSCs is that the membrane depolarisation can only spread in one direction, as the voltage-independent inactivation of VGSCs does not allow further influx of sodium ions. The spread of membrane depolarisation then continues along the axonal membrane until it reaches the synaptic terminal of the neuron in the NTS. Behind the wave of opening VGSCs, the membrane potential reaches its peak (positive) amplitude. As the membrane potential approaches this peak amplitude, the potassium permeability of the axonal membrane increases, increasing the efflux of potassium ions (K^+) from the cell, slowing the rate of depolarisation. In addition, the positive membrane potential begins to open voltage-gated potassium channels (VGPCs). VGPCs operate in a similar but opposing manner to VGSCs, having an inverse relationship to the membrane voltage, and allowing the rapid diffusion of potassium ions out of the cell (along their electrochemical gradient) through the opened pore domain. As the peak amplitude of the AP is approached, the efflux of potassium ions slows the depolarisation caused by the influx of sodium ions. As the VGSCs become inactivated, the efflux of potassium ions continues, rapidly repolarising the axon to restore the original membrane potential. However, the inactivation of VGPCs is slower than that of VGSCs, and due to this property, the cell membrane becomes slightly hyperpolarised as potassium ions continue to efflux along their electrochemical gradient. The resting potential of the membrane is then actively restored through the activity of Na^+/K^+-trans-membrane transporter (requiring the breakdown of ATP).

As the membrane repolarises, the inactivation of sodium channels is reversed as the pore of the VGSC returns to its resting (closed) state, obscuring the binding domain for the inactivating peptide motif, causing release of the motif and restoration of the 'resting' state of the VGSC. When a sufficient number of channels at the nerve terminus have returned to a closed but active state, another AP can be initiated. Until the resting state of the VGSCs has been restored, no new AP can be initiated, and this period of time is referred to as the absolute refractory period. However, following the restoration of VGSCs to a resting state, the potential for the initiation of a new AP is reduced due to the hyperpolarisation of the membrane. In this relative refractory period, a new AP can be initiated, but requires a greater generator potential. Therefore, a larger and persistent generator potential causes an increased frequency of AP firing. In this way, the analogue

input, or 'strength' of stimulus, is encoded as a digital signal, i.e. the frequency of AP firing.

It should be noted that if the generator potential is not of sufficient magnitude, the membrane potential will be gradually restored to its resting voltage, and no 'information' will be sent from the nerve terminus.

The above description describes the spread of an AP along the axon of an unmyelinated nerve fibre. However, populations of airway afferent nerve fibre may also be coated with a myelin sheath, formed by Schwann cells wrapping around the axon (*Fig. 1.2*). These sheaths allow an AP to be conducted at much greater speeds along the axon. Schwann cells provide a highly insulated sheath, which inhibits the exchange of ions along those portions of the nerve. At portions of the axon however, there are gaps of approximately 1μm, known as the nodes of Ranvier, where there is a great density of the ion channels and pumps involved in AP propogation. An AP 'jumps' from node to node in a process of saltatory conduction that is enabled by insulating effect of the myelin sheath, which reduces dissipation of the electrical signal. Saltatory conduction occurs at much greater speeds than the spread of activating Na^+ channels, and therefore myelinated nerve axons have greater velocity of AP propogation to the central terminals.

Once the AP reaches the central terminus of the peripheral nervous system, synaptic vesicles containing neurotransmitter chemicals migrate and fuse with the membrane. Vesicle fusion results in release into and diffusion across the synaptic cleft of the neurotransmitters, which then activate receptors on the termini of second order neurons of the CNS.

1.1.2 CNS processing – input, 'urge-to-cough', and output signals

The circuits and mechanisms by which airway afferent input signals are integrated and organised in the CNS to form the cough response are complex, and are not fully elucidated (Fong *et al.*, 2004). It is generally agreed that the afferent input signals which cause defensive cough are input to second order neurons within the NTS (Widdicombe & Undem, 2002). This comes from evidence that neurotransmitter antagonists injected into a discrete site of the NTS can inhibit the cough response (Canning, 2009).

A model of the brainstem centres that act in concert to integrate airway afferent input and form motor cough responses has been proposed by Shannon *et al.* (2004). The input signal from vagal airway afferent neurons is passed to relevant second order NTS neurons, which relay the signal into the Bötzinger-Ventral Respiratory Group (Böt-VRG),

a core network of neural centres that processes input from airway afferents. Importantly this centre receives input from both afferent neurons that regulate cough, but also those known to regulate breathing patterns (*see Introduction 1.2*). In addition to the Böt-VRG network, the Pontine Respiratory Group (PRG), has been implicated with an essential role in the formation of a cough reflex, although the nature of interaction/regulation of the Böt-VRG and PRG networks is not clear. Neurons from these core neurons synapse onto respiratory bulbo-spinal pre-motor neurons (I-Aug and E-Aug).

However, one interesting feature of cough is that there is a level of both unconscious and conscious control over this reflex. The afferent input, or sensation, of a need to cough can be consciously suppressed, at least to some extent. That a complete cough movement can be consciously mimicked in the absence of any urge-to-cough testifies to the conscious control over the cough reflex. However, this conscious control is not complete, as the conscious control over the cough reflex is not absolute.

1.1.3 Motor innervation of cough

The signal to cough encoded from the NTS is patterned from the respiratory bulbo-spinal pre-motor neurons (I-Aug and E-Aug) onto inspiratory and expiratory laryngeal motor neurons, which have soma within the thoracic and lumbar dorsal root ganglia (Bongianni *et al.*, 1998; Giraudin *et al.*, 2008). Upon excitation from pre-motor neurons, APs are generated and travel to the peripheral termini of these motor neurons, which are located within intercostal, diaphragm, abdominal and glotteal muscles (Undem & Weinreich, 2005; Giraudin *et al.*, 2008). Chronologically and anatomically coordinated activation of these neurons by the core networks in the CNS causes contraction of the inspiratory muscle groups following by expiratory muscle groups, in a manner to form a coherent cough reflex (Bongianni *et al.*, 1998).

1.2 Airway afferent sensory nerves

Afferent nerves innervating the airways collectively form part of a neural network, conveying relevant information to the CNS, where it is used to regulate breathing patterns, airway autonomic tone, and cough. A wide variety of conditions and pharmacological ligands at the nerve termini can activate or modulate the activity of afferent neurons. For example, stimuli that induce cough include mechanical stimulation (punctate touch, distension) (Canning, 2004), mucus (Basser *et al.*, 1989), irritants including cigarette smoke, dust and chemical fumes/noxious gasses (Lee *et al.*, 2007; Belvisi *et al.*, 2011), inflammatory mediators including PGE_2 and bradykinin (Kawakami *et al.*, 1973; Choudry *et al.*, 1989; Maher *et al.*, 2009; Grace *et al.*, 2012), and changes in luminal osmolarity (Lowry *et al.*, 1988; Koskela *et al.*, 2005), pH, and changes in temperature (McGarvey *et al.*, 1998; Wong *et al.*, 1999). This wide variety of stimuli activates one or more of a range of specific receptors expressed on the surface of airway terminating neurons. Activation of these receptors, which may be ligand-gated ion channels, or metabotropic receptors that indirectly activate ion channels, causes the influx of cations necessary to induce action potentials.

Specific categories of nerve fibre type have been described based on information from multiple studies collating functional and anatomical information about individual neurons. This categorisation delineates subtypes of airway afferent according to their responsiveness to specific stimuli, the location of termini within the airway structures, the location of neuronal soma, and axonal conduction velocity. This work has been greatly advanced through the pioneering of techniques such as single-fibre nerve recording, which can be used to record action potential firing in a single nerve fibre, both *in vivo*, and also in *ex vivo* lung/trachea/vagal nerve preparations (Coleridge & Coleridge, 1984). Delineation of airway afferents into these categories must be interpreted with a degree of caution, as the data about these various types of fibre has often been obtained in multiple species, using a variety of both *in vivo* and *in vitro* techniques. In particular, most studies of airway afferent fibre responsiveness have been conducted in multiple non-human species (mostly guinea pig, rat, cat and rabbit) (Coleridge & Coleridge, 1984). A summary of the nerve fibre categories and attributes is given in Table 1.1, and their role in the cough reflex and other airway functions is discussed in more detail below.

	Slowly adapting stretch receptors	Rapidly adapting stretch receptors	Aδ nociceptors	Cough receptors ('Polymodal Aδ')	C-fibres
Conduction velocity (myelinated)	$18 m.s^{-1}$ (+)	$14\text{-}23 m.s^{-1}$ (+)	$\sim 6 m.s^{-1}$ (+)	$4\text{-}6 m.s^{-1}$ (+)	$<1 m.s^{-1}$ (-)
Mechanical sensitivity	High (stretch, touch)	High (stretch, touch)	Low (touch only)	High (touch only)	Low
Chemical sensitivity	-	-	+++ [BK, capsaicin, acid]	+ [CA, low Cl]	+++ [BK, PGE$_2$, capsaicin, CA, others…]
Somatic origin	Nodose ganglia	Nodose ganglia	Jugular ganglia	Nodose ganglia	Jugular & nodose ganglia
Location of airway termini	Extra- & intra-pulmonary; ASM	Extra- and intra-pulmonary	Extra- and intra-pulmonary	Extra-, few intra-pulmonary	Extra- and intra-pulmonary

Table 1.1 Characteristics of airway vagal afferent fibre types thought to be involved in the cough reflex
Abbreviations: ASM: airway smooth muscle, BK: bradykinin, PGE$_2$: Prostaglandin E$_2$, CA: citric acid
Information taken from (Fox et al., 1993; Riccio et al., 1996; Kajekar et al., 1999; Schelegle & Green, 2001; Canning, 2004; Mazzone, 2005; Canning, 2006a; Nasra & Belvisi, 2009)

Slowly adapting stretch receptors

Slowly adapting stretch receptors (SARs) have myelinated axons, and therefore conduct APs at comparatively fast velocities of approximately 18m.s^{-1} (Canning *et al.*, 2006). These fibres are involved in the regulation of tidal breathing patterns; during inspiration and exhalation, as the smooth muscle begins to approach maximum tidal distension or compression, SARs begin to fire (Schelegle & Green, 2001). At either extreme, a burst of prolonged AP firing is observed, which inputs to the correct second-order neurons in the CNS to inhibit further inhalation or exhalation, as determined by the type of input stimulus (hyper- or hypo-inflation) (Schelegle & Green, 2001). When stimulated by a sustained mechanical deformation, these fibres are slower to 'adapt' to the new mechanical conformation of their receptors, and therefore firing in response to hyper- or hypo-inflation is prolonged compared to other types of mechano-transducing fibre (Coleridge & Coleridge, 1984). The receptors mediating mechano-transduction in airway afferents are currently unknown; as Taylor-Clark *et al.* (2006) state, "The 'physical hypothesis' for mechanical nerve activation is that mechanical stimulation physically alters a mechano-gating protein leading to the opening of a cation channel in the terminal membrane."

SAR termini are found within the smooth muscle layer of the trachea, bronchi, bronchioles and smaller diameter airways – essentially within any airway structure that possesses smooth muscle (Bartlett *et al.*, 1976). Whilst SARs do not respond directly to stimuli that provoke cough, this does not preclude the possibility that SAR input to the CNS may indirectly modulate cough by affecting how the CNS responds to stimuli from other airway afferent fibres firing in response to a cough stimulus (Canning, 2006a; Canning & Mori, 2011).

Rapidly adapting stretch receptors

Rapidly adapting receptors (RARs) possess myelinated axons, and therefore have fast-conducting action potentials, in the range of 14-23m.s^{-1} (Fox *et al.*, 1993; Riccio *et al.*, 1996). RARs are activated by mechanical stimuli including hypo- and hyper-inflation of the lung, and also by punctate touch, but are relatively insensitive to chemical stimuli (Bergren & Sampson, 1982; Pack & DeLaney, 1983; Ho *et al.*, 2001; Widdicombe, 2003). By contrast to SARs, RARs nomenclature relates to their rapid adaption (cessation of AP generation) in the presence of persistent stimulation. Whereas SARs are thought to play a greater role in tidal breathing, activation of RARs can elicit cough responses. Whilst RARs are generally considered to be insensitive to chemical pro-tussive stimuli such as capsaicin and bradykinin, they may be activated by these stimuli indirectly via the mucus production and bronchoconstriction induced by these stimuli (Widdicombe, 2003).

RARs mostly originate in the nodose ganglia, and protrude sub-epithelial termini that are found throughout the airways, but are particularly abundant in the larger structures of the upper airways (Mortola *et al.*, 1975; Sant'Ambrogio *et al.*, 1978; Canning, 2004; Nasra & Belvisi, 2009). Given their exquisite sensitivity to even light punctate touch it has been suggested that their primary role is therefore in evoking defensive cough to clear obstructive matter and maintain airflow (Nasra & Belvisi, 2009).

'Cough' receptors

The suggestion of a specific 'cough receptor' type of airway afferent fibre was suggested by Canning *et al.* (2004) to describe "a subpopulation of myelinated, capsaicin-insensitive polymodal afferent neurones that are not readily classified as either rapidly or slowly adapting receptors [and] are primarily responsible for regulating cough evoked from the trachea or larynx". These fibres have similar characteristics to RARs, including myelination of axons and cell bodies originating from the nodose ganglia. The conduction velocity of cough receptors is however slower from RARs, mostly in the range of approximately 4-6m.s^{-1} as opposed to 14-23m.s^{-1}. In addition, whilst these fibres are, like RARs, mechanically activated in that they are exquisitely sensitive to light punctate touch, they are by contrast unresponsive to stretch of the airway structures (i.e. changes in pulmonary volume and bronchoconstriction). They are also unresponsive to certain chemical stimuli such as capsaicin and bradykinin, similarly to RARs. However in contrast to RARs the cough receptors are responsive to other chemical stimuli such as CA and changes in pH (Canning, 2004; Mazzone, 2004).

Currently the identification and characterisation of 'cough receptors' has been carried out exclusively in the anaesthetised guinea pig, and the existence of these fibre types, and their relevance to the cough reflex in other species, including humans, is unclear (Canning, 2004; Mazzone, 2004)

C-fibres

C-fibres possess non-myelinated axons, and are the slowest conducting nerve fibre type innervating the airways, conducting APs at a velocity of under 1m.s^{-1} (Fox *et al.*, 1993; Riccio *et al.*, 1996; Mazzone, 2004). C-fibres originate in both the nodose and jugular ganglia, and protrude termini to sites in both intra- and extra-pulmonary airways. They are activated predominantly by chemical stimuli, including capsaicin, bradykinin, PGE$_2$ and CA, but are also mildly sensitive to stimulation by punctate touch, although are not

activated by changes in pulmonary volume or bronchoconstriction (Coleridge & Coleridge, 1984; Mohammed *et al.*, 1993; Riccio *et al.*, 1996; Lee & Pisarri, 2001; Undem, 2004). As C-fibres are unresponsive to changes in airway pressure and bronchoconstriction, the activation of these fibres by chemical stimuli is via a direct effect on these nerves rather than indirect result of bronchoconstriction (Coleridge & Coleridge, 1984; Undem, 2004). In addition, a subset of C-fibres are activated by a multitude of inhaled irritants (cigarette smoke, air pollution), aromatic compounds in food and perfume (mustard oil, wasabi, cinnamon) and endogenous inflammatory mediators (PGE_2, bradykinin) (Bessac & Jordt, 2008; Nassenstein *et al.*, 2008; Taylor-Clark *et al.*, 2008; Grace *et al.*, 2012).

Whilst all C-fibres are unmyelinated and respond to capsaicin and other chemical stimuli, there are two distinct populations of C-fibre, termed 'pulmonary' and 'bronchial' C-fibres, which respectively innervate the intra- and extra-pulmonary airways and possess unique phenotypes (Coleridge & Coleridge, 1984; Springall *et al.*, 1987; Kummer *et al.*, 1992; Undem, 2004): Bronchial C-fibres originate in the jugular ganglia, primarily innervate the larger conducting (extra-pulmonary) airways, and are mostly not stimulated by ATP, and express neurokinins such as substance P (SP) and calcitonin gene-related peptide (CGRP). By contrast, pulmonary C-fibres originate in the nodose ganglia, primarily innervate the lower (pulmonary) airways, are stimulated by ATP, and mostly do not express substance P or CGRP.

Stimuli that cause C-fibre firing in anaesthetised animals also evoke cough in conscious animals (and humans). However, cough evoked by C-fibre activation is abolished in anaesthetised animals. This means that it is not possible to directly demonstrate that activation of bronchial or pulmonary–terminating fibres evoke cough. It is thought that, as mechanically-evoked cough is stimulated by nerve fibres with receptive fields in the upper airways, that bronchial C-fibres are most likely to cause cough (Undem *et al.*, 2002). In concordance with this hypothesis, it has been shown that activation of pulmonary C-fibres may inhibit cough in anaesthetised animals (Widdicombe, 1995; Widdicombe & Undem, 2002). Pulmonary C-fibre stimulation promotes the reflexes of apnoea, shallow/rapid breathing, and bradycardia (Coleridge & Coleridge, 1984). It is therefore thought that the afferent input by pulmonary C-fibres that act to modify the breathing pattern opposes afferent inputs that would normally evoke cough, at the level of the CNS, resulting in inhibition of the cough response (Tatar *et al.*, 1994; Karlsson & Fuller, 1999).

In addition to a possible role in evoking cough, activation of bronchial C-fibres can cause local axonal reflexes, causing release of neuropeptides such as CGRP, SP, and Neurokinin A (NKA) from C-fibres at both peripheral and central termini (Barnes, 1986; 1995). It has been shown in animal models that peripheral release of neuropeptides can contribute to inflammatory processes in the lung, causing bronchoconstriction, mucus production and oedema (Nasra & Belvisi, 2009). In addition, neuropeptides may act in a paracrine fashion on both peripheral and central nerve termini to alter the expression of receptors on sensory nerves and second order neurons in the NTS to potentiate the responsiveness to future chemical cough stimuli (Fischer *et al.*, 1996; Undem *et al.*, 1999; Myers *et al.*, 2002; Bonham *et al.*, 2006; Sekizawa *et al.*, 2008; Lieu & Undem, 2011; El-Hashim *et al.*, 2013).

Additionally, a small number of airway C-fibres have cell bodies residing in thoracic dorsal root ganglia (DRG), although what input these fibres contribute to the cough reflex is unclear, as bilateral vagotomy abolishes the cough response to capsaicin (Coleridge & Coleridge, 1984; Nasra & Belvisi, 2009).

Aδ nociceptors

Aδ-nociceptors are a population of airway afferents that originate in the jugular ganglia, protruding myelinated axons that conduct APs at velocities of $4\text{-}6\text{m.s}^{-1}$, from termini located in both the intra-and extra-pulmonary airways (Canning *et al.*, 2006). Aδ-nociceptors express the ligand-gated Transient Receptor Potential Vanniloid type 1 (TRPV1) ion channel, and so are chemically sensitive to agents such as capsaicin, bradykinin and PGE_2, but differ from C-fibres in that they do not express substance P or tachykinins (Riccio *et al.*, 1996; Kajekar *et al.*, 1999; Yu, 2005). Additionally these fibres possess low responsiveness to mechanical stimulation.

Nerve fibre types involved in the cough reflex

The use of single fibre recording techniques, both *in vitro* and *in vivo* (in anaesthetised animals), has allowed researchers to determine which fibre types are activated by particular stimuli. However this information does not necessarily translate to cough; or rather, afferent nerve firing does not necessarily equate to generation of a cough response. For example, the tussive stimulus capsaicin evokes C-fibre firing in anaesthetised animals, but anaesthesia abolishes capsaicin-evoked cough. This highlights the complex neural integration of inputs from multiple nerve fibres that is regulated at both conscious and unconscious levels of the CNS. Furthermore, it

highlights the difficulties in making conclusions about which fibres mediate cough in conscious animals from single fibre studies in anaesthetised animals. Therefore, whilst there are many similarities in the stimuli that can evoke cough in animal models and humans, it remains unclear whether the characteristics of nerve fibre sub-types as determined in animal models are the same as those of human airway afferent nerves.

However, current dogma suggests cough evoked by chemical stimuli, such as capsaicin and bradykinin, is evoked primarily by bronchial C-fibres, with some possible contribution from RARs and/or cough receptors, whereas cough evoked by mechanical stimuli such as bronchoconstriction and mucus is mediated primarily by cough receptors and/or extra-pulmonary RARs (Canning, 2009; 2011). However this summary undoubtedly greatly oversimplifies the processes involved in evoking cough, as there is evidence that all fibres which innervate the airways may be involved in the regulation of cough at some level, via integration of their respective signals in the CNS (Mazzone, 2005; Canning, 2011). What is more, the phenotype of airway afferent nerves may change under certain conditions (*see Introduction 1.3.4*), such as those found in respiratory infections and diseases, leading to a dysfunction of the normal cough reflex.

1.3 Chronic cough; burden, causes and mechanisms

1.3.1 Problematic cough – incidence and health burden

A European Respiratory Community Health survey (Cerveri *et al.*, 2003) suggests that as much as 12% of the general population may suffer from excessive, or problematic, cough. In addition cough is one of the most common complaints for which patients seek medical care (Schappert & Rechtsteiner, 2011). Short-term problematic cough is a frequent symptom of influenza infection, but cough may also present as a problematic and persistent symptom common to many respiratory diseases (Irwin *et al.*, 2006).

Under normal physiological conditions in healthy individuals, cough is an essential defensive reflex that is initiated by relevant and appropriate stimuli acting on sensory nerves in the airways. An occasional cough is therefore a necessary function to maintain normal airflow to the airways. However, in certain conditions, excessive cough can become a problematic and distressing symptom. In these situations, the cough reflex becomes hyperresponsive, with coughs being evoked by normally innocuous stimuli, leading to unproductive bouts of coughing that cannot be consciously suppressed (Pratter, 2006b). Frequently the impact that such coughing can have on patients quality of life may be underestimated, however it should be borne in mind that coughing can vary from a relatively gentle 'clearing of the throat' to a vigorous and explosive 'line of last defense' mechanic (Irwin, 2006a). It is reported that during vigorous coughing, intratracheal expiratory velocities of up to 500 miles per hour, or about 5/7 the speed of sound, and high intrathoracic pressures of up to 300 mmHg may be reached (Irwin, 2006c). Whilst in a defensive cough, for example to expel an inhaled foreign object, this explosive release of air is highly effective and desirable, on a recurring basis this manoeuvre can cause a variety of adverse effects, both physical and psycho-social (Irwin, 2006c). The physical adverse effects include a long list of symptoms affecting multiple bodily systems including cardiovascular, gastrointestinal, genitourinary and respiratory (Irwin, 2006c). These effects can be serious and immediate medical concerns, including brady/tachyarrythmia (Omori *et al.*, 1984; Reisin *et al.*, 1994), diaphragmatic rupture (George *et al.*, 2000) and loss of consciousness/cough syncope (Mccann & Bruce, 1949; Kerr & Derbes, 1953; Skolnick & Dines, 1969). Patients with long-term excessive cough may also suffer ongoing psychosocial issues, such as self-consciousness at inability to voluntarily control cough and at apparent symptoms that may include excessive sweating, cough syncope and urinary incontinence (among others) (Irwin & Curley, 1991; French, 1998; French *et al.*, 2002). Such symptoms may significantly reduce the patients quality of life and lead to social withdrawal (French, 1998).

In order to assess both the impact of chronic cough on patients lives, and also to better understand the nature of how the cough reflex is altered in chronic coughers, a range of methodologies to assess cough in the clinic have been devised.

1.3.2 Assessment of cough in the clinic

Study design in the clinical evaluation of cough can vary widely, depending on the hypothesis under examination. However in the main the methodology employed can be described as belonging to one of 3 main categories; cough questionnaires, ambulatory cough measurements, and cough reflex sensitivity testing.

Cough questionnaires

Cough questionnaires are retrospective subjective analyses that seek to assess the severity and/or impact of cough on patients' lives. The Leicester Cough Questionnaire (LCQ) (Birring *et al.*, 2003), the Cough-specific Quality of Life Questionnaire (CQLQ) (French *et al.*, 2002), and the Chronic Cough Impact Questionnaire (CCIQ) (Baiardini *et al.*, 2005) are validated cough-specific questionnaires that have been designed with the aim of evaluating the impact of chronic cough on patients' quality of life.

The LCQ contains 19 items that examine the physical, psychological and social impacts of cough. Each question is rated on a 7-point Likert* scale, and a low LCQ score indicates poorer quality of life. The CQLQ contains 28 items that examine how cough impacts on patients in terms of physical complaints, psychosocial issues, emotional well-being, health anxieties, and functional abilities. These questions are rated on a 4-point Likert* scale, and a high CQLQ score represents poorer quality of life. The CCIQ contains 25 items that examine the practical, emotional, social and physical aspects of the impact of cough on patients. These questions are rated on a 5-point Likert*. scale, and a high CCIQ score represents poorer quality of life.

The CCIQ is the least validated of the three questionnaires, having been validated in a heterogeneous sample of patients with chronic cough associated with asthma, gastroesophageal reflux disease (GERD), post-nasal drip syndrome (PNDS) and chronic

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.* *'Likert scale' refers to a format for quantifying qualitative data, by generating a score from a collection of responses that are scored along a range (Guttman, 1944).*

obstructive pulmonary disease (COPD), that was also a mix of smoking status (current, ex-, never) (Baiardini *et al.*, 2005). The LCQ and the CQLQ were validated using patients with unexplained chronic cough (French *et al.*, 2002; Birring *et al.*, 2003; 2006; Kelsall *et al.*, 2008), showing a decrease in quality of life of these patients. Similarly, patients with chronic cough associated with cystic fibrosis, asthma, and COPD were also found to have low quality of life scores (Smith *et al.*, 2006; Marsden *et al.*, 2008; Polley *et al.*, 2008; Fathi *et al.*, 2009; Field *et al.*, 2009). Additionally, the LCQ and the CQLQ have been directly compared in groups of patients with asthma, COPD or chronic cough, and overall were found to correlate in their assessment of the impact of cough on patients quality of life (Kalpaklioglu *et al.*, 2005; Polley *et al.*, 2008).

Ambulatory cough counting

Ambulatory cough counting is an objective measure that seeks to quantify cough in terms of numbers, duration, and frequency during a patients 'normal' routine. Ideally, a patient would wear a recording device and microphone, which would record cough sounds made by the subject as they go about their normal daily routine. The recording system would receive input from an open field or contact type microphone, possibly supplemented with additional hardware, for example accelorometers or bands to measure chest compression. The recording would then be analysed by an algorithm designed to isolate cough sounds made by the subject from background noise to accurately quantify cough number, duration and frequency. Unfortunately such an idealised system has yet to be fully realised (Smith, 2010). Probably the main difficulty has been in the design of software which can accurately discern cough from background noises (Smith, 2010). In terms of recording hardware, advances in commercially available audio equipment (eg. MP3 players) have made possible portable and unobtrusive microphone/recording devices that can be worn by patients and make recordings over extended periods of time. There are multiple such systems in development, each with various types of hardware and custom software for recording analysis, which have been recently reviewed by Smith (2010). Generally these systems may have good correlation with manual counting over shorter periods of time, for instance 1 hour (Barry *et al.*, 2006; Paul *et al.*, 2006; Birring *et al.*, 2008). But currently these systems are either unable to record for longer periods of time, or suffer from decreased correlation with manual counting as time increases. Problems with software analysis include difficulties with differentiating between cough sounds and background noises and speech. Other systems currently in development, which utilise sensors such as a contact microphone or chest compression band, promise improved accuracy over longer recordings, although these systems remain to be validated in larger scale studies (Smith, 2010; Vizel *et al.*, 2010).

Cough reflex sensitivity testing

Assessment of cough reflex sensitivity is an objective measure of cough evoked by inhaled stimuli. This technique is a laboratory research/diagnostic tool, and differs from cough questionnaires and ambulatory cough counting, as it does not directly assess the impact or severity of spontaneous cough in 'normal' settings. The technique does however allow an empirical pharmacological assessment to be made. Subjects inhale a designated amount of a series of increasing concentrations of an aerosolised agent that evokes cough, and are instructed to cough freely. This instruction to cough freely is important, as it serves to remove the conscious decision not to cough (Morice *et al.*, 2007a), resulting in the technique being closer to a measure of the afferent input that causes the 'urge-to-cough' (Davenport, 2008).

Results from cough reflex sensitivity assessments are usually presented as $\log[C_2]$ and $\log[C_5]$ values, or rather, as the concentrations of tussive stimulus required to evoke 2 or 5 coughs (respectively) from the subject (Morice *et al.*, 2007a). C_2 and C_5 values can either be interpolated from a concentration-response curve, or determined by selecting the lowest concentration of the tussive agent to evoke the required number of coughs (Morice *et al.*, 2007a). These values can be compared between individuals (i.e. healthy vs chronic cough patients with different underlying disorders), or within the same individual pre- and post-treatment. C_2 and C_5 measurements within individuals have been found to be reproducible at periods of up to 6 months, demonstrating the reliability of this technique for clinical trials of anti-tussives or interventions aimed at treating chronic cough (Dicpinigaitis, 2003).

Patients reporting with chronic cough have generally been demonstrated to have a lowered cough threshold (indicating increased sensitivity) to tussive stimuli, such as capsaicin and CA. This includes chronic cough patients with COPD, asthma, and GERD as underlying respiratory conditions, and also has been observed in patients with unexplained (idiopathic) chronic cough (Choudry & Fuller, 1992; Ferrari *et al.*, 1995; Wong & Morice, 1999; Doherty *et al.*, 2000).

Comparison of QoL, ambulatory recording and cough reflex measures

The choice of clinical methodology for assessment of cough will depend on the hypothesis a researcher wishes to test, and in this regard, subjective and objective measures of cough each offer different advantages. For example, quality of life studies provide a quantitative examination of the impact of cough on patients' lives. It may be argued that this is the most important statistic to determine; as lowered quality of life is

the reason patients' seek medical care. However subjective measures provide information about the patients perception of the impact of cough, they do not provide information on how the cough reflex is altered in disease, nor on how cough therapies work. By contrast, ambulatory cough measurements hold much promise for the future to provide objective measures of the severity of cough, and empirical data on recovery following treatments. However the available hardware/software must be able to monitor cough over longer periods (24h+) of time, and larger studies with such systems are still required to further validate this technique (Irwin, 2006a; Smith, 2010). Cough reflex sensitivity testing is a validated technique that is particularly useful in the initial assessment of anti-tussive therapy in the clinic, due to the controlled conditions of the assessment, reproducibility of measurements over time, and the empirical pharmacology of the data produced (Dicpinigaitis, 2003; Irwin, 2006a; Morice *et al.*, 2007a). In addition, the use of different pharmalogical stimuli to evoke cough enables probing of the mechanisms by which the cough reflex is altered, and also may be relevant to understanding the mechanisms by which potential anti-tussives have their action (Irwin, 2006a; Smith, 2010).

To date, data on comparisons of the utility and concordance of the various techniques for assessing chronic cough (and its impact) are limited, with only a few studies directly comparing the different methodologies. The earliest such comparative study was carried out by Birring *et al.* (2006), whom compared cough frequency (recorded over 6h, manually counted) to cough reflex sensitivity to capsaicin and to quality of life (assessed by LCQ) in 20 "chronic coughers". The authors observed that cough frequency correlated with a lowered quality of life (as determined by LCQ), but unexpectedly also correlated with increased C_2 and C_5 to capsaicin; implying that sensitivity to capsaicin *decreases* as cough frequency increases. There are however, some issues with the methodology/study design that may explain this counter-intuitive result: the population selected was highly heterogeneous, including a small number (20) of patients with a variety of causes of cough (cough variant asthma, eosinophilic bronchitis, GERD, post-viral, unexplained, beonchiectasis, chronic bronchitis and COPD, n=1-5 per group). In addition, the population included subjects with cough duration of >3 weeks as 'chronic' coughers, only 8 subjects had cough reflex sensitivity assessed, and ambulatory measurements were taken for only 6h. Together these issues make it difficult to interpret this data, especially when compared to similar comparative studies that have utilized longer (24h) ambulatory recordings, have a better-defined chronic cough subject population, and larger numbers of subjects. Generally, 24h ambulatory cough recordings have found that frequency of cough inversely correlates with $\log[C_2]$ and/or $\log[C_5]$ values (to citric acid) in asthmatics (non-selected for cough) (Marsden *et al.*, 2008), in COPD patients with chronic cough

(Smith *et al.*, 2006), and also in a larger population of chronic coughers albeit with various causes (Decalmer *et al.*, 2007). Also in these studies – as well as a study by Kelsall *et al.* (2008) – increased cough frequency was moderately well associated with decreased quality of life (assessed by LCQ), and also with symptom scoring (via questionnaire or visual analogue scoring).

Taken together these studies seem to indicate that objective measures of cough generally agree with subjective measurements of cough. The choice of study design may therefore be tailored on the type of hypothesis under examination. For example, cough reflex sensitivity testing may be better suited to initial clinical trials examining the efficacy of therapies for cough, whereas ambulatory cough counting and symptom scoring may require larger populations.

1.3.3 Causes of chronic cough

Cough is one of the most common symptoms for which patients seek medical care (Schappert & Rechtsteiner, 2011). The American College of Chest Physicians (ACCP) guidelines on the diagnosis and management of cough give a diagnostic workflow to identify the cause(s) of cough, based on the reported duration of cough symptoms. Firstly a patient history and medical examination will be undertaken. The duration of symptoms is therefore divided into 3 categories, with cough of less than 3 weeks termed 'acute cough', cough lasting between 3-8 weeks 'sub-chronic cough', and cough persisting longer than 8 weeks is referred to as 'chronic cough'. There are a variety of possible causes of cough, or rather, disease states that are associated with problematic cough. Upon identification of a possible cause, treatments will generally be directed at treating the underlying condition.

The most common cause of acute and sub-chronic cough are respiratory infections, particularly viral infections (common cold) (Irwin *et al.*, 1990). Typically post-viral cough resolves itself shortly after clearance of the infection without requiring additional treatment, but may persist for several weeks following clearance of other symptoms (Irwin *et al.*, 2006). For this persistent cough, treatments may be attempted, however there is limited evidence that current cough therapies have efficacy in this patient population (Pratter, 2006a). Cough can also be a symptom of other types of respiratory infection, including pneumonia and *Bordatella Pertussis* ('whooping cough'). In these cases, the infection itself will be considered more hazardous to patient health, and treatments will therefore be aimed at treating the infection first and foremost.

Whilst acute and sub-chronic cough are frequently caused by short-term infection of the upper respiratory tract, which resolves upon resolution of the underlying condition, long-term unresolved coughing can be a symptom of a range of conditions, including lung cancer, PNDS, GERD, COPD and asthma (Irwin *et al.*, 2006). Normal clinical strategy for the treatment of cough associated with a respiratory infection or disease would be to treat the underlying condition associated with the onset of chronic cough (Irwin *et al.*, 2006). However, successful treatment of the underlying condition is not always possible, and in these cases, and where the cause of cough cannot be defined, this is not a viable clinical strategy for the treatment of cough. This includes, for example, patients with inoperable lung tumours, and also patients with chronic respiratory diseases that are associated with chronic cough, such as asthma and COPD, where treatments do not completely reverse the disease conditions, and cough continues to be a distressing symptom.

COPD

Chronic Obstructive Pulmonary Disease (COPD) is a prevalent and debilitating respiratory disease, primarily of the airways. It is a leading cause of death and disability worldwide, and disease incidence is predicted to continue increasing, such that COPD is predicted to be the third leading cause of death by 2020 (Vestbo *et al.*, 2013). COPD is characterised by irreversible and progressive reduction of airflow, measured as a decline in lung function through spirometry (Barnes & Stockley, 2005; Rabe *et al.*, 2007; Paredi *et al.*, 2010). Current treatments provide essentially only moderate symptomatic relief and do not halt progression of the disease. The airflow limitation in COPD is accompanied by an abnormal inflammatory response to noxious inhaled particulates or gases, for example tobacco smoke, which is thought to be one of the primary causative agents for the initiation of COPD (Barnes & Stockley, 2005; Rabe *et al.*, 2007; Salvi & Barnes, 2009). Typically such exposures must take place over a long duration before the symptoms of COPD appear – hence most diagnoses of COPD are made when patients are in middle age.

Chronic cough is often one of the first complaints that patients present with prior to a diagnosis of COPD: such that the recent GOLD strategy for diagnosis, management and treatment of COPD notes that patients presenting with chronic cough accompanied by a decline in actual compared to predicted spirometry values should be considered for a diagnosis of COPD (Vestbo *et al.*, 2013). Indeed, cough was found to be the most commonly experienced symptom, as reported by 70% of 3265 COPD sufferers interviewed, occurring daily in 46% of the same population (Rennard *et al.*, 2002).

One of the primary characteristics of COPD is an abnormal inflammation of the airways that is unresponsive to standard anti-inflammatories, including the gold-standard treatment of corticosteroids (Barnes, 2013). It is thought that chronic exposure to noxious gases/particles drives inflammation in the lungs (Decramer *et al.*, 2012). Cigarette smoke (CS), for example, stimulates multiple pulmonary immune cells (in particular macrophages, but also neutrophils, and $CD4^+$ T_h1 and $CD8^+$ T_c lymphocytes) to release many inflammatory mediators, which recruits further inflammatory cells (in particular neutrophils, but also macrophages and $CD4^+$ T_h1 and $CD8^+$ T_c lymphocytes) to the airways (Barnes, 2008). It is thought that this positive feedback loop, along with repeated stimulation provided by chronic CS-exposures, drives a persistent and progressive inflammation. This inflammation, along with exposure to the damaging components of CS, causes destruction of the alveolar structure, and enhances mucus production (via goblet cell hyperplasia and hypertrophy) and fibrosis, resulting in reduction in the surface area for gas exchange, reduced elastic recoil, and increased airflow obstruction (Hogg *et al.*, 2004; Barnes, 2008; Lai & Rogers, 2010). The exact proportion of these structural changes may vary between patients, as COPD is an umbrella term that covers several inter-related lung diseases, including chronic bronchitis, small airways disease (SAD) and emphysema (Barnes, 2004a; Sturton et al., 2008). Whatever the proportion in an individual patient, these structural changes contribute to the reduction in airflow, as assessed by spirometry.

The mechanism by which cough becomes problematic in COPD is unclear. There is some evidence, however, that components of both the inflammatory response and CS exposure may cause modulation of the cough reflex, both at the level of sensory innervation and the CNS (expanded on in *Section 1.3.4)*. Normal clinical strategy for the treatment of cough associated with respiratory disease would be to treat the underlying condition associated with the onset of chronic cough (Irwin *et al.*, 2006). However, due to the ineffectiveness of anti-inflammatories for COPD and the progressive nature of the disease, this is not a viable strategy for the treatment of cough in COPD.

Asthma

Defining asthma is somewhat difficult, as there is no single definitive genetic or environmental cause or trigger for development of this disease (Hargreave & Nair, 2009). However, broadly speaking, asthma is a chronic inflammatory disease of the airways, characterised by sudden but transient decreases in airflow associated with dyspnea, cough and wheeze, which are generally fully reversible by bronchodilator treatment. It has been estimated that 300 million people worldwide may suffer from asthma, with the

highest incidences in the Americas, Europe, and Australia of 5-10% of the population (Masoli *et al.*, 2004).

Sudden bronchospasm of airway smooth muscle in asthmatics ('asthma attack') causes breathlessness and wheeze. Bronchospasm may be triggered by many stimuli, which in normal subjects would be innocuous, including dust and other allergens, pollen, air pollution, exercise, and cold air (Eder *et al.*, 2006). This airway hyperresponsiveness is driven either by chronic airway inflammation and/or structural changes to the airways induced by this inflammation (Lommatzsch, 2012). Whilst the chronic inflammation in asthma also involves an abnormal activation of multiple immune cells, it is different from the inflammation observed in COPD, in that it is mostly suppressed by anti-inflammatory therapy, and involves different cell types, including $CD4^+$ T_h2 cells, mast cells, and eosinophils (Barnes, 2008).

Asthma is one of the most frequent causes of chronic cough, and almost all asthma patients report cough as a symptom, with many reporting persistent and problematic cough (Niimi, 2011). Indeed, so common is the association that the ACCP guidelines recommend that in non-smoking patients presenting with chronic cough, and in the absence of other causative diagnoses, asthma should always be considered as a cause (Dicpinigaitis, 2006). It is further recommended that chronic cough patients be trialed on standard asthma therapeutics (inhaled bronchodilators and/or steroids), even in the absence of a diagnosis of asthma by decreased predicted % FEV_1, methacholine inhalation challenge. Thus chronic cough patients whom do not display asthmatic symptoms but are successfully treated with asthma therapy are diagnosed with cough-variant asthma (CVA) (Dicpinigaitis, 2006). Whilst CVA sufferers do not display the symptoms of classic asthma, a similar inflammatory phenotype and structural changes (fibrosis, airway thickening) are present in both conditions (Niimi *et al.*, 2000; De Diego *et al.*, 2005; Matsumoto *et al.*, 2007).

The exact mechanisms by which chronic cough develops on a background of asthma are unclear. Similarly to COPD, one of the hallmarks of asthma is chronic inflammation of the airways (Barnes, 2008), although in asthma this inflammation is generally well-controlled by the use of steroids, one of the mainstays of treatment (Dicpinigaitis, 2006). The successful treatment of the cough in all CVA patients (Pavord, 2004), and in the majority of chronic coughing asthmatics (Dicpinigaitis, 2006) with anti-inflammatories suggests a causative association between airway inflammation and modulation of the cough reflex. Severe asthmatics are a sub-group of asthma subjects whose symptoms are refractory to anti-inflammatory treatment with the gold-standard therapy of inhaled or oral steroids

(Pakhale *et al.*, 2011), and chronic cough may continue to be a distressing symptom to these patients in particular, further strengthening the association between inflammation and chronic cough.

Unexplained (idiopathic) chronic cough

Formerly known as idiopathic chronic cough, the 2006 ACCP guidelines on the diagnosis and management of cough advises that chronic cough of indeterminate cause be referred to as 'unexplained' chronic cough (Irwin *et al.*, 2006). This name change was selected to emphasise that unexplained cough is a 'diagnosis of exclusion', and thus is a label for chronic cough caused by any of a range of conditions that are not as yet implicated in causing cough (Pratter, 2006c).

Chronic cough may be the only presenting symptom of multiple respiratory disorders, including asthma, eosinophilic bronchitis and GERD (Irwin *et al.*, 1990; Lee *et al.*, 2001). A difficulty with making a diagnosis of unexplained chronic cough is that physicians must carefully rule out these potential causes. Therefore, considering only data from more recent studies in several specialist cough clinics in the UK, US, Australia and Japan, Chung and Pavord (2008) estimate that unexplained chronic cough may account for between 7 and 46% of patients presenting with chronic cough.

Similar to chronic cough in asthma and COPD, unexplained chronic cough patients describe bouts of coughing brought on by normally innocuous stimuli, such as exposure to perfumes or aerosols, sudden changes in temperature of inspired air, and during talking (McGarvey *et al.*, 2009; Morice *et al.*, 2011).

It has been argued that many unexplained chronic cough patients may be better described as having 'cough reflex hypersensitivity syndrome' or 'airway sensory hyperreactivity syndrome' (Chung, 2011; Millqvist, 2011). This is due to the observation that unexplained chronic cough patients frequently have a lowered threshold to tussive stimuli such as capsaicin and CA in the absence of any causative factor (Chung, 2011; Millqvist, 2011). The mechanisms underlying the modulation of the cough reflex in unexplained chronic cough and/or cough hypersensitivity syndrome are unclear (Pratter, 2006c; Chung, 2011; Millqvist, 2011).

1.3.4 Mechanisms underpinning chronic cough

There are a number of potential mechanisms that may contribute to coughing in respiratory diseases, including excess airway mucus production, airway inflammation, and the inhalation of noxious particles/fumes (e.g. cigarette smoke) (Smith & Woodcock, 2006).

The accumulation of excess mucus in the small airways has been shown to correlate with disease progression in both COPD and asthma, as indicated by a decline in respiratory flow (FEV_1) (Hogg *et al.*, 2004; Hesselink *et al.*, 2006). However, in both asthma and COPD subjects, measures of FEV1 do not correlate with cough reflex sensitivity to capsaicin (Doherty *et al.*, 2000), suggesting that mucus production does not play a role in the modification of the chemical stimulus-evoked cough reflex. Whilst mucus production may not alter the cough reflex via modification of the sensitivity of airway afferents, excess mucus production is likely to evoke increased coughing via direct mechanical stimulation of airway afferents (Smith & Calverley, 2004). This cough is likely productive in the sense that it serves to loosen and remove excess secretions to maintain airflow, and as such may not be a sensible target for anti-tussive therapy.

Levels of inflammatory mediators and cells are increased in chronic respiratory diseases associated with chronic cough, such as COPD and asthma (Bhowmik *et al.*, 2000; Gompertz *et al.*, 2001; Biernacki *et al.*, 2003; Montuschi *et al.*, 2003). Inflammatory mediators that are upregulated in these conditions, such as PGE_2 and BK, have been demonstrated to evoke cough in humans, and in animal models can directly activate sensory nerves (Kawakami *et al.*, 1973; Choudry *et al.*, 1989; Grace *et al.*, 2012).

The inhalation of noxious particles and fumes, in particular cigarette smoke, have been linked with the development of chronic cough, with for example, current cigarette smoking being the leading risk factor associated with chronic cough in the general EU population (Cerveri *et al.*, 2003). Inhalation of noxious particles and fumes, from occupational exposures, as well as cigarette smoking, can increase the level of airway inflammation seen in both asthma and COPD patients (Barnes, 2008). In addition, inhaled irritants such as CS have been shown to evoke cough in humans, and in animal models CS can directly via activate airway C-fibres and RARs (Lee *et al.*, 1989; Kou & Lee, 1990; Lai & Kou, 1998; Bessac & Jordt, 2010).

Airway inflammatory mediators have been shown to increase the production of oxidants by multiple airway cells. Noxious fumes such as cigarette smoke can also contain high levels of oxidizing agents, further increasing the oxidative stress in the airways (van der

Vaart, 2004). Increases in oxidative stress may be important in the development of chronic cough, as multiple oxidants, for example hypochlorite, hydrogen peroxide and 4-hydroxynonenal, have been shown to directly activate airway-terminating C-fibres or vagal afferent neurons in guinea pigs (Bessac *et al.*, 2008; Taylor-Clark *et al.*, 2008). Additionally, 8-isoprostane, a marker of oxidative stress, has been shown to be associated with increased cough reflex sensitivity and lower quality of life scores (assessed by LCQ) in asthma patients (Koskela *et al.*, 2012).

It has been suggested that in chronic respiratory conditions, the general milieu of increased pro-tussive endogenous and exogenous chemical stimuli may play a role in directly evoking frequent cough. In particular, the Transient Receptor Potential Vanilloid type 1 (TRPV1) and TRP Ankyrin type 1 (TRPA1) channels have been implicated with a role in mediating the responsiveness of airway nerves to a range of stimuli. TRPV1 and TRPA1 belong to a superfamily of TRP channels, which are 6 transmembrane domain, non-selective, but Ca^{2+}-preferring ion channels, with a pore region between the 5th and 6th transmembrane regions (Clapham, 2005; Szallasi *et al.*, 2007). TRPV1 is a ligand-gated, cation permeable (Ca^{2+} preferring but also Na^+) channel expressed on Aδ and C-fibres, and is a key receptor mediating afferent chemosensitivity (Canning, 2009). Capsaicin is a direct and specific exogenous activator of TRPV1; therefore as the cough reflex threshold to capsaicin is decreased in asthma, COPD and unexplained chronic cough patients, TRPV1 has been implicated with a key role in these conditions (Choudry & Fuller, 1992; Wong & Morice, 1999; Doherty *et al.*, 2000). TRPA1, another ligand-gated Ca^{2+} permeable channel, is also present on subsets of chemosensitive sensory neurons, and is also implicated with a role in chronic cough, as it is directly activated by reactive oxygen species (ROS) and cigarette smoke constituents, such as reactive aldehydes acrolein (Bessac & Jordt, 2010; Grace & Belvisi, 2011). Indirect activators (via metabotropic receptors) of TRPV1 and/or TRPA1 include mediators that are increased in inflammatory conditions, such as bradykinin, PGE_2, leukotriene (LT) B4, and hydroxyeicosatetraenoic (HETE) and hydroperoxyeicosatetraenoic (HPETE) acids (Hwang *et al.*, 2000; Carr & Undem, 2003; Clapham, 2003; Adcock, 2009; Maher *et al.*, 2009).

However, in addition to direct activation, there are several lines of evidence supporting a role for indirect sensitisation of the cough reflex to subsequent pro-tussive stimuli. Generally these mechanisms may be sub-divided into modulation of receptors expressed on airway afferent nerves at either the peripheral or central nerve terminals.

Peripherally, it has been suggested that TRPV1 activity may be modulated, either via phosphorylation of an intracellular loop of the channels, or via a reduction in inhibition of TRPV1/TRPA1 by phosphatidylinositol 4,5-bisphosphate (PIP_2) as a consequence of modified intracellular signaling pathways activated by e.g. inflammatory mediators (Ma & Quirion, 2007; Petrus *et al.*, 2007; Zhang *et al.*, 2008b; Adcock, 2009). However, whilst *in vitro* research has demonstrated that these mechanisms are feasible, direct evidence of the relevance of activation of these pathways *in vivo* are lacking. Another suggested mechanism of sensitisation is an upregulation in TRPV1 and/or TRPA1 expression in afferent sensory neurons, either in fibres expressing these receptors already (increasing sensitivity), or *de novo* expression in normally chemically insensitive afferent fibres (Undem *et al.*, 1999; Myers *et al.*, 2002; Zhang *et al.*, 2008a; Lieu *et al.*, 2012). In human subjects, increased densities of CGRP- and SP-positive nerves have been observed in the pulmonary epithelium of unexplained cough and CVA subjects (O'Connell *et al.*, 1995; Lee *et al.*, 2003). Furthermore, Groneberg *et al.* (2004) have further found that an increased density of TRPV1-expressing nerves observed in chronic cough subjects correlates with a lowered cough reflex sensitivity to capsaicin. These findings suggest an increase in the numbers of chemosensitive nerves may be important in the development of chronic cough.

Sensitisation of sensory afferent nerve may also occur at the central terminals. The release of neuropeptides in the ganglia and NTS may enhance the activity of and modulate the receptor expression of airway-terminating afferent fibres, as well as second order neurons in the NTS (Fischer *et al.*, 1996; Bonham *et al.*, 2006; Sekizawa *et al.*, 2008; Lieu & Undem, 2011; El-Hashim *et al.*, 2013).

The mechanisms by which cough reflex sensitivity is enhanced in chronic cough patients are not fully understood, but are likely to involve either an increased expression or activity of TRPV1 (and possibly TRPA1), probably driven by interactions between multiple mediators associated with airway inflammation (*Fig. 1.4*).
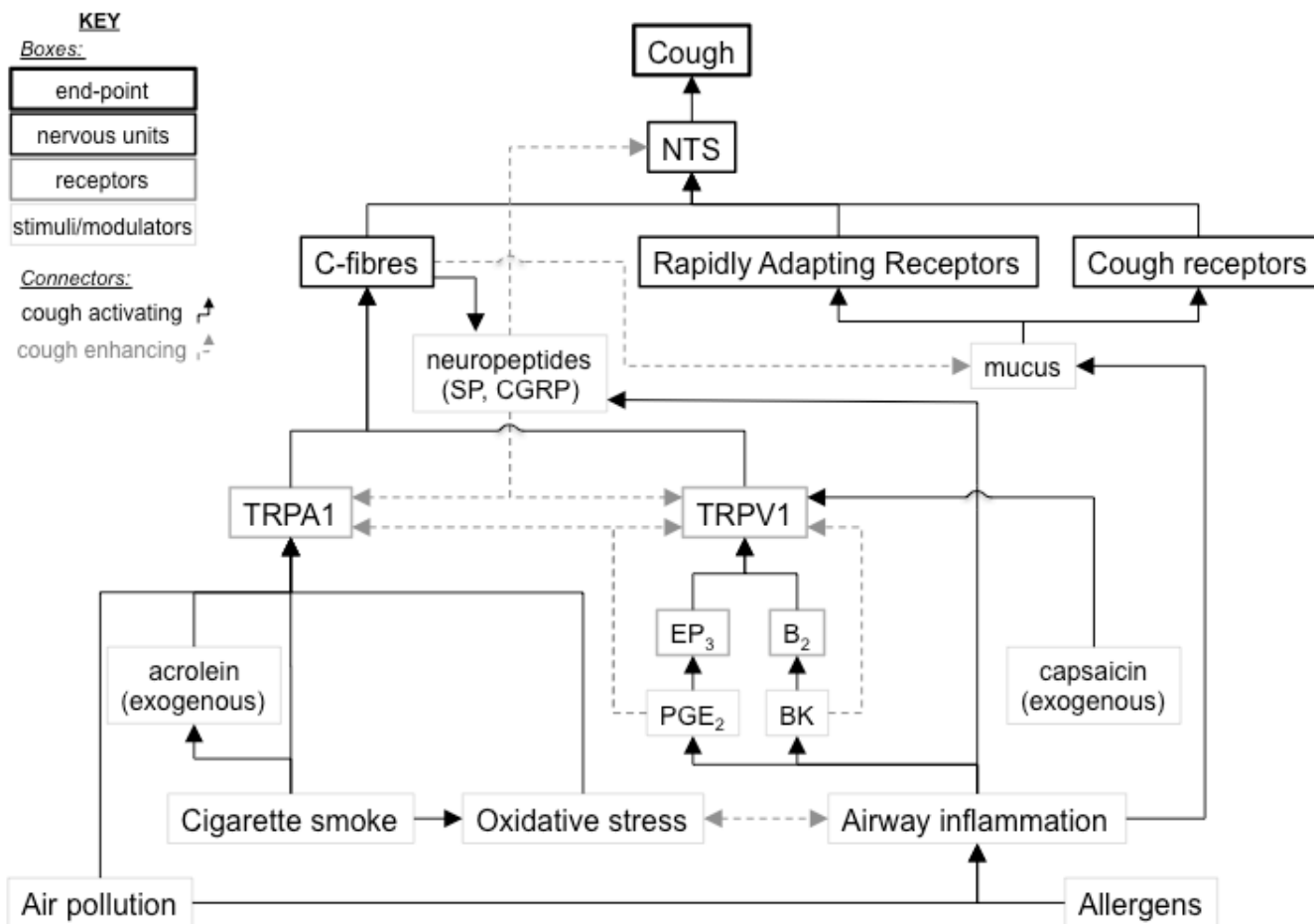
Figure 1.4 Schematic of cough activating and sensitising pathways
Cough is initiated by activation of TRPV1/A1-expressing chemically-sensitive C-fibres (or possibly Aδ-nociceptors), or of mechanically-sensitive RARs/cough receptors. Components of cigarette smoke and air pollution, or exogenous inhaled chemicals such as capsaicin and acrolein can activate TRPV1 or TRPA1 and evoke cough via C-fibres directly. Additionally these stimuli, as well as allergens, can contribute to inflammatory processes, elevating the levels of mediators such as PGE_2 and BK, which can directly activate TRPV1 and/or TRPA1, and also sensitise these receptors to further stimuli. Cough responses can also be modulated by the release of neuropeptides, which may enhance TRPV1/TRPA1 responses by modulating the activity and/or expression of these channels on C-fibres and RARs. Abbreviations: RAR; Rapidly Adapting Receptor, SP; Substance P, CGRP; Calcitonin Gene-related Peptide, TRPV1/A1; Transient Receptor Potential Vanniloid type1/Ankyrin type1, EP_3; Prostaglandin E receptor type 3, B_2; Bradykinin receptor type B_2, PGE_2; Prostaglandin E type 2, BK; Bradykinin. Figure adapted from Canning et al. (2004).

1.4 Current cough therapeutics

Pharmacological therapies aimed at treating chronic cough can be broken into three main categories based on their intended method/site of action, into drugs that treat an underlying component of disease (i.e. inflammation, mucus, reflux), drugs targeting the peripheral nervous system, and those that dampen the urge-to-cough by targeting the CNS.

Drugs that successfully treat an underlying condition in subjects with chronic cough are often successful at restoring a normal cough reflex. For example, proton pump inhibitors, used to treat reflux, are said to have a high success rate in chronic cough patients with GERD (Irwin, 2006b). Additionally, for many asthmatic subjects, anti-inflammatories and bronchodilators often provide good control of chronic cough in line with other symptoms (Dicpinigaitis, 2006).

However, where current therapies are ineffective, such as in COPD patients and severe asthmatics, or where the underlying condition cannot be identified (unexplained chronic cough), peripheral- or CNS-targeting therapies that directly inhibit cough are required.

The most effective anti-tussive medications currently available are centrally-acting compounds, including drugs such as codeine, morphine and dextromethorphan, that act on receptor types including opioid (μ-, δ- & κ-opioid) and/or σ- receptors as agonists, and additionally dextromethorphan acts as an NMDA receptor antagonist (Bolser, 2006b). However, a recent paper by Smith *et al.* (2006) demonstrated that codeine had no significant benefit over placebo, as assessed by objective cough monitoring. Furthermore, another study by Paul *et al.* (2007) comparing dextromethorphan, diphenhydramine (anti-histamine/anti-tussive non-prescription cold medication) and honey to placebo found that honey provided the greatest symptomatic relief, as assessed by cough questionnaire (on a Likert scale). Whilst this study was conducted in children with upper respiratory tract infections rather than chronic cough patients (whom may therefore not have the same modified cough reflex sensitivity), that the most efficacious anti-tussive (dextromethorphan) was outperformed by honey highlights the lack of efficacy of current cough medications.

Whilst there are currently no effective peripherally acting anti-tussive therapies available, targeting the PNS is attractive for two reasons. Firstly they generally are not associated with the same adverse effects observed with many CNS-targeting compounds, such as nausea, sedation and addictiveness (Bolser, 2006a). In addition, peripherally acting drugs may be more specific in their targeting, and hold potential to, for example, inhibit

the heightened sensitivity to chemical agents, whilst leaving the 'normal' defensive cough reflex to mechanical stimuli intact (Nasra & Belvisi, 2009). Interestingly, a recent small clinical trial of a novel compound that may fit into this class has been recently reported; Abdulqawi *et al.* (2013 – ERS abstract *In Press*) have demonstrated that an oral P_2X_3 antagonist was successful at reducing cough rates and severity in unexplained chronic cough patients. P_2X_3 receptors, which are activated by ATP, are present on airway sensory nerves (Undem & Nassenstein, 2009). Currently however, the role that P_2X_3 receptors play in regulating the cough reflex is unclear.

A recent meta-analysis on the efficacy of all types of existing cough therapies concluded that, of all clinically-evaluated compounds, only centrally-acting opioid-based drugs and dextromethorphan have any significant anti-tussive effect (Yancy *et al.*, 2013). However, codeine and dextromethorphan, whilst moderately effective, were associated with significant side-effects at therapeutic doses including sedation, nausea (including vomiting), drowsiness, and dizziness (Bolser, 2006b). No peripherally-acting cough therapies were found to be have any significant anti-tussive effect (Yancy *et al.*, 2013). In fact, so low is the demonstrable level of efficacy of all types of non-prescription, or over-the-counter (OTC) cough medications, compared to the low, but real risk of severe adverse effects, that these products for infants were withdrawn from the US market in October 2007 (Shehab *et al.*, 2010; Vassilev *et al.*, 2010).

The market for OTC anti-tussive drugs has been estimated to be worth approximately US\$3.6 billion in the US, and GB£100 million (Dicpinigaitis, 2011). Additionally, it is estimated that in the EU, as much as 12% of the general population may suffer from chronic cough (Cerveri *et al.*, 2003). Considering that no existing anti-tussive has been demonstrated to be both effective and safe, it is apparent that there is a significant unmet clinical need for novel anti-tussives.

1.5 Outline of thesis

1.5.1 Aims

There are currently no available therapies for cough that are both effective and safe. Frequently the most effective therapies are unsafe due to their action in the CNS, whereas many peripherally acting therapies are safe but ineffective. There are therefore two potential routes to novel safe and effective cough therapies; either designing novel centrally acting anti-tussives that have improved safety profiles, or designing peripherally acting compounds that have improved efficacy. Due to the problems associated with safety in centrally acting drugs, and our lack of understanding of the CNS processes involved in mediating the cough reflex, this thesis will primarily focus on examining the efficacy of novel peripherally acting anti-tussive compounds.

The drug development process can be long and costly, taking approximately a decade and incurring costs of US$1 billion for a new compound to go from discovery to clinical usage (DiMasi *et al.*, 2003). This thesis therefore aims to examine the efficacy of two existing compounds, which are clinically approved for other uses, as well as a novel compound that is currently in the lead development stage of the drug discovery pipeline (Meyers *et al.*, 2011). Where these compounds are found to be effective at inhibiting cough, their mechanism(s) of action will be examined.

The classes of compound evaluated in this thesis for their anti-tussive potential are β-adrenergic receptor agonists, methylxanthines and Fatty Acid Amide Hydrolase (FAAH) inhibitors. Various β_2-adrenergic receptor agonists, and the methylxanthine compound theophylline have been prescribed for decades for the relief and treatment of the symptoms of asthma, as bronchodilators that act on airway smooth muscle to cause relaxation of constricted airways. By contrast, FAAH inhibitors are a novel class of compound, and currently are not clinically approved for use in patients.

1.5.2 Strategy of Investigation

The potential of each of the anti-tussive compounds will be examined using a range of *in vivo* and *in vitro* animal models of cough and sensory nerve activation:

- The efficacy of the compounds will be examined using an *in vivo* guinea pig model of tussive stimuli-evoked cough.

- The peripheral action of the compounds on airway sensory afferent nerves will be examined using an *in vivo* model of single airway fibre firing, and/or an *in vitro* model of calcium influx into isolated airway-terminating afferent neurons.

- The mechanism of action of the compounds will be examined using a range of relevant pharmacological tools in an *in vitro* preparation of vagus nerve tissue from guinea pigs, or knock-out mice, as appropriate.

- Furthermore, key experiments with the isolated vagus nerve technique will be repeated using human tissue, in order to confirm that the compounds sensory nerve activity and mechanism of action is relevant to humans.

- Finally, where possible, the efficacy of the compounds will be examined in a guinea pig CS-exposure model that displays enhanced cough and sensory nerve responses to tussive stimuli. This will hopefully provide a more realistic indication of the likely efficacy of these compounds in the clinic, where any potential anti-tussive would be expected to inhibit an enhanced cough reflex.

1.5.3 Animals models of cough and sensory nerve activation

In the evaluation of novel anti-tussives, appropriate non-human models are useful to validate the effectiveness of compounds prior to clinical trials (Mackenzie *et al.*, 2004). As will be apparent from *Section 1.2*, animal models of evoked cough and sensory nerve activation have been used extensively to derive our knowledge of the airway afferent nerves involved in the cough reflex. These same models can be used to examine the efficacy of new anti-tussives, as well as their activity on the sensory nerves that evoke cough.

Evoked cough

In cats, dogs, rabbits and guinea pigs a cough response resembling the human reflex can be evoked by inhalation of the same chemical stimuli (capsaicin and CA) that are used to evoke cough in humans in clinical studies (Mackenzie *et al.*, 2004). Which of these animals possesses a cough reflex most similar to humans is debatable; however, the European Respiratory Society Guidelines on the assessment of cough state "The most useful animal model of cough is the conscious guinea pig" (Morice *et al.*, 2007a). This recommendation is based on the cost efficiency of using larger animals such as dogs, cats and rabbits – the guinea pig is the smallest species with a cough response similar to that observed in man (Mackenzie *et al.*, 2004). Whilst they are commonly used in experimental research, rats and mice, are unsuitable for cough studies, as rats have

anatomically divergent afferent cough input compared to man, and mice do not cough (Korpáš & Tomori, 1979; Morice *et al.*, 2007a).

The most compelling evidence supporting the use of the guinea pig as a suitable model species for the evaluation of cough therapeutics is that guinea pigs and human subjects have similar cough reflex sensitivity to the most commonly used tussive stimuli (capsaicin and CA) used in clinical cough studies (Laude *et al.*, 1993).

Sensory nerve activation as a proxy for cough

Models of sensory nerve activation have been developed in the guinea pig to examine the mechanisms by which the cough reflex is initiated (*see Introduction 1.2*). These models may be used to examine the action of anti-tussives on the activation of sensory nerves involved in evoking cough.

Three different models of sensory nerve activation were selected for use in this thesis, which together allow a comprehensive examination of the action of compounds on sensory nerve activation:

- Recording of the depolarisation in whole isolated vagus nerve trunks induced by tussive stimuli (*see Methods 2.3*). This *in vitro* technique allows relatively high-throughput, cost-efficient pharmacological evaluation of the effect of anti-tussives on sensory nerve activation by the same stimuli that evoke cough *in vivo*. Furthermore, the same technique can be used to examine sensory nerve activation in human and mouse vagus nerves. This allows the translation of results into a relevant human tissue, and also the use of genetically modified knock-out mouse tissue. Whilst mice do not cough, in this preparation murine vagus nerves respond similarly to the same tussive stimuli as human and guinea pig vagus nerves.
- Recording of calcium influx induced by tussive stimuli in isolated airway-terminating single afferent neurons (*see Methods 2.4*). This *in vitro* technique allows determination of the effects of compounds specifically on individual airway-terminating neurons, rather than on all the nerve fibres in the vagus nerve, as in the isolated vagus nerve preparation.
- Recording of *in vivo* single afferent nerve fibre firing evoked by aerosolised inhaled tussive stimuli (*see Methods 2.5*). This technique allows determination of the effect of compounds on individual nerve fibre types (e.g. C-fibre). Compared to the isolated vagus nerve and neuron techniques, this technique uniquely

allows determination of the effect of compounds acting on the nerve termini within the airways, and assesses action potential firing as an end-point.

The principles and methodology of each of these techniques is discussed further in Chapter 2.

Modeling the disease-modified cough reflex

Using experimental animals, such as the guinea pig, *in vivo* models of enhanced capsaicin- and CA-induced cough and sensory nerve activation can be generated by exposing animals to cigarette smoke (Karlsson *et al.*, 1991; Bergren, 2001a; Lewis *et al.*, 2007). It is thought that these models provide a good system to study COPD-enhanced cough, as they display a similar phenotype to COPD patients, induced by a relevant causal agent (Bolser, 2004).

In the first published model of *in vivo* CS-enhanced cough responses, guinea pigs were exposed to cigarette smoke for 1h, twice daily, over two weeks, demonstrating enhanced cough sensitivity to capsaicin and CA (Karlsson *et al.*, 1991). In a more chronic model of 90 days (30min exposure/day), Bergren *et al.* (2001a) demonstrated similarly enhanced capsaicin-, and also BK-induced cough responses in guinea pigs.

More recently, in the model developed by the Respiratory Pharmacology group (NHLI, Imperial College), guinea pigs exposed to CS for 1h, twice daily, for 8 days, showed enhanced capsaicin and CA cough responses. These enhanced cough responses corresponded with enhanced capsaicin and CA responsiveness of isolated vagus nerves from CS-exposed guinea pigs (Wortley *et al.*, 2011). This model aims to mimic the enhanced cough reflex sensitivity observed in chronic cough patients, particularly chronic cough in COPD, where CS is the predominant risk factor for development for the disease.

1.5.4 Outline of thesis structure

This thesis has been organised in a modular fashion, with Chapters 3, 4, & 5 examining the anti-tussive efficacy and mechanisms of action of each class of compound separately using *in vivo* and *in vitro* models of cough and sensory nerve activation. In chapter 6, the efficacy of each compound will be examined in a guinea pig model of enhanced sensory nerve activation and cough induced by CS-exposure.

2 Methodology

2.1 Human and animal tissue; ethics & welfare

Male Dunkin Hartley guinea pigs and male C57Bl/6 mice were purchased from Harlan (Bicester, UK). Experiments were approved by the Imperial College London Ethical Review Process committee, and performed in accordance with the UK Home Office guidelines for welfare based on the Animals (Scientific Procedures) Act 1986, under a Home Office project licence (PPL 70/7212).

Human vagus, as part of whole or partial lung tissues, was received from either the Harefield Hospital (HH: London, UK), or from various locations in the US via the International Institute for the Advancement of Medicine (IIAM: NJ, US). Tissue received from HH was either recipient or donor tissue unsuitable for transplant. Tissue received from IIAM was donor tissue unsuitable for transplant. Tissue received from all locations was approved by local Human Tissue Ethics Committee.

2.2 *In vivo* cough challenge and cough counting

Guinea pigs were exposed to an aerosolised tussive agent (capsaicin or CA) for set periods of time, with the numbers of coughs recorded. At a set time before cough was evoked/recorded, animals could be dosed with a test compounds in order to evaluate the anti-tussive effect of the compound on either naïve, or on CS-enhanced cough.

2.2.1 Cough challenge apparatus

Two Perspex plethysmography chambers (Buxco, US) were connected to a central Aerogen nebuliser (Buxco, US) as illustrated in *Figure 2.1*. A conscious, unrestrained guinea pig was placed into each of the chambers, so that the two animals could be simultaneously challenged with aerosolised solution of tussive agent, dispersed from the nebuliser into the two chambers. The aerosolised tussive agent was administered for 5 minutes, after which the nebuliser was set to remove the aerosol from the chambers. The number of coughs was recorded for 10 minutes in total, with the recording time commencing at the start of nebulisation, and continuing for 5 minutes after the end of nebulisation.
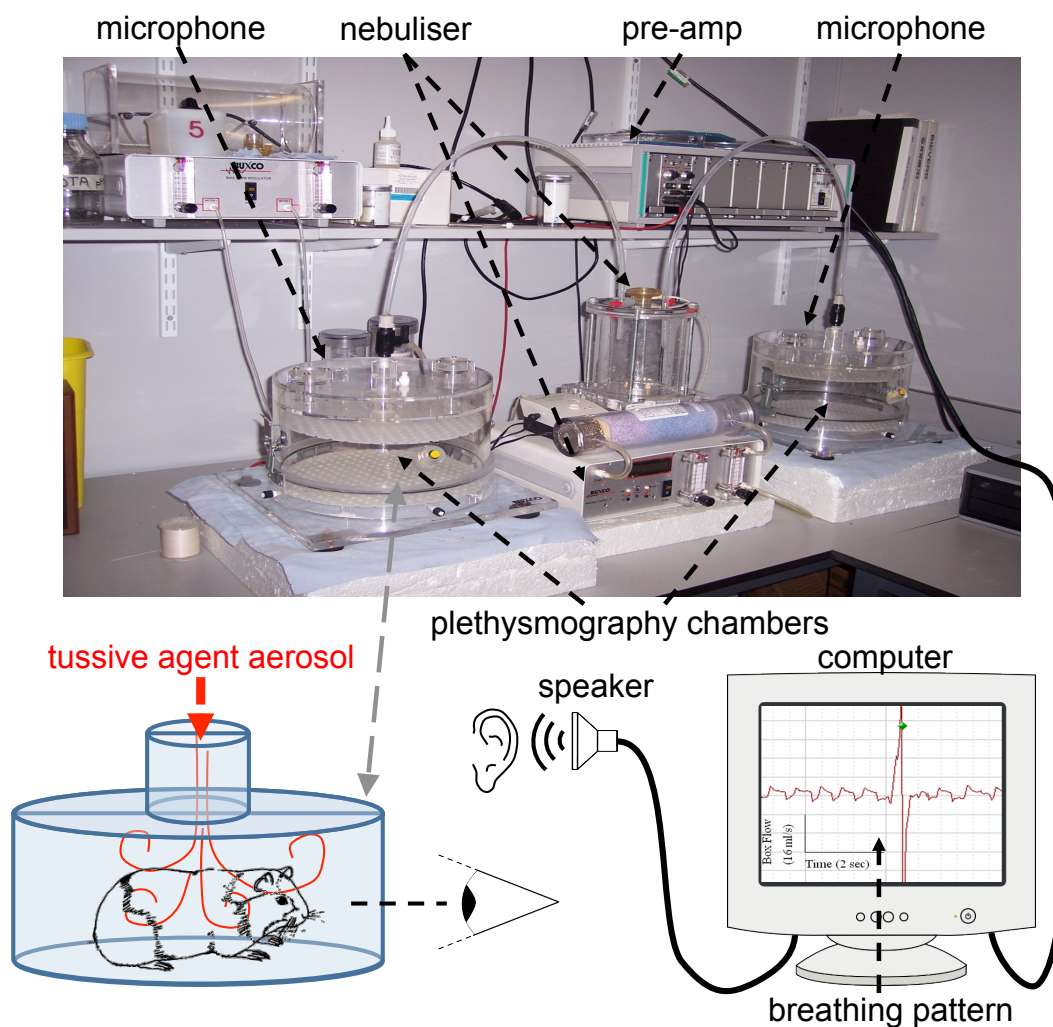
Figure 2.1 Diagram of apparatus for objective cough counting
Animals were placed in plethysmography chambers (see picture top left, cartoon bottom left), into which an aerosol of tussive agent was nebulised). Plethysmography chambers were fitted with a microphone and pressure transducer connected to a computer to visualise breathing patterns (top right). Coughs were manually counted by listening to the speaker output from the microphones, confirmed by observing the characteristic posture of the animals and spike in breathing pattern on the computer monitor.

2.2.2 Data Recording

Microphones, present in both chambers, were connected to an amplifier, and thence to both a speaker and a computer running cough analyser software (Buxco, USA). In this way, coughs were recorded by software analysis of an explosive cough signature, and confirmed by a trained observer comparing the sound output to the characteristic opening of the mouth, and the altered stance and posture of the animal.

2.3 Isolated vagus nerve recordings

In vivo cough experiments in guinea pigs can be expensive, and require the use of multiple animals per group for a single concentration of a drug. The isolated vagus recording system described in this section has some advantages in the pharmacological evaluation of potential anti-tussives, allowing multiple drugs and concentrations to be evaluated in a single animal. Another significant advantage is that isolated vagus nerve from multiple species, including guinea pigs, humans and mice can also be used. Thus guinea pig tissue can be used to parallel cough experiments, human tissue can be used to determine whether findings translate to humans, and the use of mouse tissue allows examination of the effectiveness of compounds in tissue from genetic knock-out animals for specific proteins (receptors/ion channels etc). Whilst mice do not cough, the vagus nerve of mice has been shown to respond to the same agents that stimulate both a response in guinea pig vagus, and also cause cough in guinea pigs (Maher *et al.*, 2009; Grace *et al.*, 2012).

2.3.1 Vagal nerve dissection

Human tissue

Human whole lungs were received either dry (Harefield Hospital within 3h of transplant) or in isotonic saline (US locations, within 48h of cross-clamp) on ice. On receipt, the whole lungs were immersed in modified KH (prepared as in previous section) solution which was bubbled with 95% O_2, 5% CO_2. The vagal nerves were identified running parallel to the trachea and bronchi, and dissected from the lungs. Extraneous connective tissue was then removed, and the resultant nerve tissue was then de-sheathed. The resultant human vagus nerve was then cut into 10-15mm long sections for use in the recording apparatus. Care was taken during dissection and for the duration of the experiments to ensure that the vagus nerve was not crushed or stretched, and remained in oxygenated KH solution.

Animal tissue

Guinea pigs and mice were sacrificed by injection of pentobarbitone (200mg.kg^{-1}, *i.p.*), following which a midline incision was made to remove the skin on the dorsal side of the thorax. The ribcage, thyroid and connective tissue and smooth muscle above the vagus nerve were then removed to reveal the vagal nerves running from the base of the skull to the heart. Both vagal nerves – caudal to the nodose ganglion and proximal to the heart –

were removed, and placed into modified KH (prepared as in previous section) solution which was bubbled with 95% O_2, 5% CO_2. The nerve tissue was then cleared of any remaining connective tissue, de-sheathed, and cut into sections 10-15mm long. Care was taken throughout dissection and experimentation to ensure that the vagus nerve was not crushed or stretched, and remained in oxygenated KH solution.

2.3.2 Apparatus/principles

Dissected pieces of vagal nerve were mounted in a grease-gap recording chamber (*Fig. 2.2*) by drawing nerves through a narrow channel (2 mm diameter, 10 mm long) within a Perspex block (custom made). Petroleum jelly was carefully injected through a side-port into the center of this channel – extending approx. 4-6mm along the channel – thus providing an area of high resistance that electrically and chemically isolated the two ends of the nerve. A hollow glass rod of 1.5mm diameter was inserted into a Ag/AgCl-pellet half-cell electrode (Mere 2 flexible electrodes, WPI, UK), with the air-spaces carefully filled with KH solution to avoid air bubbles. The end of the glass rod was then positioned to made contact with one end of the nerve, with a similarly prepared electrode making contact with the opposite end. The electrodes were connected to a DAM50 amplifier unit (WPI, UK), which amplified the DC potential signal (x10 gain, high filter 1KHz, 5Hz sample rate). The amplified signal was then recorded on a chart recorder (LectroMed 2, LectroMed, UK [now Digitimer, UK]). One end of the nerve was constantly perfused with KH solution at a rate of 2ml.min^{-1} to allow delivery of stimuli and test compounds; the other end remained in a chamber containing KH. KH was bubbled with 95% O_2/5% CO_2 and maintained at 37°C using a thermal jacket and water bath.

KH and compounds dissolved in KH were placed in containers situated above the Perspex chamber, and were perfused through a water jacketed section of rubber tubing, with an in-line switch to select which solution to perfuse (*Fig. 2.2*).

This system does not directly measure action potentials or firing of the nerve, instead it indirectly measures the compound depolarisation of the membranes of all the nerve fibres within the vagus nerve, by recording the difference in the ionic potential of the two ends of the nerve. In the nerve ending that is exposed to perfusate containing an activating ligand at sufficient concentration to cause depolarisation there is an influx of predominantly sodium ions from the extracellular KH solution (from high Na$^+$) in to the axon cytosol (to low Na$^+$) (*see Introduction 1.1.1*); however, the net effect of the movement of ions during depolarisation of a nerve is a lowered concentration of positive ions in the KH solution within the glass tube near the Ag/AgCl electrode, which leaves the Cl- ions in a 'freer' state to react with the electrode pellet. The difference between the

increased potential on the electrode at this perfused end of the nerve, and the unaltered equilibrium at the opposite end of the nerve is amplified and recorded by the chart recorder.
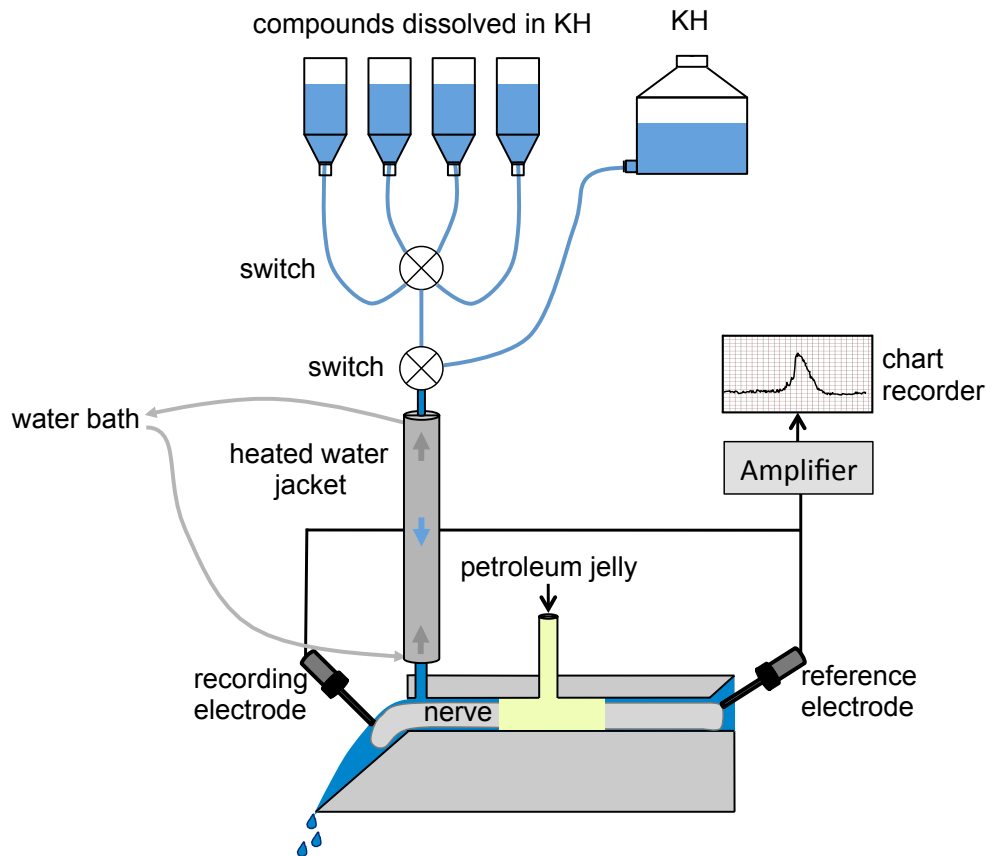
Figure 2.2 Diagram of apparatus for isolated vagus nerve depolarisation recordings
Vagus nerve dissected from guinea pigs or mice was drawn into a channel in a perspex chamber filled with KH solution. KH was constantly perfused onto the left side of the nerve through a section of water-jacketed tubing, maintaining the perfusate at 37°C. A series of switches allowed changing of the perfusate from KH alone to various compounds or combinations of compounds dissolved in KH. Ag/AgCl half-cell electrodes were placed in contact with both sides of the vagus nerve, which were electrically isolated by injecting petroleum jelly into the centre of the channel. The signal from the electrodes was passed through an amplifier and the resulting signal was output onto a chart recorder.

There are three potential caveats to note when considering the results obtained using this apparatus: firstly, that the depolarisation measured is not necessarily equal to firing of the nerve fibre, as a depolarisation may not be of sufficient magnitude to generate an action potential. Secondly, the vagus nerve supplies innervating fibres not just to the airways, but also to other mid-line organs such as the heart and gut. Thirdly, the cell bodies and nerve termini are not present in the isolated vagus trunks, and there may be differences between the receptors and associated signaling proteins in the plasma membranes of the nerve terminus and the nerve axon.

However, despite these caveats, it should be noted that there is a good correlation between the compounds that activate the vagus nerve in this apparatus, also cause cough *in vivo*, whilst those that inhibit induced depolarisation also inhibit induced cough (Birrell *et al.*, 2009; Freund-Michel *et al.*, 2010; Grace *et al.*, 2012). This system is also amenable to pharmacology that would be difficult & expensive *in vivo*, and has the further advantage that as the nerve is isolated the effects of potential anti-tussive drugs may be considered for their effect on sensory nerves, separate from any bronchodilator effects. Furthermore, it should be noted that where practicable key experiments were performed using calcium imaging of isolated airway-terminating neuronal cell bodies, recording of action potentials in *in vivo* single chemosensitive airway-terminating C-fibres, and most importantly, *in vivo* cough.

2.3.3 Measurements/recording protocol

The following protocol was used to measure either depolarisation/stimulation, or inhibition of stimulation of the vagus nerve, with variations in incubation timings and concentrations of drugs.

For examining a compound for potential anti-tussive activity, the following protocol was used to assess inhibition of depolarisation by a stimulating agent such as capsaicin. Two to three control stimulations were performed (incubation with drug for 2 min, 'wash' to baseline with KH, repeat) to give two consecutive depolarisations of consistent magnitude. Following wash of the final control stimulation, the test compound was incubated (by constant perfusion) for at least 10 min (incubation times varied for each compound – see relevant results chapter for details). Following incubation, both stimulating agent and test compound were perfused onto the nerve for 2 min, following which the nerve was perfused with KH alone for 10 min to 'wash' any remaining compound(s) from the nerve. The nerve was then perfused with stimulating agent alone for 2 min, with this final stimulation serving to demonstrate that the test compound can at least be partially washed off, and that the nerve remains viable at the conclusion of the experiment. Only one concentration of any single test compound was examined on any single piece of nerve, from any one individual animal or patient.

2.3.4 Data recording

The chart recorder was calibrated using a 1mV pulse from the pre-amplifiers to set a scale of 1cm:0.1mV. A measurement was taken of the distance from the baseline prior to

application of stimulating agent (i.e. capsaicin) to the peak of a response following application to calculate the magnitude of depolarisation. For inhibition studies, the average magnitude of two consistent control stimulations (within 10% of each other) was taken. The magnitude of depolarisation in the presence of test compound(s) was then compared to the control stimulation to give percentage inhibition, according to the equation given in *Methods 2.9*.

2.4 Isolated neuron recordings

The general premise of this technique was to assess the effect of potential anti-tussives on intracellular calcium increases in isolated primary airway-terminating single chemosensitive neurons. Whilst this is also an *in vitro* technique, it allowed examination of the effect of the test compounds in only neurons that had airway terminating processes, rather than on all the nerve fibres in the vagus nerve, as in the isolated vagus recordings. This methodology used in this section is similar to that described in Dubuis *et al.* (*In Press*), and Grace *et al.* (2012).

2.4.1 Staining of airway-terminating neurons

Male Dunkin Hartley guinea pigs (250-400g) were *i.n.* dosed with $1ml.kg^{-1}$ of solution of DiI (DiIC$_{18}$ 3 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbo-cyanine perchlorate, 0.15 $mg.ml^{-1}$). DiI was dissolved in 100% ethanol and diluted on the day of use to give a final vehicle of 2%v/v ethanol in 0.9% saline. DiI is a lipophilic fluorophore that passively integrates into the outer lipid layer of plasma membranes. Once in the airways, the dye enters the outer layer of the plasma membrane of any cells with which it comes in contact, including neurons with termini in and very proximal to the airways (e.g. within epithelium). The dye then diffuses laterally in 2 dimensions across the whole cell membrane, including in a retrograde direction along nerve axons, to the cell bodies in the sensory ganglia that supply the airways; primarily nodose and jugular neurons (Hofmann & Bleckmann, 1999). Guinea pigs were dosed with DiI 2-3 weeks prior to experimentation; this allowed time for the dye to reach the plasma membrane around the cell bodies, with a reported rate of diffusion along axolemma of approximately 6mm per day (Lukas *et al.*, 1998), but reduced the potential for trans-neuronal diffusion of the dye into the membranes of non-airway terminating neurons (Hofmann & Bleckmann, 1999; Lieu *et al.*, 2012).

2.4.2 Ganglia cell dissection and neuron dissociation

Guinea pigs were killed by injection of sodium pentobarbitone (200 $mg.kg^{-1}$ *i.p.*). The skin was removed from the head of the animal, and the sternocleidomastoid and trapezius muscles were removed. Then the jawbone was cut and remaining connective tissues were removed from the lateral sides and dorsal portion of the skull. A hole was made in the skull at the intersection of the interparietal and parietal bones, which was expanded to remove a section of the skull by cutting laterally across the parietal bones, and then along the frontal ridge above the eyes. The cerebellum was then removed by carefully cutting its connections to the optical and aural nerves, and the spinal cord. Inverted 'V'

shaped cuts were then made in the squamosal and occipital bones immediately anterior and posterior to the tympanic bulla. The tympanic bulla on one side of the animal was then levered partially out of the skull, whilst carefully cutting any connective tissues, to reveal the nodose and jugular ganglia lying on the occipital bone underneath the tympanic bulla. The jugular and nodose ganglia were identified by both their position and their characteristic shapes, as described below and illustrated in *Fig. 2.3*. The nodose ganglion is more distal to the cerebellum, and is ovoid in shape with a slightly striated appearance. It is connected directly to the jugular ganglion, which is approximately 4mm more caudal to the cerebellum, and possesses a more triangular shape, with a 'starburst' of connected nerves. The membrane covering the jugular ganglion was carefully shredded to allow the removal of the ganglion, which was then placed in ice cold HBSS
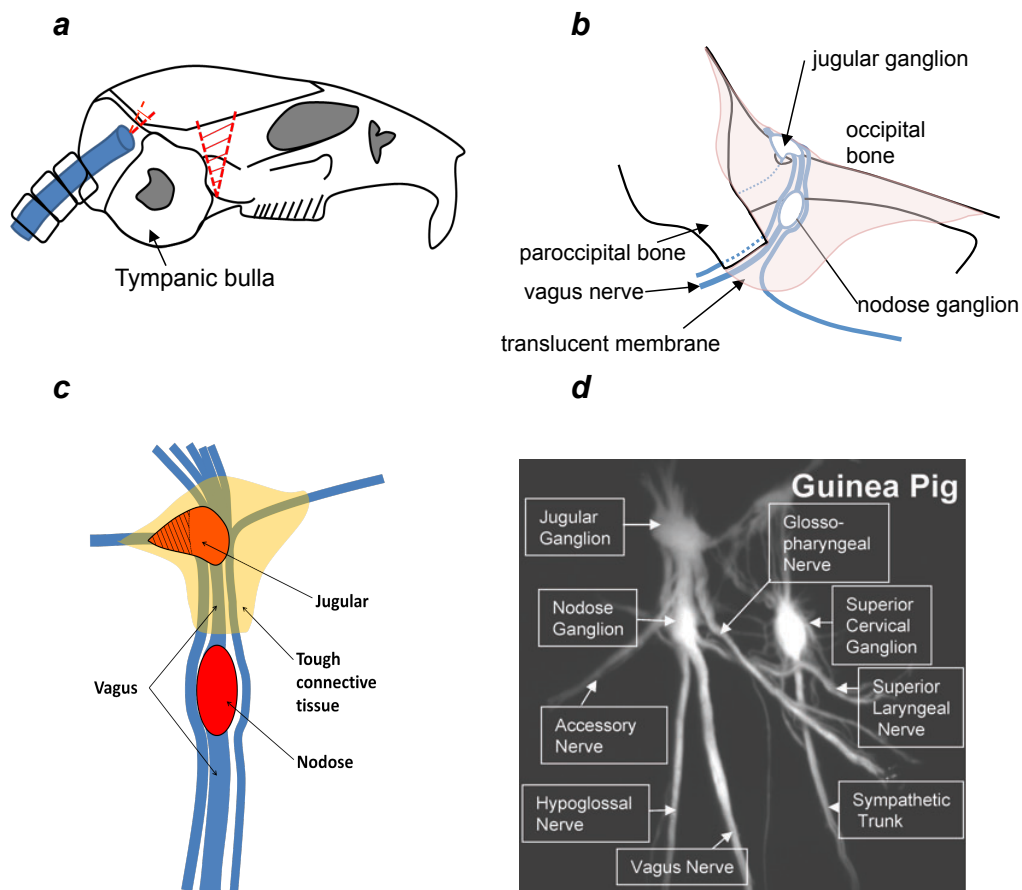
Figure 2.3 Anatomy of guinea pig vagal nerves and ganglia
[A] Location of cuts to remove the ear bone (tympanic bulla). [B] location of jugular and nodose ganglia once the ear bone has been removed. [C] Illustration, and [D] photograph of the shape of the dissected nodose and jugular ganglia (highlighted in red in the illustration). [A-C] reproduced from Dubuis et al. (2013) Curr. Prot. Pharmacol. In press [D] Photograph reproduced from Nassenstein et al. (2010) J Physiol 588 (23) 4769–4783.

2 Methodology

(mM: KCl, 5.33; KH$_2$PO$_4$, 0.441; NaCl, 138; Na$_2$HPO$_4$.$_7$H$_2$O, 0.3; glucose, 5.6; HEPES, 5; pH 7.4). The remaining jugular ganglion underneath the opposite tympanic bulla was then collected in the same manner.

The jugular ganglia were placed onto a glass petri dish in HBSS at room temperature, and were freed of connective tissues under a microscope at 2x magnification. The ganglia were then cut into quarters to expose the inside of the ganglion. In order to isolate sufficient neurons, both ganglia from a single animal were digested together during the following enzymatic digestion process. A two-step process of incubation with papain and then collagenase/dispase enzymes was used to break down extracellular matric proteins and collagen. Ganglia were then placed in Papain Activating Solution (PAS; L-Cysteine, 0.04%^{w}/$_v$; EDTA, 0.5mM; CaCl$_2$, 1.5mM; in HBSS) which had been pre-incubated with Papain (200U.ml^{-1}) for 30 min at 37°C. The papain solution containing the ganglia was gently agitated every 5min, and after exactly 30min was centrifuged for 2min at 1400rpm and 22°C. The papain solution was then removed carefully, and the ganglia were incubated in a collagenase/dispase solution (collagenase IV, 10mg.ml^{-1}; dispase II, 12mg.ml^{-1}; in HBSS) for 40min at 37°C, again with gentle agitation every 5 min. Cells were then centrifuged again for 2min at 1400rpm and 22°C to allow removal of supernatant. A trituration process was then used to break up any remaining larger (visible) clumps of cells. Cells were resuspended in HBSS, and glass Pasteur pipettes with successively smaller tip sizes (ranging from 1-0.3mm) was used to agitate larger clumps of cells until they could no longer be seen. The solution of cells was then pipetted into L15 medium containing Percoll (20%^{v}/$_v$), which was then centrifuged for 9min, at 1400rpm and 22°C in order to separate cells from any remaining larger cellular fascia. Supernatant was aspirated and the pellet was resuspended in L15 medium, before another being centrifuged again for 3min at 2300rpm and 22°C. The supernatant was then removed, and cells were resuspended in complete F-12 medium (containing 10%^{v}/$_v$ FBS, 100U.ml^{-1} penicillin, and 0.1mg.ml^{-1} streptomycin). Plastic cell culture dishes (PA, US) used for fluorescence microscopy (hereafter referred to as fluorodishes) were prepared 24h previously, by applying (0.01%^{w}/$_v$) poly-d-lysine for 2h, followed by laminin (31.25µg.ml^{-1}) for 22h, both in HBSS, to the base of the fluorodishes 24h prior to isolation of neurons, in order to aid cell adherence. The remaining laminin solution was then removed and the base of the dish was washed with HBSS, before 20µl of resuspended cells in complete F-12 was carefully placed into the centre of the fluorodish. The cells were incubated for 2h at 37°C (95% O$_2$, 5% CO$_2$) to allow adherence to the Poly-D-Lysine/laminin-coated centre of the fluorodish, before a further 2ml of complete F-12 media was added and cells were incubated overnight at 37°C (95% O$_2$, 5% CO$_2$).

2.4.3 Fluorescence imaging dyes

Within 24h of isolation, neurons were loaded with the calcium-binding fluorophore Fura2. Fura2 was selected to avoid overlapping excitation and emission wavelengths with the DiI already present in the cell membranes of the airway terminating neurons (*Table 2.1*).

Dye name	Localised to	Responds to	Max excitation λ	Max emission λ
DiI	Plasmalemma	n/a	550nm	570nm
Fura2 (Ca^{2+}-bound)	Cytoplasm	Ca^{2+} binding	340nm	520nm
Fura2 (unbound)	Cytoplasm	Ca^{2+} binding	380nm	480nm

Table 2.1 Maximum excitation/emission wavelengths for imaging dyes

Fura2 is a ratiometric Ca^{2+}-binding dye with two excitation/emission profiles for its Ca^{2+}-bound and -unbound states (*Table 2.1*). Both Ca^{2+}-bound and Ca^{2+}-unbound excitation wavelengths were used in fast sequence to generate the two respective emission profiles, and the levels of the two emissions related, to give an arbitrary unit of Ca^{2+}-bound Fura2 dye as a proportion of the total Fura2. The use of a ratiometric dye negates the variability associated with local differences in concentration of the dye and thickness of the cell. Cells were incubated with 3µM Fura2-AM in extracellular solution (ECS – KCl, 5.4; NaCl, 136; $MgCl_2$, 1; $CaCl_2$, 1.8; NaH_2PO_4, 0.33, D-glucose, 10; HEPES, 10; all mM in dH_2O; pH to 7.4 at 37°C with NaOH) for 40 min at room temperature in the dark. Fura2-AM is the esterified form of Fura2, which is hydrophobic, allowing passive movement through cell membranes into the cytoplasm. Once in the cytoplasm, intracellular esterases present in the cytosol cleave the hydrophobic AM (acetoxymethyl ester) group, leaving Fura2, which is then unable to pass back out through the cell membrane. The Fura2-AM solution was then aspirated, and the cells were incubated with ECS for a further 30min in the dark at room temperature, to allow the dye to be fully de-esterified (Takahashi *et al.*, 1999).

2.4.4 Apparatus/recording principles

Once neurons had been isolated from jugular ganglia and loaded with fluorescent dyes, they were then placed onto the stage of an inverted epi-fluorescence type microscope (Zeiss Axiovert 200, Carl Zeiss Microscopy, UK). The microscope was mounted on an anti-vibration table (TMC 63-500, Scientifica, UK) and the microscope stage was surrounded by an incubation chamber (XL-3 Incubator, Carl Zeiss Microscopy, UK) maintained at 37°C and 5% CO_2. An epi-illuminator (HBO 50 100W Mercury/Xenon arc

lamp, Carl Zeiss microscopy, UK) and a tungsten-halogen lamp with heat filter (HXP 100, Carl Zeiss microscopy, UK) were used as light sources for bright-field phase contrast illumination. Sets of dichromatic mirrors and filters (given in *Table 2.2*) were used to excite the fluorescent dyes and record emissions at the appropriate wavelengths (*see Table 2.1*), with an Optoscan monochromator (Cairn Research, UK) used to rapidly change the wavelength of the excitation light.

Dye name	Excitation filter	Mirror	Emission filter
DiI	BP 531/40	BS 565	BP 593/40
Fura2 (Ca^{2+}-bound)	BP335/7	BS 410	BP 520/10
Fura2 (unbound)	BP 387/11	BS 410	BP 520/10

Table 2.2 Filters and mirrors used to excite and record emissions from fluorescent dyes (Carl Zeiss Microscopy, UK)

Neurons were observed using an air objective 20x magnification lens (LD Plan-Neofluar KORR air objective 0.4*f* aperture 7.9mm focal distance, Carl Zeiss Microscopy, UK). A cooled fast-acquisition CCD camera was used to view and record images from the microscope on a computer using the Simple-PCI software suite (Hamamatsu Corp., USA). Simple-PCI was also used to control the filters and shutter during the experimental protocol. Focus was locked during the experimental recording, as variations in focus may cause differences in the light intensity recorded. In order to avoid photo-bleaching – degradation of the dye when exposed to light for longer periods – exposure times of 200ms (Ca^{2+}-bound) and 10ms (Ca^{2+}-unbound) were used for excitation of Fura-2 during the experiment. The software was set to alternate the filters to expose the neurons in sequence to the excitation light for Ca^{2+}-bound Fura-2, then immediately afterwards (within 100ms) to Ca^{2+}-unbound Fura-2, recording the emission from each excitation as an image. One image was taken of emission from Ca^{2+}-bound and Ca^{2+}-unbound every 5s (or 30s during longer incubation periods) during the experimental protocol described below. In this way, two parallel series of both Ca^{2+}-bound and Ca^{2+}-unbound Fura-2 emissions were recorded over the course of the experimental protocol.

Within the incubation chamber, a perfusion system for the delivery of ECS containing the different compounds to be used was set up. This custom-made delivery system was designed to rapidly switch between perfusion of different compounds, allowing a complete change of the solution bathing the cells in 3s, whilst maintaining a constant volume of 600µl in the fluorodish (*Fig. 2.4*). All solutions were maintained at 37°C and 5% CO_2 during the experimental protocol described in the following section.
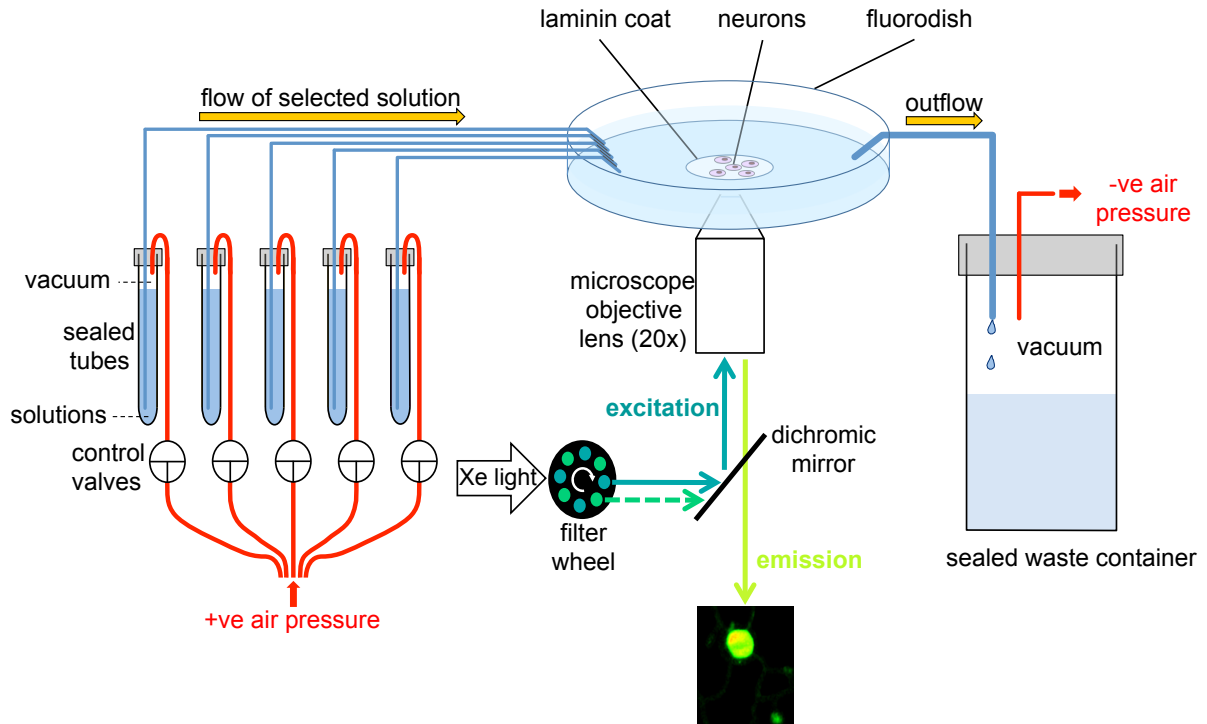
Figure 2.4 Diagram of apparatus for $[Ca^{2+}]_i$ imaging of isolated neurons
Isolated jugular ganglion neurons adhering to a laminin-coated fluorodish were placed onto a microscope stage. Airway-terminating neurons were identified by shape, size and presence of DiI staining. The microscope was set to record a series of time lapse images of Fura-2 calcium dye emission, whilst ECS alone, or ECS containing dissolved compounds was perfused from sealed tubes into the fluorodish by positive air pressure. Solutions were removed from the opposite side of the fluorosdish to a sealed waste container using negative air pressure, maintaining a volume of 600 µl in the fluorodish. All equipment illustrated, except filter wheel, dichromic mirrors and sealed waste container were within an incubator hood maintained at 37°C and 5% CO_2.

2.4.5 Measurements/recording protocol

Fluorodishes of isolated jugular ganglion cells were mounted on the microscope stage, and neurons were focused in the field of view using bright-field illumination at 20x magnification. Cells were then excited with the correct wavelength of light for DiI, in order to identify those cells that contained the dye, and were therefore the cell bodies of airway-terminating neurons. The field of view was then adjusted as required to fit as many airway-terminating cells in one field as possible. Once the field of view had been selected, the focus was adjusted and locked for the recording protocol. The Simple PCI software was then set to record a series of time-lapse images of Fura-2 emissions as described in the previous section.

Neurons were first exposed to ECS containing 50mM of potassium chloride (K_{50}: KCl, 50; NaCl, 91.4; $MgCl_2$, 1; $CaCl_2$, 2.5; NaH_2PO_4, 0.33, D-glucose, 10; HEPES, 10; all mM in

dH_2O; pH to 7.4 at 37°C with NaOH) for 15s, which induces a depolarisation of the cell membrane, via an increase in intracellular calcium. Neurons were then perfused with ECS ('wash') until Fura-2 emission levels returned to baseline. This initial stimulation with K_{50} was used as a control response, to which all further responses were normalised. Neurons were then perfused with the tussive agent for 30s, to induce an increase in intracellular calcium, which was washed to baseline by perfusion with ECS. A second response to the tussive agent was then induced in the same manner, before the potential anti-tussive drug being tested was perfused and then incubated with the cells for an appropriate period (incubation times varied for each compound – see relevant results chapter for details). Following incubation, both tussive agent and test compound were perfused together for 30s, following which any response was washed by perfusion with ECS for 5min. The neurons were then perfused with the tussive agent alone for 30s, with this final tussive agent stimulation serving to demonstrate that the test compound can at least be partially washed off. Finally, after a wash to baseline with ECS, another K_{50} stimulation was performed, and this final stimulation with K_{50} was compared to the initial K_{50} response, in order to confirm that the neurons remained viable at the end of the experiment. Only one concentration of any single test compound was examined on any single piece of nerve, from any one individual animal or patient.

2.4.6 Data recording

As described in *Methods 2.5.4*, data was recorded by Simple-PCI software as two parallel set of images (image stacks) showing, respectively, emissions of Fura-2 excited at the relevant wavelengths for Ca^{2+}-unbound and Ca^{2+}-bound forms of the dye. In order to quantitate the levels of emission from individual cells, each cell was individually selected using ImageJ (64-bit, version 1.43u, NIH, USA), along with a representative equivalent size area of background. The software then quantified the light emission level of each selected area for each image in the sequence (each frame), and the data was output to MS Excel (version 14.2.5, Microsoft, USA). The emission value for each cell then had the background light level from the same frame subtracted. For each cell, this left two series of quantified numbers for emission levels of Ca^{2+}-unbound and Ca^{2+}-bound forms of Fura-2. The light emitted by Ca^{2+}-bound Fura-2 was then divided by the light emitted by Ca^{2+}-unbound Fura-2 to generate a sequence of values for each frame in the recording. The use of this ratio corrects for any degradation of the dye due to photo-bleaching over longer recordings, and also for inter-experimental differences in loading efficiency of Fura-2. The sequence of ratios was then input to the Origin software package (version 8.6, OriginLabs Corp., USA), along with the time-stamp for each frame, in order to generate a graph of emission ratio over time. The graph was then used to

calculate Area Under the Curve (AUC) for each response to tussive agent stimulation. AUC was preferred over peak magnitude to take into account the multiphasic shape of the calcium responses.

All AUC values for each cell were then normalised as a percentage of the size of the initial K_{50} response for the same cell, as described in *Methods 2.9.1*.

2.5 *In vivo* single afferent fibre recording

The general premise of this technique was to assess the effect of potential anti-tussive compounds on the *in vivo* firing activity of a single afferent airway-terminating C-fibre in response to capsaicin. This technique has the advantage of the compounds acting on the nerve termini, where action potentials would normally be evoked by tussive stimuli, and measuring nerve firing as an end-point, compared to the isolated vagus nerve and neuron experiments described previously.

2.5.1 Surgery and apparatus

Male Dunkin Hartley guinea pigs (400–750g, Harlan, UK) were anaesthetised with urethane (*i.p.* 1.5g.kg^{-1}), supplemented with additional urethane as required. The animal was then ventilated, and surgery was performed to setup the nerve fibre recording system, as described below and in Adcock *et al.* (2003), and summarised in *Figure 2.5*. The trachea was cannulated to maintain blood gases and pH at physiological levels by artificial ventilation (Ugo Basile small animal ventilator – tidal volume 10ml.kg^{-1}, 50-60 breaths.min^{-1} room air). A nebuliser (Aerogen nebuliser, Buxco) was connected via a side arm in line with the ventilator and tracheal cannula, for the delivery of aerosolised vehicle or drugs to the airways. The right carotid artery was cannulated (passed to the ascending aorta/aortic arch) to continuously measure systemic arterial blood pressure and heart rate using the Spike2 software data acquisition system via a CED Micro1401 interface. The same software also recorded tracheal pressure from a pressure transducer connected to a side arm of the tracheal cannula. Body temperature was monitored with a rectal thermometer, and maintained at 37ºC with a heated blanket and control unit (Harvard Apparatus, UK). Animals were paralysed by vecuronium bromide, initially administered at a dose of 0.10 mg kg^{-1} (*i.v.* into cannulated right jugular vein), followed every 20min with 0.05mg kg^{-1} *i.v.* to maintain paralysis. The depth of anaesthesia was assessed throughout the experimental protocol by monitoring heart rate and blood pressure.

A cervical incision was made to locate the cervical vagus nerves, which were dissected free from the carotid artery, sympathetic and aortic nerves and then cut at the central end. The left vagus nerve was cleared of its surrounding fascia for sensory nerve fibre recording. The skin and muscle in the neck around the cervical incision were tied to a metal ring to form a well, which was filled with light mineral oil. Thin filaments of nerve
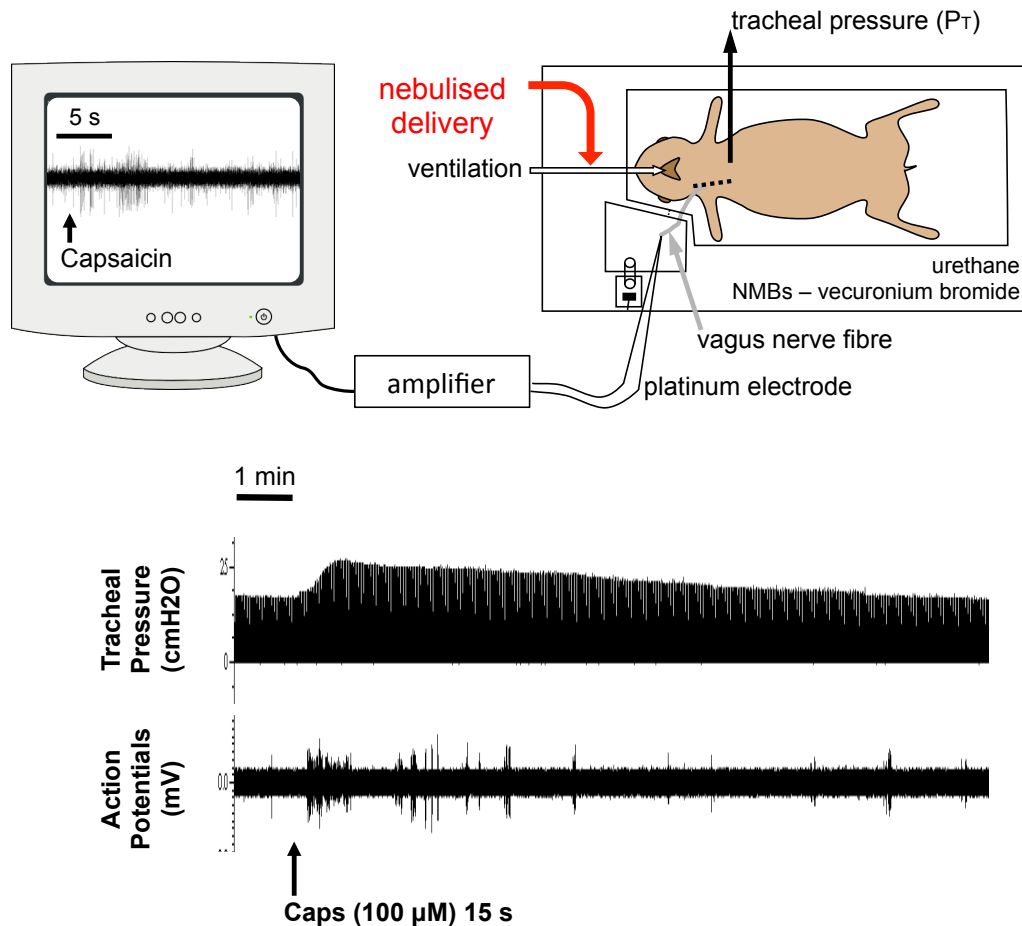
Figure 2.5 Diagram of apparatus for single fibre nerve recordings
Top: Anaesthetised guinea pigs were carefully dissected to reveal the vagus nerves, which were both cut posterior to the vagal ganglia. The left vagus nerve was teased, and a single nerve was placed onto a platinum recording electrode, connected to a digital oscilloscope, outputted to Spike2 software on a computer to record nerve action potentials. Animals were ventilated and tracheal pressure was monitored via an intra-tracheal cannula. Nebulised drugs were delivered via a side-arm of the tracheal cannula. Bottom: representative traces showing effect of capsaicin on tracheal pressure and action potentials.

were teased from the vagus nerve, under a binocular microscope, until a single nerve fibre was left. Bipolar Teflon-coated platinum electrodes (exposed at the tips) were used for recording purposes, with the single nerve fibre positioned on the recording electrode, and the reference electrode placed on the nerve fascia. Electrodes were connected to a pre-amplifier head-stage (Digitimer NL100K) to record action potentials. The signal was amplified (Digitimer NL104), filtered (Digitimer NL125) and passed through a Humbug noise reducer (AutoMate Scientific) before input into the Micro1401 interface and the Spike2 software and monitored on a digital storage oscilloscope (Tektronix DPO 2012). The software allows pulse train counting over selected time periods. The input signal is also fed through an audio amplifier to a loud speaker.

Conduction velocities were measured to distinguish slow-conducting non-myelinated C-fibres from fast-conducting myelinated Aδ-fibres. Conduction velocity was measured by stimulation of the vagus nerve close to the thorax with bipolar silver electrodes, using a supra threshold voltage at 0.5ms, 1Hz (Grass stimulator) and relating the time from stimulus to response to the measured distance between stimulating and recording electrode.

2.5.2 Nerve fibre identification

As discussed in *Introduction 1.2*, sensory nerves innervating the lungs have been classified as belonging to one of several categories of nerve fibre type. Single vagal nerve fibres in the recording apparatus described here can be identified as belonging to one of three groups of airway sensory nerve endings, i.e., slowly adapting stretch receptors (SARs), irritant receptors (rapidly adapting stretch receptors, RARs, Aδ-fibres) and pulmonary/bronchial C-fibre receptors using several criteria (Adcock *et al.*, 2003). These criteria include pattern of spontaneous discharge, response to hyperinflation and deflation, adaptation indices (AIs), response to capsaicin aerosol administration and conduction velocities. As a rule, a receptor that possessed no obvious pattern to the spontaneous activity (often very sparse), did not respond to hyperinflation/hyperdeflation but responded to capsaicin aerosol was used for experimentation. At the conclusion of the experimental protocol, verification of a C-fibre was confirmed by determination of conduction velocity as described above.

2.5.3 Measurements/Recording protocol

After surgery, and identification of a capsaicin-sensitive fibre that was quiescent to overinflation/deflation, as described above, the following protocol was used to record inhibition of capsaicin-evoked responses by the potential anti-tussive compounds.

After a baseline recording of at least two minutes, capsaicin (100µM) was administered by aerosol for 15 s, and the changes in nerve fibre firing, intratracheal pressure and blood pressure were recorded until baseline levels were re-established. After an interval of 10 min, vehicle (0.1% DMSO in PBS) was administered by aerosol for 1min while recording variables. After another interval of 10 min, the capsaicin aerosol (15 s, 100 µM) was repeated (internal vehicle control response). After another 10 min interval, either vehicle (0.1% DMSO in PBS) or anti-tussive compound (*see Sections 3.2.2 & 4.2.2*) was administered. A final capsaicin aerosol (15 s, 100 µM) was administered 20min after vehicle/drug administration.

2.5.4 Data Recording

Action potentials and tracheal pressure were recorded using the Spike2 software package. This software package was used to output data into MS Excel (Microsoft, USA), which was then used to calculate % inhibition of capsaicin responses, as described in the equation in *Methods 2.9.1*.

2.6 *In vivo* cigarette smoke exposures

Guinea pigs were exposed to cigarette smoke (CS) from research cigarettes (3R4F - with filters removed; University of Kentucky, KY) for 1h, twice daily for 8 days, as shown in *Figure 2.6*. Animals had *ad libitum* access to food and water at all times, except during 1h CS exposures.
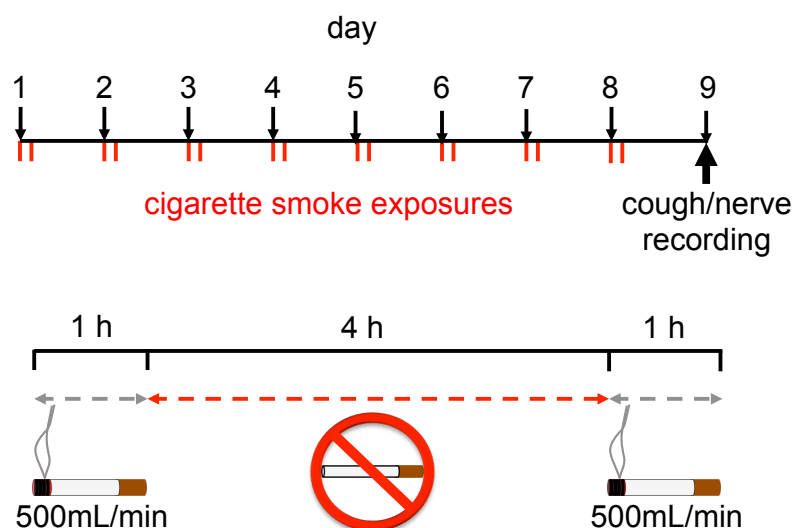
Figure 2.6 Diagram of timings of cigarette smoke exposures
Animals were exposed to cigarette smoke twice daily (top panel), with a 4 hour gap between exposures (bottom panel), before animals were used for either in vivo cough studies or in vitro vagus nerve recordings.

The smoke was generated using a negative pressure system to draw air through lit research cigarettes, as illustrated in *Figure 2.7*. A pump was used to draw air, at a constant flow-rate of 1.5L.min^{-1}, through a pinch-valve system. The pinch valve system was set to draw from a lit cigarette for 2s, with room-air being continuously drawn into the chamber for the remaining period (4s). This generated a mock 'puff' on the cigarette, with each 6s 'cycle' drawing 50ml of smoke followed by 100ml of room air. When the lit cigarette burned to the writing on the lower end, a new cigarette was placed in the pinch-valve and re-lit manually. The air and smoke from the pinch valve system was drawn into a Teague chamber (255L volume) housing the animals, where a fan at the bottom of the chamber dispersed the smoke evenly throughout. Air extracted from the chambers was vented from the facility. For air-exposed negative-control comparator groups, the same protocol was applied, except the cigarette was not lit. Each exposure period of one hour consisted of 50 minutes of 'active' smoke exposure, with 10 minutes at the end allocated for venting of cigarette smoke, when the flow was increased to maximum and set to draw room air only.

Tobacco Smoke Particulate (TSP) levels were assessed at the midpoint of each exposure, to ensure consistency in the levels of cigarette smoke particulates. To assess TSP, a volume of air was drawn from the exposure chamber, over 1min, through a borosilicate glass microfiber filter (Pallflex, Pall Corporation, UK). The TSP value was calculated as the ratio of the change in filter weight compared to the volume of air drawn through the filter. Animals were weighed prior to the first exposure and on cull days.
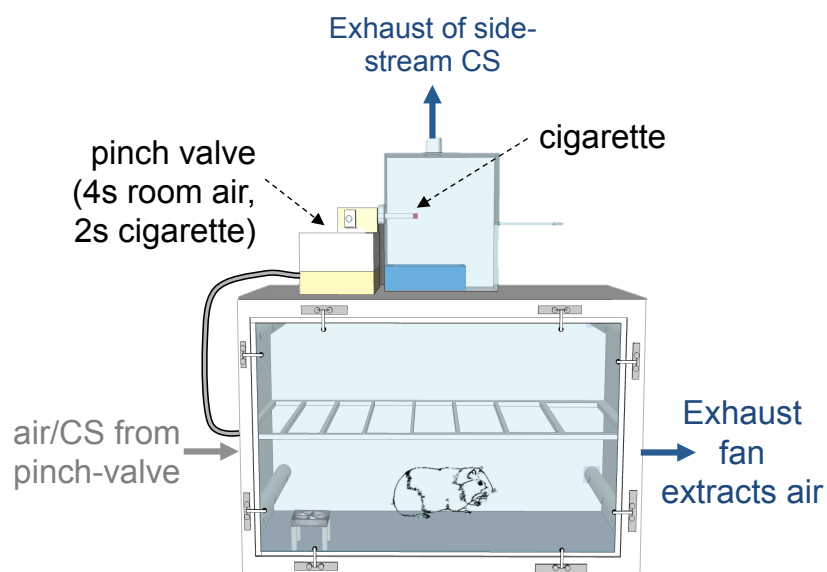
Figure 2.7 Diagram of apparatus for CS exposures
Animals are housed in open cages (not shown), which are placed in a larger chamber, sealed except for inlet and exhaust fixings. A lit cigarette is placed in one of the pinch valve openings, and air is then drawn by negative pressure alternately from the room (4 s) or through the cigarette (2s). The air and CS passing along tubing into the chamber containing the animals, and is then distributed by a small fan. The exhaust fan then pulls the air/CS mixture across to, and then out of, the other side of the chamber.

2.7 Provision and genotyping of knock-out mice

2.7.1 Knock-out mice

Mice homozygous for mutated alleles of the β_2-adrenergic receptor were kindly provided by Professor Harding, NHLI, Imperial College (see Nikolaev *et al.*, 2010).

Mice homozygous for mutated alleles of the β_3-adrenergic receptor were kindly provided by Professor Lowell, via Johns Hopkins University, US (see Mantzoros *et al.*, 1996).

Mice containing at least one mutated AC6 allele were kind gifts provided by Dr Hammond of the University of California, San Diego. Breeding pairs of mice containing at least one mutated allele were bred in-house at Imperial College to generate a colony of *adcy6*$^{-/-}$ mice. Mice were confirmed to be homozygous for the mutant (KO) allele by amplifying the KO sequence using PCR and visualising the product on an agarose gel by electrophoresis.

2.7.2 DNA extraction

DNA was extracted from tail tips taken from mice using an extraction kit ('Extracta DNA prep for PCR', Quanta, UK) according to the manufacturers instructions. Briefly 0.5cm of tail was removed and placed into an eppendorf tube, and 75µl of extraction buffer was added to each tube. The tail samples in buffer were then heated (95°C; 30min) before being allowed to cool to room temperature. Then 75µl of stabilization buffer was added to the sample tube, and the concentration of DNA in the samples was assessed by spectrophotometry at wavelengths of A260/A280 on a GeneQuant RNA/DNA quantifier (Amersham Pharmacia, UK). Samples were then adjusted to 10ng.ml^{-1} by dilution with nuclease-free water.

2.7.3 Genotyping of samples by Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is used to amplify a select sequence of DNA between a pair of primers. Nuclease-free water was added to a reaction mixture containing 50ng extracted DNA sample (5µl), reaction buffer (5µl), 0.2mM dNTPs (1µl), 2mM MgCl$_2$ (2µl), 1.25U Taq polymerase (0.2µl), and forward and reverse primers (1µl each), to give a total reaction volume of 25µl (reagents purchased from Applied Biosystems, UK). The reaction tubes were placed into an ABIprism 96 well plate (Applied Biosystems, UK), and the PCR reaction was run in an ABIprism 7000 PCR machine (Applied Biosystems, UK). The

samples were heated to 94°C (2min) to denature the DNA strands, followed by 40 cycles of three alternating temperatures to allow annealing of primers, extension of primers by the Taq Polymerase enzyme, and denaturing of DNA strands to allow further amplification upon repeating of the temperature cycle. The temperatures and timing of the cycles are specific to the primers used, and the length of the expected DNA product; details of forward and reverse primers and reaction times and temperatures are given in the methods section of the relevant chapter. The number of cycles was selected to maximise the amplification of the DNA product without increasing non-specific Taq polymerase activity that can occur with greater numbers of cycles (Kramer & Coen, 2001). The PCR reaction was stopped by cooling the reaction to 4°C.

2.7.4 Visualisation of PCR product by electrophoresis

The PCR product was visualised alongside a relevant DNA ladder by agarose gel electrophoresis. The samples were placed into wells on an agarose (2%) gel in Tris Borate EDTA (TBE) buffer containing $0.05\mu g.ml^{-1}$ Safeview Nucleic Acid Stain (NBS Biologicals Ltd, UK). A voltage of 80V was applied to the gel for 1h, and the stained DNA was then visualized under UV light and photographed. The DNA ladder used was dependent on the size of the expected product.

2.8 Measurement of mRNA levels to assess gene expression

2.8.1 Principles of gene expression assessment

In order to assess the expression of specific target genes in vagal ganglia cells, quantitative, or real-time polymerase chain reaction (q-PCR) was performed on RNA extracted from mouse ganglia cells.

DNA is essentially a stable repository of all the information required to make any of the proteins of any cell type in an individual organism. DNA encodes the information required to make proteins in sequences of nucleotide base pairs. In order for a protein to be expressed in a cell, the DNA sequence encoding that protein must be transcribed to messenger RNA (mRNA). Like DNA, mRNA encodes information in nucleotide base pairs (with some differences), but unlike DNA can leave the nucleus. This allows mRNA to bind with ribosomes in the cytosol and on the endoplasmic reticulum (via leader sequences on the 5' end of the mRNA). Ribosomes translate mRNA sequences into amino acid sequences, thereby creating proteins. Therefore assessing the expression of specific mRNAs in a cell type can be used as an approximate proxy for the expression of a protein in the same cells. The major caveat to this is that protein expression can be suppressed via other regulatory processes in the cell, despite the expression of it's corresponding mRNA sequence. However, in general, whilst levels of mRNA expression do not always correlate well with levels of protein expression, expression of a specific mRNA gives an indication that a specific protein is expressed in a cell type (Gry *et al.*, 2009; Vogel & Marcotte, 2012).

In order to assess the level of gene expression in ganglia cells, RNA was first extracted from those cells by dissolution of the cell membranes, according to a protocol designed to ensure that the RNA is not degraded or contaminated (*see Methods 2.8.2*). Once the levels of mRNA had been normalised to a known level, all the mRNA sequences in a sample were then transcribed into complementary DNA sequences (cDNA), using a reverse transcription enzyme (*see Methods 2.8.3*). The production of cDNA provides a more stable transcript of the original mRNA sequences that can be used with the primers and probes for DNA used in PCR reactions. The expression of target mRNA was then determined by measuring levels of the target cDNA transcript using q-PCR (*see Methods 2.8.4*). This technique involves the use of a reporter dye attached to a specific antisense oligonucleotide probe that binds to the target cDNA strand. As DNA polymerase replicates the cDNA strand, the reporter dye is displaced from the probe, causing enhanced fluorescence of the dye. The level of fluorescence emitted is proportional to

the number of times the cDNA target is replicated, so that the relative expression of the target cDNA can be determined.

2.8.2 RNA extraction

RNA was extracted according to a method previously described by McCluskie et al. (2004). Mouse vagal ganglia were dissected similarly to the method described for guinea pig ganglia dissection (*see Methods 2.4.2*), except in the mouse the jugular and nodose ganglia are fused into a single vagal ganglia instead of being separate entities. The pair of vagal ganglia from each mouse were dissected, cleared of connective tissue and then cut into quarters and transferred to a 2ml Eppendorf tube. All tubes used had been autoclaved in order to ensure that they did not contain active ribonucleases, which would otherwise cause the catalytic degradation of RNA. TRI Reagent (1ml, Sigma, UK) was added to the tubes, which were then mixed by several inversions. RNA, DNA and protein dissolve in TRI Reagent due to its guanidine thiocyanate and phenol composition. Sample tubes were centrifuged (15,000 x g; 15min; 4°C) to remove insoluble material (i.e. extracellular membranes). The supernatant (containing dissolved RNA, DNA and protein) was transferred to a new 2ml Eppendorf tube left to stand (5min, room temperature) to ensure complete dissociation of nucleoprotein complexes. Chloroform (0.2ml, Sigma, UK) was added, and the tubes were vigorously shaken (15s) and left to stand (15min, room temperature) before centrifugation (15,000 x g; 15min; 4°C). Following centrifugation, the solution separated into 3 phases: a dense organic phase containing proteins, an interphase containing DNA, and an aqueous phase containing the RNA. The aqueous phase was removed to a new Eppendorf tube, and was then mixed with approximately 1:10 (v/v) isopropanol (Sigma, UK) and left to stand (5min, room temperature) before being centrifuged (12,000 x g; 10min; 4°C). The resultant supernatant was transferred to a new Eppendorf tube and more isopropanol was added (to give a total of 0.5 ml isopropanol). Samples were inverted once to mix, left to stand (5min, room temperature) and centrifuged (12,000 x g; 10min; 4°C), causing the RNA to precipitate as a pellet. The supernatant was then removed (NB samples were kept on ice from this point forward), 70% v/v ethanol was added to the tube and mixed (5s vortex), centrifuged (12,000 x g; 5min; 4°C), and the supernatant was then removed before the pellet was left to air dry (5min).

RNA pellets were dissolved in 50µl nuclease-free water using a vigorous pipetting action. A small sample was then taken and diluted 1:10 in nuclease-free water in a UV-transparent 96-well plate. The purity and concentration of the RNA samples was measured on a BioTek PowerWave XS microplate spectrophotometer (BioTek) at

wavelengths A260/A280. Samples were then accordingly adjusted to 0.05mg.ml^{-1} using nuclease-free water.

2.8.3 cDNA synthesis

RNA samples (0.5µg in 10µl) were reverse transcribed using TaqMan reverse transcription reagents (Applied Biosystems, UK). The reverse transcriptase master mix contained TaqMan RT buffer (5µl), MgCl$_2$ (5.5mM; 11µl), deoxyNTP mixture (500µM per NTP; 10µl), random hexamers (2.5µM; 2.5µl), RNase inhibitor (0.4 U.µl^{-1}; 1µl) and MultiScribe reverse transcriptase (1.25 U.µl^{-1}; 1.25µl). The final reaction mixture (adjusted to 50µl with nuclease-free water) was incubated using a thermal cycler (10min; 25°C, then 30min; 48°C and 2min; 95°C - model 480; Perkin Elmer, US) to generate cDNA transcripts. cDNA samples were frozen at -80 °C until required.

2.8.4 TaqMan real-time PCR

The cDNA transcripts were prepared for real-time PCR amplification. Each reaction mixture contained TaqMan universal master mix (containing Taq DNA polymerase; 12.5µl), sample cDNA (7.5ng in 3µl), Assay on Demand reagent (contains target gene specific primers and probe; 1.5µl) or 18S internal control (1.5µl) (all reagents from Applied Biosystems, UK). The final reaction mixture was adjusted to 25µl with nuclease-free water, and amplified using an ABI PRISM 7000 TaqMan machine (Applied Biosystems). The amplification protocol comprised 1 cycle for 2 minutes at 50°C, 1 cycle for 10 minutes at 95°C and 40 cycles of 95°C for 15 seconds and 60°C 1 minute. The principle of the TaqMan real-time PCR amplification process is described briefly below.

The premise of the q-PCR technique is that it allows measurement of the levels of specific target cDNA sequences by using fluorescent probes for specific sequences of DNA present in the target gene. These probes are short antisense oligonucleotide sequences that specifically bind to the target cDNA, and have a fluorescent reporter dye attached to their 5' end and a quencher dye attached to their 3' end. In close proximity the quencher dye reduces the fluorescence emitted by the reporter dye, and in the intact probe, the proximity of the two ends with the reporter and quencher dyes bound, is such that the fluorescence emitted by the reporter dye is significantly reduced. In the reaction mixture, if the target cDNA is present, the probe anneals to the specific target sequence on the cDNA of interest, downstream of the primer sequence. The reaction mixture also contains primers for the cDNA of interest and DNA polymerase. The primers bind to

specific sequences on the target cDNA, which allows the binding of DNA polymerase, which then (under the reaction conditions) begins to copy the cDNA downstream of the primer. As the new antisense cDNA strand is extended to the probe, the 5' nuclease activity of DNA polymerase cleaves the reporter and quencher dyes. Without binding to the probe to hold the quencher dye in proximity, the fluorescent emission of the reporter dye increases. The probes and primers are provided in excess, so that the level of fluorescence of the reporter dye increases with each new copy of the cDNA target that is transcribed. In addition to the genes of interest, the levels of RNA expression of a control target were also assessed in the same samples in parallel q-PCR reactions. The control gene selected was 18S ribosomal RNA, a so-called 'housekeeping gene', which is abundantly present in all eukaryote cells and is commonly used as a control for RNA quantification (Suzuki *et al.*, 2000). The fluorescence from the reporter for the target cDNA probe can be compared to the fluorescence from the reporter for the control cDNA probe to give the relative expression of the gene of interest in the sample.

2.8.5 Analysis of q-PCR results

Results were analysed using ABI PRISM 7000 Sequence Detection System software program (Version 1.2.1, Applied Biosystems, UK). A threshold was set manually on the amplification plot within the geometric (linear) phase of amplification, following guidelines supplied by Applied Biosystems. The threshold cycle (C_t) is the cycle number at which the level of fluorescence from a particular reporter exceeds the set threshold, and so C_t values are inversely proportional to the initial copy number, such that a lower C_t value denotes a higher concentration of the target in the initial sample. C_t values given by reporter dye emission from target probes were normalised to the C_t value for the emission from the 18s control probe to give the relative expression of the target mRNA (ΔC_t). In order to take into account the exponential amplification reaction, the expression data was transformed and is presented here as $2^{-\Delta Ct}$.

2.9 Data analysis and statistics

2.9.1 Data Manipulation and Statistical Analysis

Statistical Analysis

The type of statistical analysis used is indicated in figures describing the relevant data. A summary of the types of data analysed with each statistical test used in this thesis is provided below:

- Where data was normalised to an internal control (i.e. isolated vagus nerve, neuron and *in vivo* single fibre experiments), statistical analysis was performed on the raw data rather than the normalised data. In these cases, the analysis used was a paired students t-test, comparing the response in the presence of vehicle or test compound to the initial control response.

- Where a comparison was made between the mean responses in two separate groups of animals (i.e. cough studies), a Mann-Whitney U test was used comparing responses in vehicle-treated to responses in drug-treated groups.

- Where a comparison was made between the mean responses of more than two separate groups of animals (i.e. cough studies with more than 1 dose/drug being tested), a Kruskal-Wallis test was used, with Dunn's post-hoc analysis to compare responses in the vehicle-treated group to responses in each of the drug-treated groups.

Normalisation/presentation of data

Data is presented throughout as mean ± standard error of the mean (SEM).

In both isolated vagus nerve and isolated neuron experiments, data is presented as percentage inhibition – that is to say the magnitude of a response in the presence of vehicle or test compound compared to the magnitude of the respective internal control response. The data is modified for presentation here in order to reduce and/or simplify the number of figures required. As noted above, statistical analysis was performed on raw data, in order to avoid altering the distribution of the data to be analysed. The equations used to calculate percentage inhibition for isolated vagus and isolated neuron datasets are provided below:

Equation for calculation of percentage inhibition in isolated vagus nerve and single fibre nerve recording datasets:

$$\% \ inhibition = 100 - \left(\frac{Response\ in\ presence\ of\ vehicle/drug}{Mean\ of\ initial\ control\ responses} \times 100 \right)$$

NB Response refers to the magnitude of depolarisation evoked by 2min incubation with the tussive agent being examined in the dataset in question.

Equation for calculation of percentage inhibition in isolated neuron calcium recording datasets:

$$\% \ inhibition = 100 - \left(\frac{Response\ in\ presence\ or\ absence\ of\ vehicle/drug}{K_{50}\ response} \times 100 \right)$$

NB Response refers to the magnitude of depolarisation evoked by incubation with K50 for 30s at the beginning of all experiments.

2.9.2 Data analysis software

Statistical Analyses

All statistical analysis was performed as described above using Graphpad Prism (version 5.0 or 6.0, Graphpad Software, USA) running on the Mac OSX 10.7 or 10.8 operating system (Apple Computers Ltd, USA).

Normalisation of data

Normalisation of vagus and neuron responses to internal controls was calculated as described in the equations above, using Excel for Mac 2011 (version 14.2.5, Microsoft, USA) running on the Mac OSX 10.7 or 10.8 operating system.

Quantification of light emission from Ca^{2+} and voltage-sensitive dyes

Quantification of light emission intensity from individual neurons was calculated using ImageJ64 (version 1.43u, NIH, USA). Area under the curve (AUC) of the light emission data from ImageJ64 was calculated using Microcal Origin (version 6.0, Microcal Software, USA).

Operating Systems

All software was running on the Mac OSX 10.7 or 10.8 operating system (Apple Computers Ltd, USA), except Microcal Origin, which was running on Windows 7 (Service Pack 1, Microsoft, USA).

3. β-adrenergic receptor agonists

3.1 Introduction/Rationale

The short-acting β_2-adrenergic receptor agonists (SABA) terbutaline and fenoterol, sometimes prescribed as bronchodilators, have previously been shown to have a direct anti-tussive effect via an action on vagal sensory nerves; inhibiting capsaicin-induced cough in guinea pigs, and also depolarisation of guinea pig and human vagus nerve (Freund-Michel *et al.*, 2010). The mechanism proposed involved an atypical cAMP/PKG-dependant pathway, where activation of the β_2-adrenoceptor led to an increase in cyclic AMP (cAMP), activating Protein Kinase G (PKG) and the downstream opening of large-conductance calcium-activated potassium channels (BK$_{ca}$) (Freund-Michel *et al.*, 2010).

The aim of this chapter was to follow up these findings by studying the impact of two structurally different, clinically relevant, long acting β_2 adrenoceptor agonists (LABAs), as well as the action of β_1-, and β_3-adrenergic receptor agonists, on sensory nerve depolarisation and cough. The secondary aim of this chapter is to examine the signaling pathways mediating the anti-tussive action of β-adrenergic receptor agonists in sensory nerves.

In order to fulfill these aims, the anti-tussive activity of two LABA compounds was investigated, namely formoterol and olodaterol, which are, in widespread clinical use and in clinical development, respectively. These compounds were evaluated for their ability to inhibit capsaicin-evoked cough and firing of sensory C-fibres in guinea pigs, and also capsaicin-induced calcium responses in guinea pig isolated airway-terminating airway neurons.

The isolated vagal nerve recording system (as described by Freund-Michel *et al.*, 2010) was then used to investigate the mechanism of action of β-adrenergic receptor agonists on sensory nerves, as a much more pharmacologically amenable and cost-effective assay system. In this preparation, the ability of the LABA compounds, as well as β_1-, and β_3-adrenergic receptor agonists to inhibit depolarisation induced by capsaicin, and range of other cough-inducing agents was assessed. Furthermore, using this preparation, the signaling of all of the β-adrenoreceptor agonists was assessed using a range of pharmacological and genetic tools. Initially it was confirmed that the β-adrenergic receptor agonists act through their respective receptor sub-types to exert their effects on sensory nerve depolarisation. Finally, the involvement of adenylyl cyclase and BK$_{Ca}$ in mediating the actions of these agonists on sensory nerves was investigated, as key parts of the signaling pathway proposed to be to be involved in β_2-adrenergic receptor agonists activity on sensory nerves.

3.2 Methods

3.2.1 Determining the effect of LABAs on capsaicin-induced cough in the conscious guinea pig

In order to examine the effect of each of the LABA compounds, male Dunkin Hartley guinea pigs were place in a Perspex chamber to receive either aerosolised vehicle (0.1% DMSO in Phosphate Buffered Saline – PBS), olodaterol (0.3 - 3µg.ml^{-1}), or formoterol (3µg.ml^{-1}) for 10min (approx. aerosolised volume 10ml), 50min prior to challenge with capsaicin. Animals were moved to a separate Perspex chamber attached to a microphone where coughs could be observed and recorded, and Penh (enhanced pause, a measure of bronchoconstriction) levels could be analysed. A baseline recording of Penh and cough was taken for 5min prior to the experiment. Capsaicin (60µM) was then administered by aerosol for 5min, during which time coughs were counted and Penh recorded, as well as for a further 5min post-exposure, using the Buxco Cough Analyser as described in *Methods 2.2* (events depicted as timeline in *Figure 3.1*).
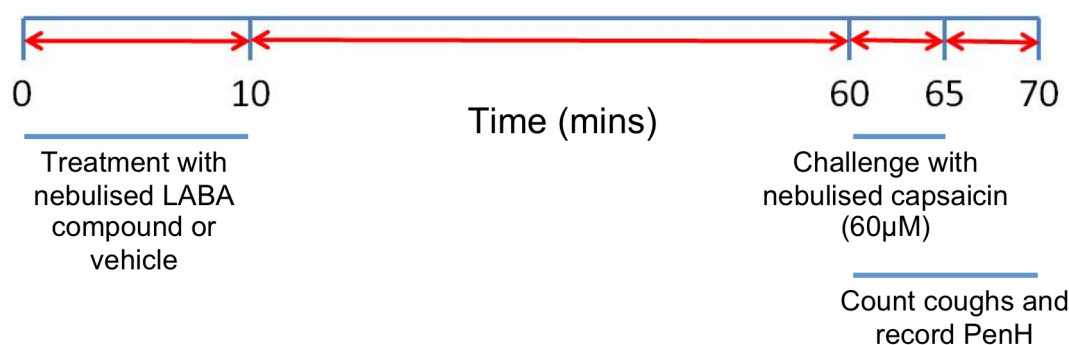
Figure 3.1 Timeline of events in the in vivo cough study
Guinea pigs were treated with nebulised vehicle, formoterol, or olodaterol (n=6) at time 0 for 10min. Guinea pigs then breathed room air with food and water ad libitum for 50min before being placed in a Buxco cough chamber – where cough and Penh were recorded for a total of 10min, 5min while capsaicin was administered and 5min after administration.

The timings, doses and vehicle used in this protocol were selected from Bouyssou *et al*. (2010), where these concentrations of these LABAs were found to be effective at preventing bronchospasm. Note however, the route of administration was changed to inhaled aerosol, as the light anaesthesia required for intratracheal dosing can impact on the cough response.

3.2.2 Determining the effect of LABAs on guinea pig airway-terminating C-fibre firing

The general premise of this technique was to assess the effect of potential anti-tussive compounds on the firing activity of a single afferent airway-terminating C-fibre in

response to capsaicin. To that end, surgery and setup was performed as described in *Methods 2.6* to allow assessment of action potentials generated by capsaicin aerosol in single afferent C-fibres of anaethetised and artificially ventilated guinea pigs. After surgery and setup, the animals were allowed to stabilise for at least 30min. After a control baseline recording of at least 2min, capsaicin (100µM) was administered by aerosol for 15s and changes in fibre activity, intra-tracheal pressure and blood pressure were continuously recorded until baseline or a steady state was re-established. After 10min, the capsaicin aerosol (15s, 100µM) was repeated. After a further 10min, either vehicle (0.1% DMSO in PBS), oladaterol (BI-1744-CL, $3\mu g.ml^{-1}$) or formoterol ($3\mu g.ml^{-1}$) were administered by aerosol to the lungs for 1min. A final capsaicin aerosol (15s, 100 µM) was administered 20min after vehicle/drug administration.

3.2.3 Determining the effect of LABAs on capsaicin-induced responses in isolated airway jugular ganglia neurons

Male Dunkin-Hartley guinea pigs were euthanised, jugular ganglia were removed, and individual jugular ganglia neurons were isolated as described in *Methods 2.4*. The jugular and nodose ganglia contain the majority of the cell bodies for airway terminating vagal neurons. Previous data in our group indicated that a greater proportion of jugular ganglia neurons respond to capsaicin (Grace *et al.*, 2012). For this reason only jugular neurons were used to examine the inhibitory effects of LABA compounds.

As described previously (*Methods 2.4.1*) guinea pigs had been i.n. dosed with DiI, a retrograde tracer dye, therefore airway-terminating neurons isolated from these animals could be identified by the DiI present in their plasma membranes. Isolated neurons were incubated (37°C, 5%CO_2) overnight in complete F12 media (containing penicillin and streptavidin), in order to allow adherence to poly-D-lysine- and laminin-coated fluorodishes. Immediately prior to imaging experiments, cells were incubated with the calcium sensitive dye Fura-2AM.

To test the effect of LABA compounds on the release of calcium in the ganglia, cells were acquired using a confocal epi-fluorescence microscope, and sensory neurons were identified by their body characteristics (20-30µM diameter) and light diffraction in phase contrast, whilst DiI emission was used to identify airway-terminating neurons. The fluorodish was connected to a pressurised perfusion system that allowed rapid change of the bath solutions as per the following protocol:

An initial control response to application of K_{50} solution (50mM hyper-potassium solution containing; 50mM KCl; 91.4mM NaCl; 1mM $MgCl_2$; 1.8mM $CaCl_2$; 0.33mM NaH_2PO_4; 10mM D-Glucose; 10mM HEPES; pH adjusted with NaOH to 7.4) was used to assess cell viability, and this response was used to normalise all subsequent responses within each cell. After perfusion with ECS to remove the K_{50} solution and allow recovery of calcium and voltage dye emissions back to baseline, cells were then stimulated twice with capsaicin (1µM) as control responses followed by a wash with ECS to recover baseline levels. The cells were then incubated with vehicle (0.1% DMSO in ECS), formoterol, or olodaterol (both 0.01, 0.1 or 1nM) for 10min, and then re-stimulated with capsaicin in the presence of test compounds. ECS was then perfused for 5min to remove capsaicin and LABA compounds, following which a final capsaicin stimulation and K_{50} application were used to confirm that capsaicin responses recovered once the drug was removed, and that the cell(s) remained viable.

3.2.4 Determining the effect of β-agonists on isolated vagus nerve depolarisation

The following experimental protocol was used to examine the effects of agonists of the different β-adrenergic receptors (subtypes $β_1$, $β_2$, and $β_3$) on isolated vagus nerve depolarisation induced by capsaicin – see *Table 3.1* for details of agonists. Following sacrifice of the guinea pig and dissection of the vagus nerve as outlined previously (*Methods 2.3*), the isolated nerve was allowed to equilibrate for 10min in the grease-gap chamber, following which capsaicin (1µM, Fluka Biochemika, Switzerland) was perfused for 2min, causing depolarisation. The nerve was then 'washed' (perfusion of KH alone) until the signal returned to baseline. Two reproducible responses to the capsaicin were recorded in this way, following which the vagus nerve was incubated with a concentration of vehicle or β-agonist compound as listed in *Table 3.1*. $β_1$- and $β_3$-agonists were incubated for 10min according to a protocol for examining efficacy in this system as

Agonist	Supplier	Target	Vehicle	Concentration	Incubation time
			(0.1%v/v in KH)	*(-log[M])*	*(min)*
Denopamine	Sigma, UK	$β_1$	DMSO	8, 7, 6, 5, 4	10
Formoterol	Sigma, UK	$β_2$	DMSO	10, 9, 8, 7, 6	20
Olodaterol	Sigma, UK	$β_2$	DMSO	10, 9, 8, 7, 6	20
BRL37344	Sigma, UK	$β_3$	dH_2O	8, 7, 6, 5, 4	10

Table 3.1: Concentrations of LABA compounds used on vagus nerve

adapted from Freund-Michel *et al.* (2010). The β_2-agonists were incubated for 20min, a time point selected for maximal efficacy from Bouyssou *et al*. (2010). The vagus was then re-challenged with capsaicin, in the presence of the vehicle or agonist. Following a 'wash' period of 10min, a final stimulation of capsaicin was used to determine tissue viability. On each piece of nerve tissue, only one concentration of any vehicle or drug was examined.

3.2.5 Examining the selectivity and mechanism of action of β-agonists using pharmacology (selective antagonists) in the guinea pig vagus

Selectivity of the agonists for the respective β-adrenergic receptors was examined using specific antagonists for each of the 3 receptors sub-types (*Table 3.2*). The protocol used to examine agonist inhibition was modified by co-incubating antagonists with a selected concentration of the agonists used previously, as follows: Two control responses to capsaicin were generated, and then vehicle (0.1% v/v DMSO) or antagonist (0.1µM, *see Table 3.2*) were incubated for 10min, prior to 20min incubation with vehicle or agonist (single concentrations selected from concentration-response data) in the presence of vehicle/antagonist. The nerve was then stimulated with capsaicin in the presence of both vehicle/antagonist and vehicle/agonist, followed by a 'wash' period of 10min, and a final stimulation with capsaicin alone.

| Antagonist | Supplier | Target | K_d at human β receptor (nM) | | | Concentration |
			β_1	β_2	β_3	
CGP20712A	Sigma, UK	β_1	1.55	776.25	6456.54	0.1µM*
ICI 118551	Sigma, UK	β_2	301.99	0.55	363.08	0.1µM*
L-748337	Tocris, UK	β_3	199.52	158.48	3.16	0.1µM[+]

Table 3.2 Range of agonists and antagonists used on guinea pig vagus
*Concentration selected from affinity values given in table from *(Baker, 2005) and [+](Candelore* et al*., 1999)*

3.2.6 Confirming selectivity of β-agonists using β-receptor knock-out mouse vagus

Wild-type and β-receptor knock-out mice (FVB/N background) were used to confirm the specificity of each β-agonist for the respective β-receptors. β_2-receptor and β_3-receptor KO mice (*Adrb2*^{-/-} and *Adrb3*^{-/-} respectively), along with age-matched controls also on an FVB/N background were kind gifts from, respectively, Professor Sian Harding (Imperial College), and Dr. Lili Barouch (Johns Hopkins Medical School). Unfortunately β_1-receptor mice could not be obtained for these experiments. The experimental procedure was

identical to the protocol used to investigate the β-agonist effect in guinea pig vagus, except that fewer sections of vagus were retrieved (2 vagal nerve sections of 10-15mm) per animal, due to the smaller animal size. The effect of each of the different β-receptor agonists was compared in the wild-type, β_2-receptor -/- and β_3-receptor -/- mice, in order to confirm that only the β_2- and β_3-agonists effect was lost in the respective knockouts.

3.2.7 The role of adenylyl cyclase in β-adrenergic receptor signaling in mouse vagus nerves

Genotyping of adenylyl cyclase knock-out mice

Mice containing at least one mutated (deletion) AC6 allele were kind gifts provided by Dr Tang of the University of California, San Diego. Breeding pairs of mice containing at least one mutated allele were bred in-house at Imperial College to generate a colony of $adcy6^{-/-}$ mice. Mice were confirmed to be homozygous for the mutant (KO) allele by amplifying the KO sequence using PCR and visualising the product on an agarose gel by electrophoresis (see *Methods 2.7.4*). One important note concerning the AC6 KO mice, however, is that the deletion of the AC6 gene, has been shown to increase the degradation of the AC5 isoform (Tang *et al.*, 2008). Therefore, as it is not possible to say that any changes in the functional responses of tissue from these mice are due to AC6 alone, these mice are subsequently referred to as AC5/6 KO mice.

DNA samples extracted from age-matched wild-type (C57Bl/6) and AC6 KO mice were amplified by PCR and visualised on agarose gel by electrophoresis, as detailed in *Methods 2.7*. Primers for the wild-type and KO AC6 allelles were obtained from Invitrogen (UK), based on the sequences (given in *Table 3.3*) published by Tang *et al.* (Tang *et al.*, 2008). Once the samples were heated to 94°C (2min) to denature the DNA strands, 40 PCR cycles of three alternating temperatures were used to amplify the wt and KO AC6 primers. Each cycle consisted of heating to 94°C for 30s, to 58°C for 45s, and 72°C for 3min. Finally the samples were maintained at 72°C for 10min, and then cooled to 4°C for at least 5min to stop the reaction.

Primer target	Primer direction	Primer sequence	Concentration
AC6 wild-type allele	Forward	GGAGACCTAGAGATGGAGTG	10µM
AC6 wild-type allele	Reverse	GCCACTTGTGTAGCGCCAAG	10µM
AC6 KO allele	Forward	AAGATCTGCTTTGTGGGTGC	10µM
AC6 KO allele	Reverse	AGCCACTGGCTCGATTCGCGTGGCG	10µM

Table 3.3 Primers used for genotyping of AC6 wild-type and knock-out mice

The samples were then separated on an agarose gel by electrophoresis for 1h at 80-100V. The expected band for the wt product was 550 base pairs (bp), and samples were run alongside a 50-2000bp ladder (Hyperladder 50bp, Bioline Reagents, UK). The expected band for the KO allele product was 2.2kbp, and samples were run alongside a 500-5000bp ladder (Hyperladder 500bp, Bioline Reagents, UK).

Primers and probes used for quantitative PCR to determine adenylyl cyclase gene (adcy) expression

Mouse ganglia cells were dissected from wild-type C57Bl/6 mice, RNA was extracted from the ganglia and reverse transcribed, and quantitative PCR (q-PCR) was performed to determine the expression levels of adenylyl cyclase mRNA expression in the ganglia (*see Methods 2.8*). The expression of adenylyl cyclase sub-types 1-10 were assessed in the mouse ganglia, and the primers and probes used for q-PCR of these targets are given in *Table 3.4*.

Target gene	Primer/Probe Assay ID (Applied Biosystems)
Adcy1	Mm01187829_m1
Adcy2	Mm00467874_m1
Adcy3	Mm00460371_m1
Adcy4	Mm00475491_m1
Adcy5	Mm00674122_m1
Adcy6	Mm00475772_m1
Adcy7	Mm00545780_m1
Adcy8	Mm00507722_m1
Adcy9	Mm00507743_m1
Adcy10	Mm00557236_m1
Rn18s (18s control)	Mm03928990_g1

Table 3.4 List of primer/probes used for q-PCR analysis of adenylyl cyclase 1-10 sub-type expression levels
Available from: lifetechnologies.com/uk/en/home/life-science/pcr/real-time-pcr/real-time-pcr-assays/taqman-gene-expression

3.2.8 The role of potassium channels in airway sensory nerve depolarisation

The role of potassium channels in the β-agonist effect on sensory nerves was investigated by examining the effect of blockers of potassium channels on β-agonist inhibition of capsaicin. The blockers (and concentrations) used were selected from a previous investigation into the effects of short-acting β-agonists on vagus nerve depolarisation (Freund-Michel *et al.*, 2010). Paxilline (1µM) was used to block large-conductance calcium-activated potassium channels (BK$_{Ca}$), clotrimazole (10µM) to block

intermediate-conductance calcium-activated potassium channels (IK_{Ca}), apamin (1µM) to block small-conductance calcium-activated potassium channels (SK_{Ca}), and glibenclamide (10µM) to block ATP-activated potassium channels (K_{ATP}). The blockers were used on guinea pig vagus nerve in a similar protocol to that used to evaluate the effect of the selective β-receptor antagonists, except vehicle (0.1% DMSO), paxilline, clotrimazole, apamin or glibenclamide were used in place of the β-receptor antagonists.

3.3 Anti-tussive potential of β-receptor agonists

3.3.1 LABA effect on naïve guinea pig cough

Conscious guinea pigs were treated with aerosolised vehicle, olodaterol or formoterol (0.3-3µg.ml^{-1}), and 1h later were challenged with an aerosol of capsaicin (60µM) to evoke cough, and number of coughs and Penh were recorded. Both olodaterol and formoterol at 3µg.ml^{-1} completely abolished the cough response to capsaicin, and olodaterol at 1µg.ml^{-1} also significantly (P<0.05) inhibited capsaicin-cough compared to the vehicle control group (*Fig. 3.2a*). The capsaicin-evoked increase in Penh observed in the vehicle group was inhibited by both olodaterol and formoterol at the effective anti-tussive dose of 3µg.ml^{-1} (*Fig. 3.2b*).
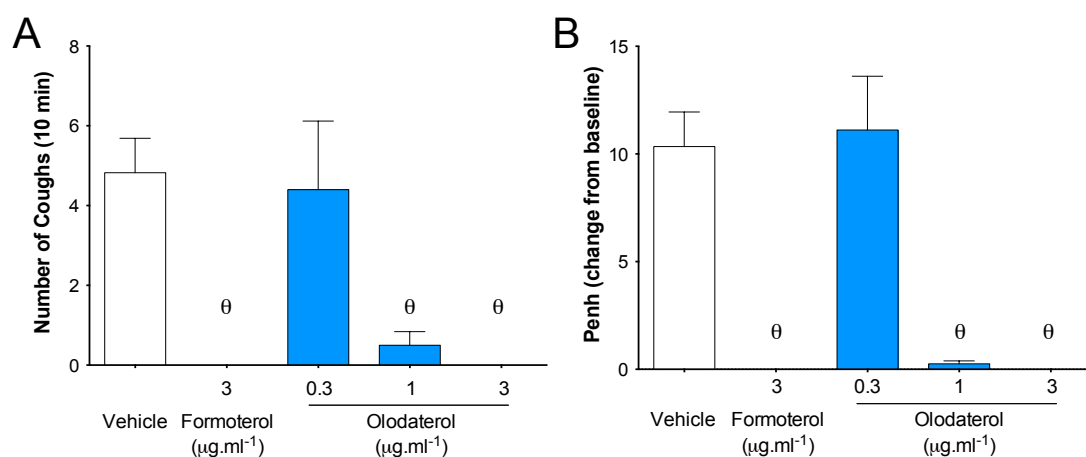
Figure 3.2 Dose response of LABA effect on guinea pig capsaicin-evoked cough and airways obstruction
*Guinea pigs were pre-treated with aerosolised vehicle (0.1% DMSO in PBS), olodaterol (0.3-3µg.ml^{-1}), or formoterol (3µg.ml^{-1}) 1h prior to 5min aerosol of capsaicin (60µM), after which **[A]** number of coughs, and **[B]** Penh, a measure of bronchoconstriction, were recorded for a 10min period (including 5min of capsaicin aerosol plus 5min post-aerosol). Data displayed as mean±SEM, n=5-17, θ p<0.05 as determined by Kruskal-Wallis with Dunn's post-test, comparing all columns to vehicle control.*

3.3.2 Effect of LABA on guinea pig airway-terminating C-fibre firing

The effect of olodaterol and formoterol on capsaicin-evoked firing of airway-terminating, single vagal afferent chemosensitive C-fibres and capsaicin-evoked bronchoconstriction was examined, as shown in the example trace below (*Figure 3.3).*
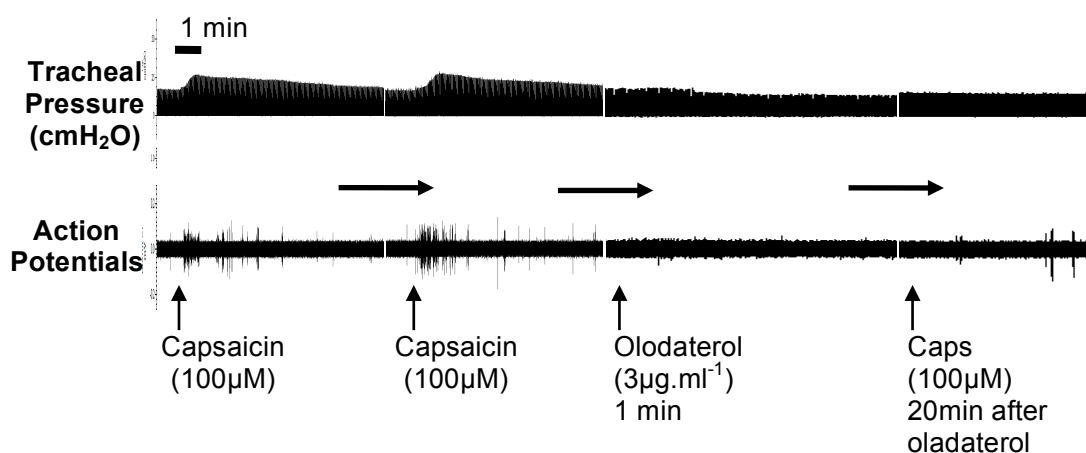
Figure 3.3 Example trace of olodaterol inhibition of capsaicin-evoked action potential in an airway-terminating chemosensitive C-fibre
Example trace showing the effect of olodaterol on capsaicin-evoked action potential firing of an airway-terminating, chemosensitive C-fibre. Lower panel shows action potential firing of the C-fibre, whilst upper panel shows tracheal pressure on same time-scale, with arrows indicating time of drug applications.

At the same dose (3μg.ml^{-1}) that inhibits the *in vivo* cough response, olodaterol and formoterol were both effective at reducing the peak firing rate and number of action potentials evoked by intra-tracheal instillation of a capsaicin aerosol (*Fig. 3.4a&b*). Baseline activity was recorded immediately prior to capsaicin aerosol, and there was little spontaneous activity in these fibres, with maximum impulse rate and total impulses of 5.67±4.26imp.s^{-1} and 1.2±0.59 impulses respectively (data not shown). Both compounds also reduced capsaicin-evoked bronchoconstriction (*Fig. 3.4c*).
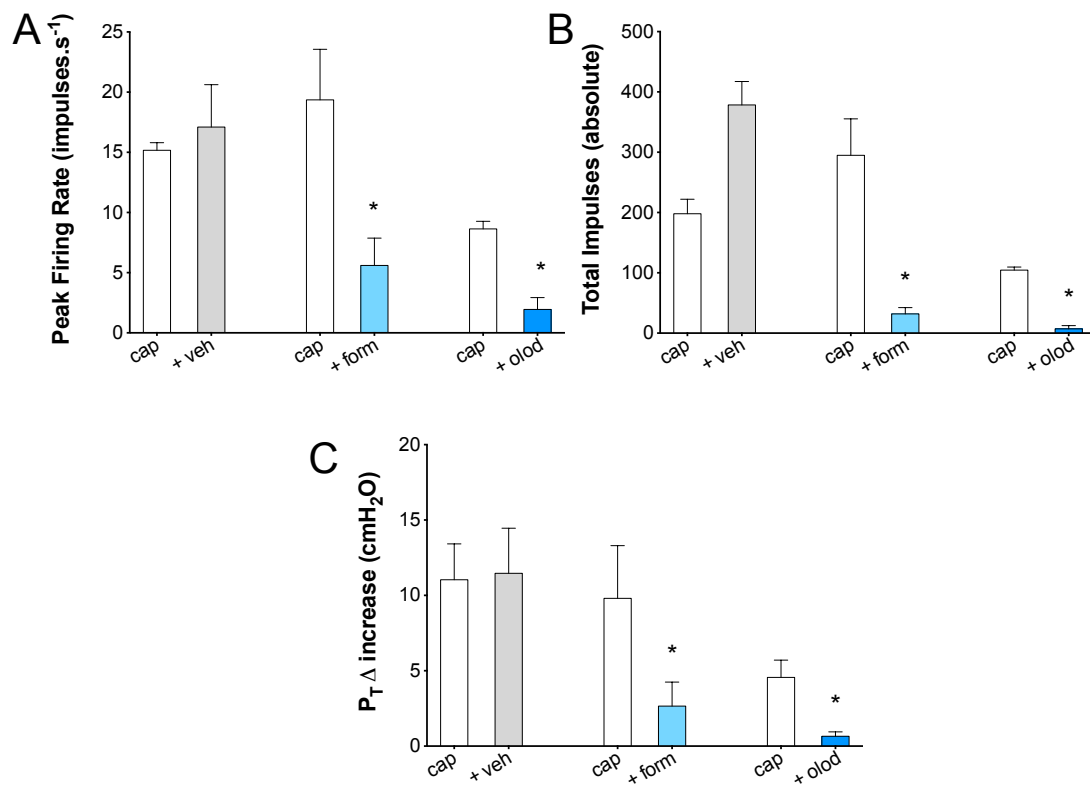
Figure 3.4 Effect of olodaterol and formoterol on capsaicin-evoked action potentials in airway-terminating chemosensitive C-fibres, and bronchoconstriction
All animals were firstly exposed to aerosolised capsaicin (cap; 15s, 100µM) alone, twice consecutively (internal control), and then aerosolised vehicle (0.1% DMSO in PBS), formoterol (form; $3\mu g.ml^{-1}$, 1min), or olodaterol (olod; $3\mu g.ml^{-1}$, 1min) was administered 10min prior to subsequent capsaicin stimulation. Single C-fibre afferent nerve responses were assessed by recording **[A]** firing rate and **[B]** total impulses. Capsaicin evoked bronchoconstriction **[C]** was also assessed. Data displayed as mean±SEM, n=3 where * $p<0.05$ as determined by paired students t-test compared to respective internal control (cap).

3.3.3 β-agonists effect on guinea pig airway isolated neurons

The effect of olodaterol and formoterol on capsaicin-induced intracellular calcium flux was examined in single chemo-sensitive airway-terminating neurons of the jugular ganglia. Both olodaterol and formoterol were effective at inhibiting the capsaicin-induced increase in intracellular calcium levels (*Fig. 3.5*).
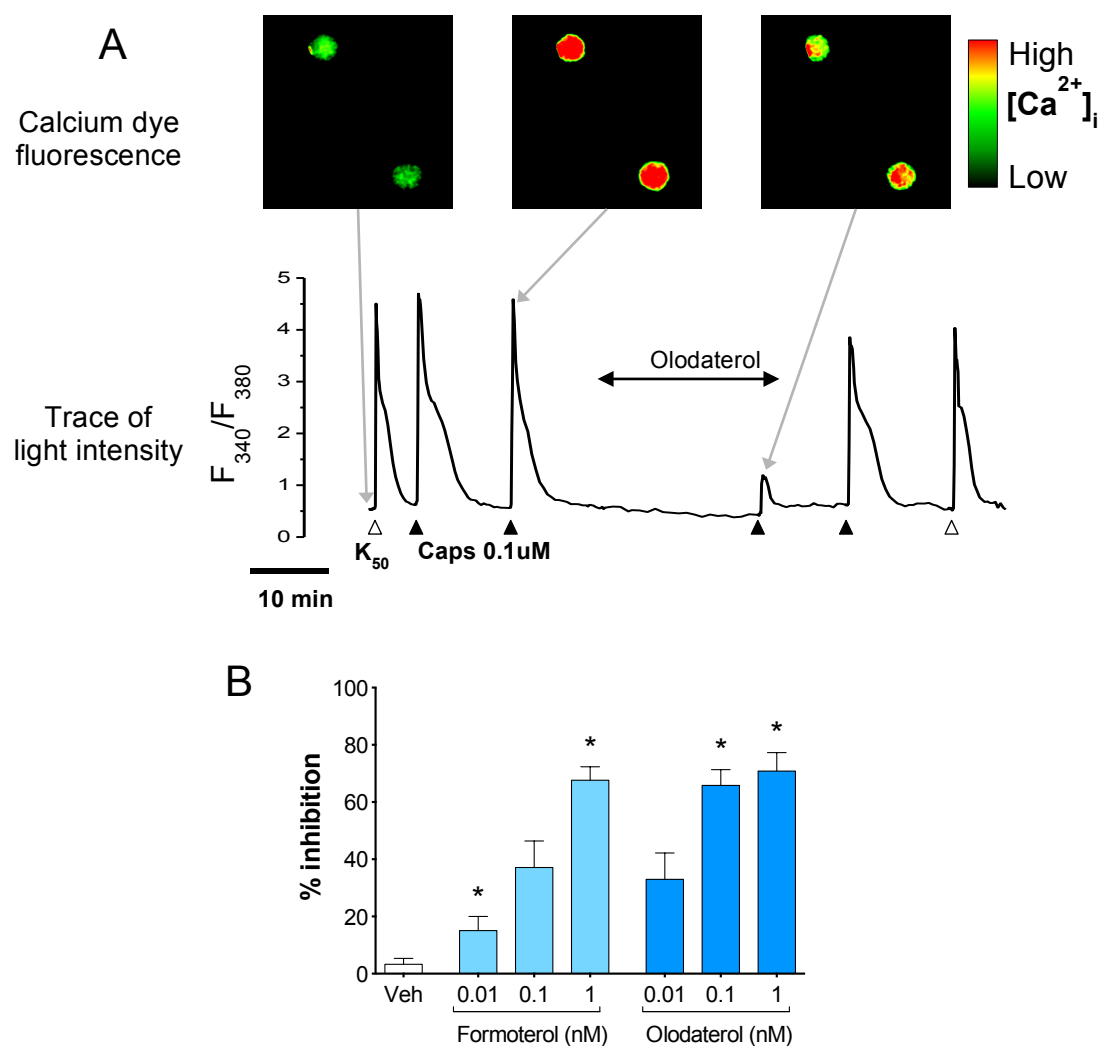
Figure 3.5 Effect of olodaterol or formoterol on capsaicin-induced intracellular calcium flux in jugular airway-terminating neurons
*[A] Representative trace of olodaterol effect on capsaicin-induced intracellular calcium levels. Open triangles indicate K_{50} (50mM KCl in ECS solution, 15s) application, closed triangles indicate capsaicin (caps; 0.1µM, 30s) application, line indicates olodaterol (1nM, 20min) incubation. Images above trace show false-coloured representation of calcium fluorescence at time-points indicated by grey arrows. [B] Summary graph shows the effect of formoterol or olodaterol (0.01-1nM) on capsaicin (0.1µM)-induced increases in intracellular calcium. Data displayed as mean %inhibition of initial capsaicin stimulations (internal control) ±SEM, n=4-10 cells from 3 guinea pigs, * p<0.05 as determined by paired students t-test compared to respective internal control.*

3.4 Mechanism of action of β-agonists

3.4.1 LABA and β₁- and β₃-receptor agonists effect on guinea pig vagus

Denopamine (β_1-receptor agonist), formoterol (LABA), olodaterol (LABA) and BRL37344 (β_3-receptor agonist) each caused a concentration-dependent inhibition of capsaicin-induced sensory nerve depolarisation (*Fig. 3.6*). Notably the LABA compounds were effective at inhibiting capsaicin-induced depolarisation by 50% at much lower concentrations (approx. 10nM) than that of the β_1- (100µM) and β_3-agonists (1µM).
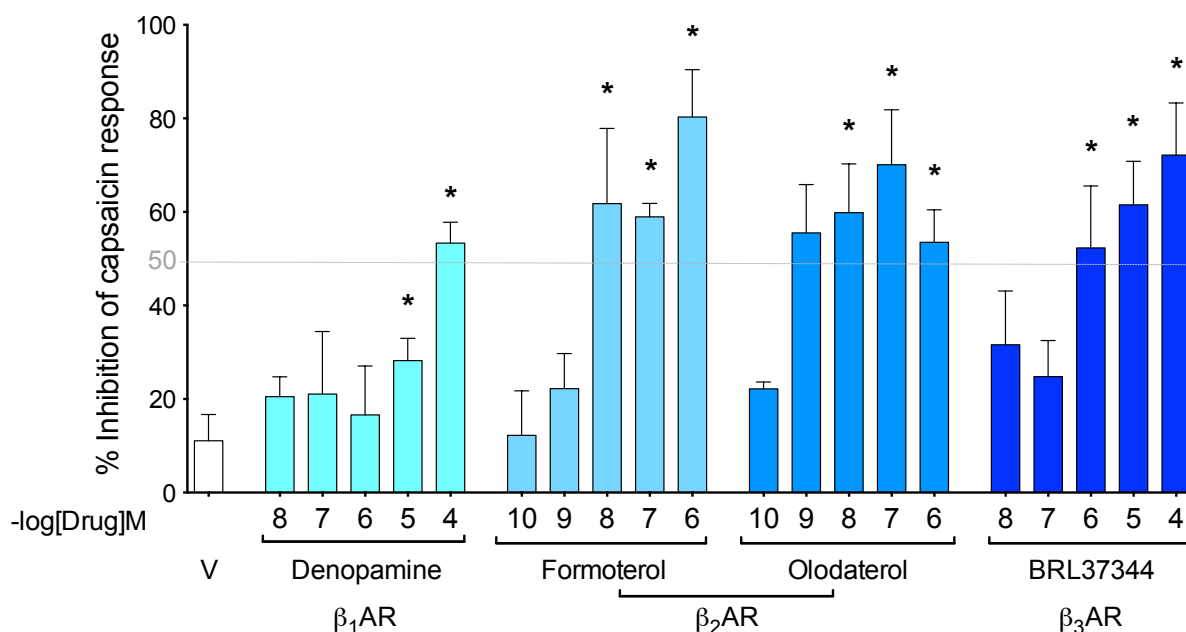
Figure 3.6 Non-cumulative concentration-response of β₁-/β₂-/β₃-agonists effect on capsaicin depolarisation of guinea pig vagus nerve
*Isolated guinea pig vagus nerve was incubated with vehicle, denopamine (β₁-adrenoreceptor agonist; 10nM-100µM; 10min), formoterol (β₂-adrenoreceptor agonist; 0.1nM-1µM; 20min), olodaterol (β₂-adrenoreceptor agonist; 0.1nM-1µM; 20min) or BRL37344 (β₃-adrenoreceptor agonist; 10nM-100µM; 10min) to examine the effect on subsequent capsaicin depolarisation. Data displayed as mean %inhibition of initial capsaicin stimulations (internal control) ±SEM, n=4-6, * p<0.05 as determined by paired students t-test compared to respective internal control.*

3.4.2 β-agonists effect on alternative tussive stimuli

A single concentration of each of the β-adrenergic receptor agonists that was effective at inhibiting capsaicin-induced vagus nerve depolarisation was evaluated for their ability to inhibit depolarisation to sub-maximal concentrations of acrolein (TRPA1 agonist) and the endogenous tussive stimuli PGE_2 and bradykinin. All of the β-adrenergic receptor agonists evaluated were effective at inhibiting all of these alternative tussive stimuli to some extent (*Fig. 3.7*), suggesting a general mechanism of action on sensory nerve depolarisation rather than activity at the capsaicin receptor TRPV1.
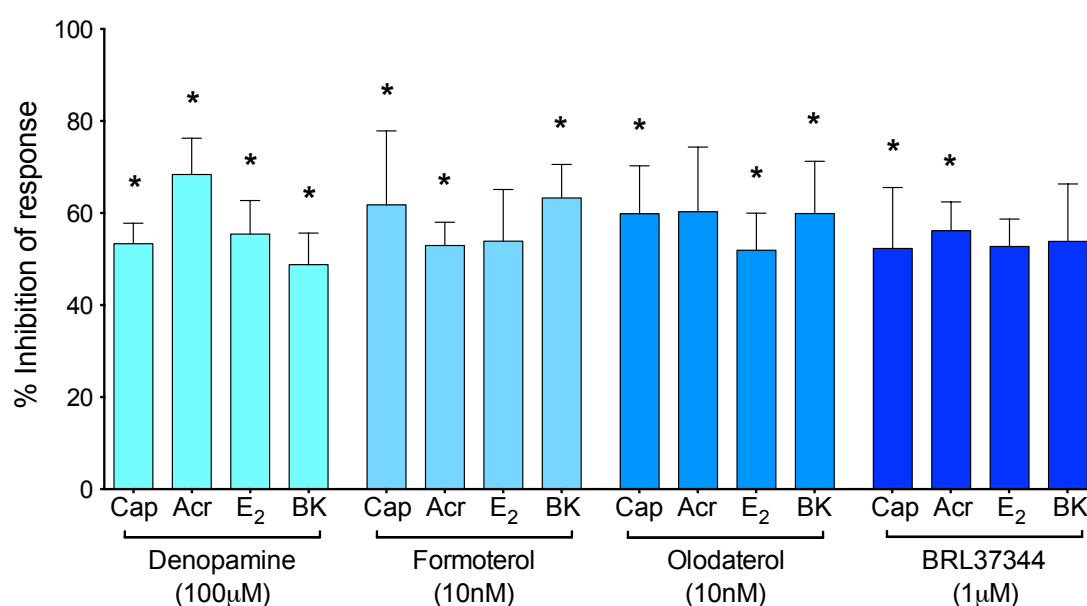
Figure 3.7 Effect of β₁-, β₂-, and β₃-agonists on tussive stimulus induced depolarisation of guinea pig isolated vagus nerve
Isolated guinea pig vagus nerve was incubated with selected concentrations of vehicle, denopamine (β₁-adrenoreceptor agonist; 100µM; 10min), formoterol (β₂-adrenoreceptor agonist; 10nM; 20min), olodaterol (β₂-adrenoreceptor agonist; 10nM; 20min) or BRL37344 (β₃-adrenoreceptor agonist; 1µM; 10min) to examine the effect on subsequent depolarisations induced by either capsaicin (1µM), acrolein (300µM), PGE₂ (10µM) or BK (3µM). Data displayed as mean %inhibition of initial stimulations (internal control) ±SEM, n=3-4, * p<0.05 as determined by paired students t-test compared to respective internal control.

3.4.3 Effect of β-receptor antagonists on β-agonists inhibition

Concentrations of denopamine (β_1), olodaterol (β_2), formoterol (β_2) and BRL37344 (β_3) effective at inhibiting capsaicin-induced vagus depolarisation by 50% were selected from _Section 3.3.1_ to be evaluated against selective antagonists of the β_1-, β_2- and β_3-receptors (0.1µM; _see Methods 3.2.5_ for antagonist names). Only the antagonist of each of the β_1-, β_2- and β_3-receptors blocked the inhibitory effect of each of the respective β-agonists (_Fig. 3.8_), indicating that the inhibition of capsaicin-induced sensory nerve depolarisation at these concentrations is mediated via activation of each of the specific receptor sub-types, and not by a crossover effect on another of the receptors.
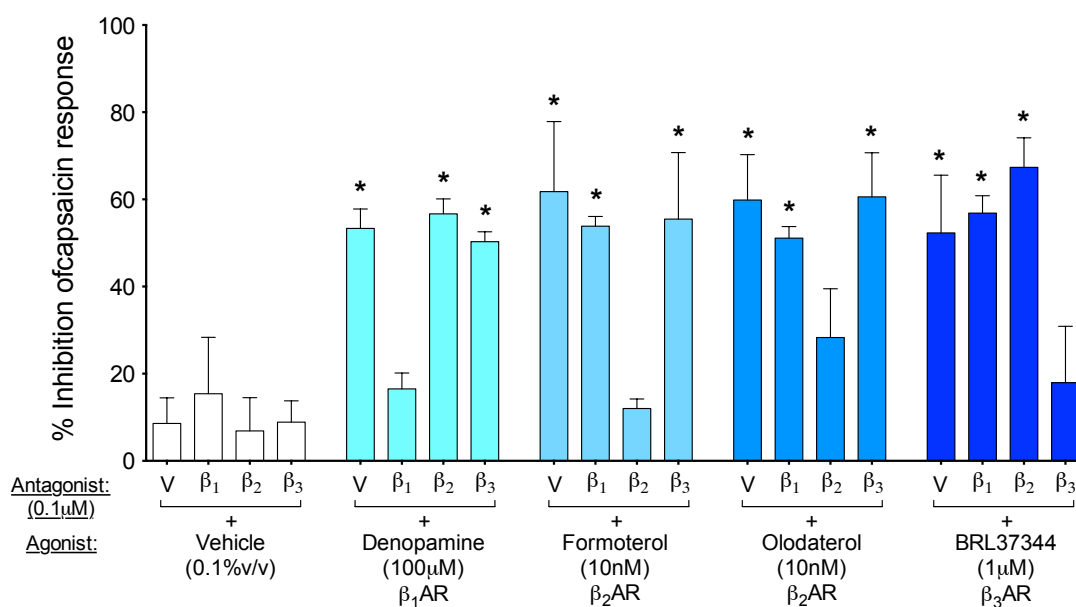
Figure 3.8 Effect of specific β-adrenoreceptor antagonists on β-adrenoreceptor agonist inhibition of guinea pig vagus nerve depolarisation
The effect of β_1- (CGP20712A), β_2- (ICI118551), β_3- (L-748337) antagonist (all 0.1µM; pre-incubated for 10min) on vehicle, denopamine (β_1-adrenoreceptor agonist; 100µM; 10min), formoterol (β_2-adrenoreceptor agonist; 10nM; 20min), olodaterol (β_2-adrenoreceptor agonist; 10nM; 20min) or BRL37344 (β_3-adrenoreceptor agonist; 1µM; 10min) inhibition of capsaicin-induced (1µM; 2min) depolarisation of guinea pig vagus nerve. Data displayed as mean %inhibition of initial capsaicin stimulations (internal control) ±SEM, n=4-6, * p<0.05 as determined by paired students t-test compared to respective internal control.

3.4.4 β-agonists effect in wild-type & knock-out mouse vagus

The inhibitory effects of the β_1-, β_2- and β_3-agonists were examined in vagus tissue from wild-type (FVB/N), β_2- and β_3-adrenoreceptor KO (*Adrb2$^{-/-}$* and *Adrb3$^{-/-}$*) mice. All of the agonists exerted an inhibitory effect on capsaicin-induced depolarisation in wild-type mice, similar to that observed in guinea pig. In the β_2- and β_3-receptor KO mice, only the inhibitory effect of, respectively, the β_2- and β_3-agonists was lost (*Fig. 3.9*). This data further confirms that the agonist tools (at the concentrations used here) act specifically on their respective receptors to exert their inhibitory effect on capsaicin-induced vagus nerve depolarisation.
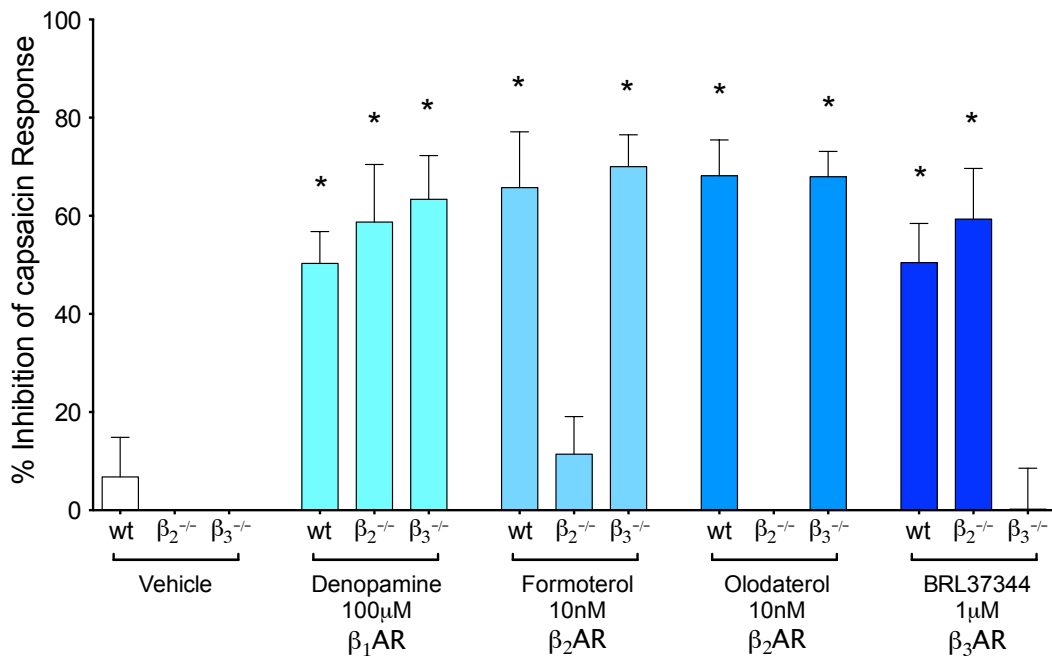
Figure 3.9 β-agonists effect on capsaicin depolarisation of vagus nerve from wild-type, β₂- or β₃-adrenoreceptor KO mice

*The effect of vehicle, denopamine (β_1-adrenoreceptor agonist; 100µM; 10min), formoterol (β_2-adrenoreceptor agonist; 10nM; 20min), olodaterol (β_2-adrenoreceptor agonist; 10nM; 20min) or BRL37344 (β_3-adrenoreceptor agonist; 1µM; 10min) on capsaicin-induced depolarisation of mouse vagus nerves taken from wild-type (wt), β_2-adrenoreceptor KO ($\beta_2^{-/-}$) or β_3-adrenoreceptor KO ($\beta_3^{-/-}$) mice. Data displayed as mean %inhibition of an initial capsaicin stimulation (internal control) ±SEM, n=4-6, where * p<0.05 as determined by paired students t-test compared to respective internal control.*

3.4.5 β-agonists effect and selectivity in human vagus

Key experiments performed on guinea pig vagus nerve were repeated on human vagus nerve tissue, in order to gauge whether the anti-tussive effects observed in guinea pig would be likely to translate to humans. The effect of single concentrations of denopamine (β_1) olodaterol (β_2), formoterol (β_2), and BRL37344 (β_3) on capsaicin-induced depolarisation was examined in the presence of vehicle, or selective β_1-, β_2- or β_3-
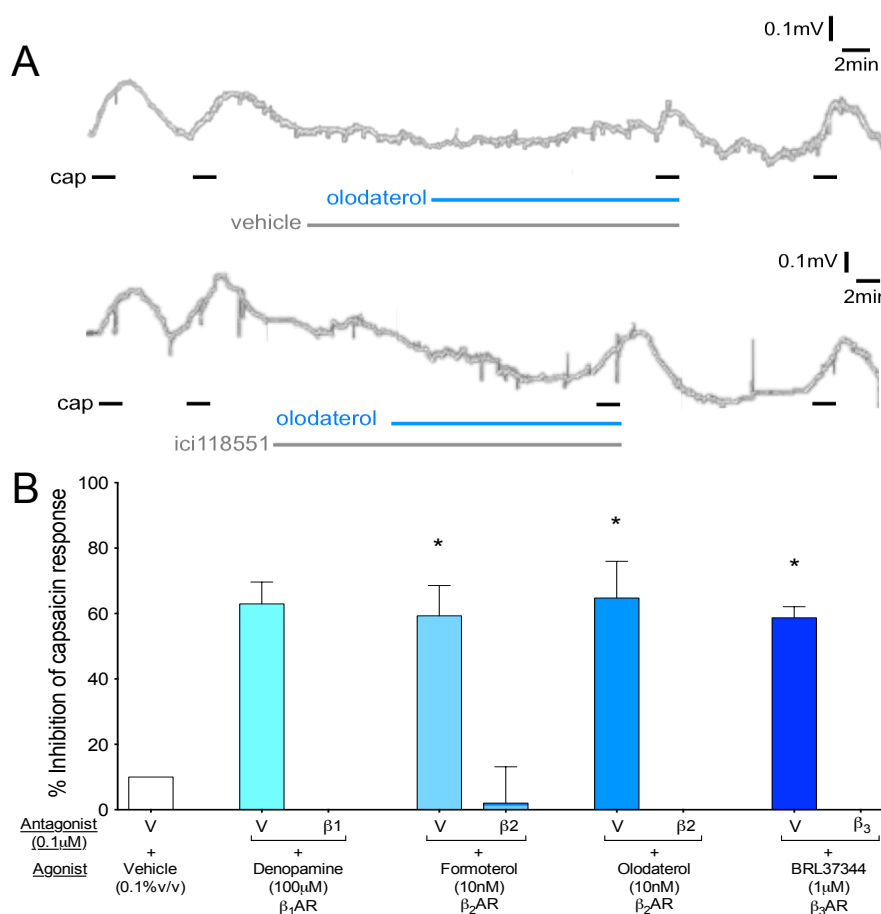
Figure 3.10 Effect of β-agonists ± respective antagonists on capsaicin depolarisation of human vagus
*[A] Representative traces of olodaterol (10nM) effect on capsaicin (cap) depolarisation of human vagus nerves, in presence of vehicle (upper; 0.1% DMSO) OR ICI118551 (lower; 0.1µM). [B] The effect of β_1- (CGP20712A), β_2- (ICI118551), β_3- (L-748337) antagonist (all 0.1µM; pre-incubated for 10min) on vehicle, denopamine (β_1-adrenoreceptor agonist; 100µM; 10min), formoterol (β_2-adrenoreceptor agonist; 10nM; 20min), olodaterol (β_2-adrenoreceptor agonist; 10nM; 20min) or BRL37344 (β_3-adrenoreceptor agonist; 1µM; 10min) inhibition of capsaicin-induced (1µM; 2min) depolarisation of human vagus nerve. Data displayed as mean %inhibition of initial capsaicin stimulations (internal control) ±SEM, n=3, * p<0.05 as determined by paired students t-test compared to respective internal control.*

receptor antagonists. Similarly to the results observed in guinea pig vagus nerve tissue, all of the β-adrenergic receptor agonists, including the LABA compounds, were effective at inhibiting capsaicin-induced depolarisation of human sensory nerves (*Fig. 3.10*). Furthermore, selective β₁-, β₂- or β₃-receptor antagonists reversed the inhibitory effect of the β-receptor agonists, demonstrating the agonists' inhibitory effects are mediated via their respective receptors in human vagus nerve (*Fig. 3.10*).

3.4.6 Involvement of adenylyl cyclase activation in β-agonists inhibitory effects

In order to identify the predominant isoform of AC in mouse vagal neurons, the relative expression of all the adenylyl cyclase sub-types was first determined by q-PCR in isolated mouse vagal ganglia neurons. AC6 appeared to be the predominant sub-type in these neurons, being approximately 4-5 times more highly expressed than any other AC sub-type in mouse vagal neurons (*Fig. 3.11*).
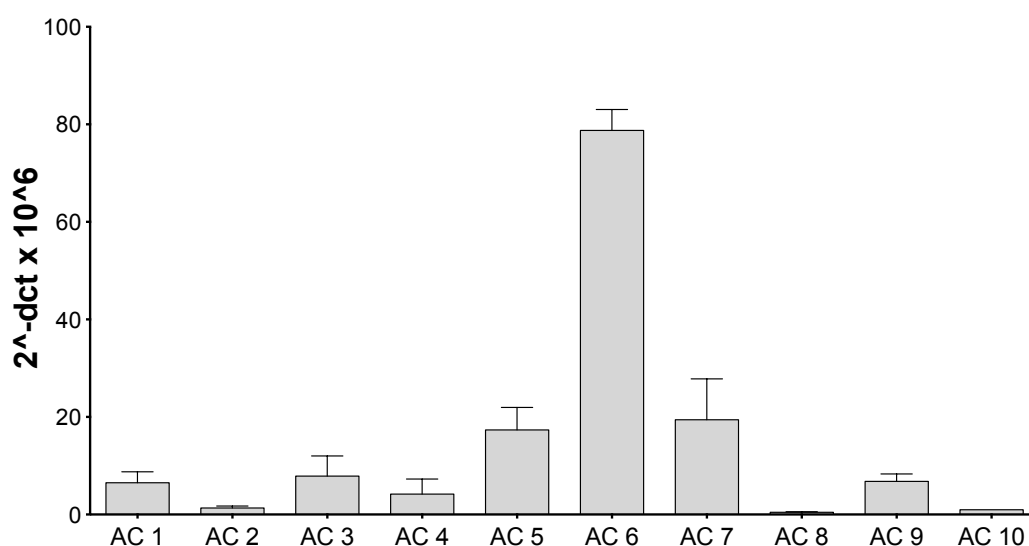
Figure 3.11 Relative expression of adenylyl cyclase sub-types in mouse vagal ganglia
Relative mRNA expression of adenylyl cyclase (AC) sub-types 1-10 in vagal ganglia from wild-type C57Bl/6 mice. Data displayed as mean±SEM, n=2-3.

Therefore in order to determine the involvement of AC in the anti-tussive activity of the β-receptor agonists, the inhibitory effect of the agonists against capsaicin-induced depolarisation was compared in wild-type mice and AC5/6 KO mice. Furthermore, forskolin was examined in the same tissues, to determine if a direct activator of AC would have a similar inhibitory effect as the β-receptor agonists. A single concentration of forskolin (30μM), similar to each of the β₁-, β₂- and β₃-receptor agonists, inhibited

capsaicin-induced vagus depolarisation in wild-type mice (*Fig. 3.12*). What is more, the inhibitory effect of forskolin, and that of each of the β-receptor agonists, was not observed in vagal tissue from AC5/6 KO mice (*Fig. 3.12*).
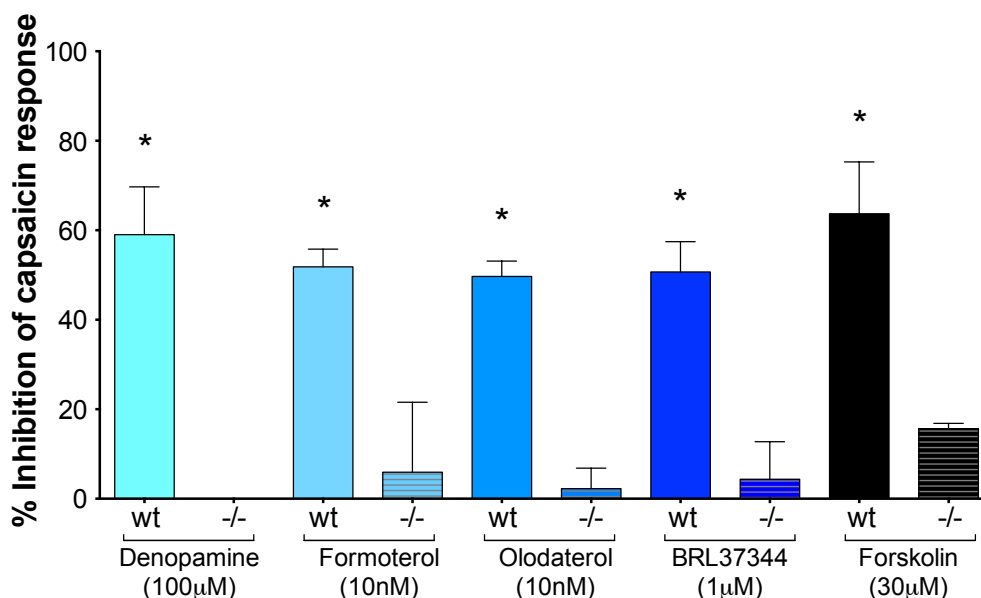
Figure 3.12 Effect of β₁-, β₂-, and β₃-agonists and forskolin on capsaicin-induced depolarisation of wild-type or AC5/6 -/- isolated mouse vagus nerve
*The effect of vehicle, denopamine (β₁-adrenoreceptor agonist; 100μM; 10min), formoterol (β₂-adrenoreceptor agonist; 10nM; 20min), olodaterol (β₂-adrenoreceptor agonist; 10nM; 20min), BRL37344 (β₃-adrenoreceptor agonist; 1μM; 10min) or forskolin (adenylyl cyclase activator; 10μM; 10min) on capsaicin-induced depolarisation of mouse vagus nerves taken from wild-type (wt), or adenylyl cyclase 5/6 knock-out (-/-) mice. Data displayed as mean %inhibition of an initial capsaicin stimulation (internal control) ±SEM, n=3-5, where * p<0.05 as determined by paired students t-test compared to respective internal control.*

Mice used for vagal tissue experiments were genotyped to confirm that they possessed either the wild-type or AC5/6 KO genotype. A representative sample of these results is shown in *Figure 3.13* below.
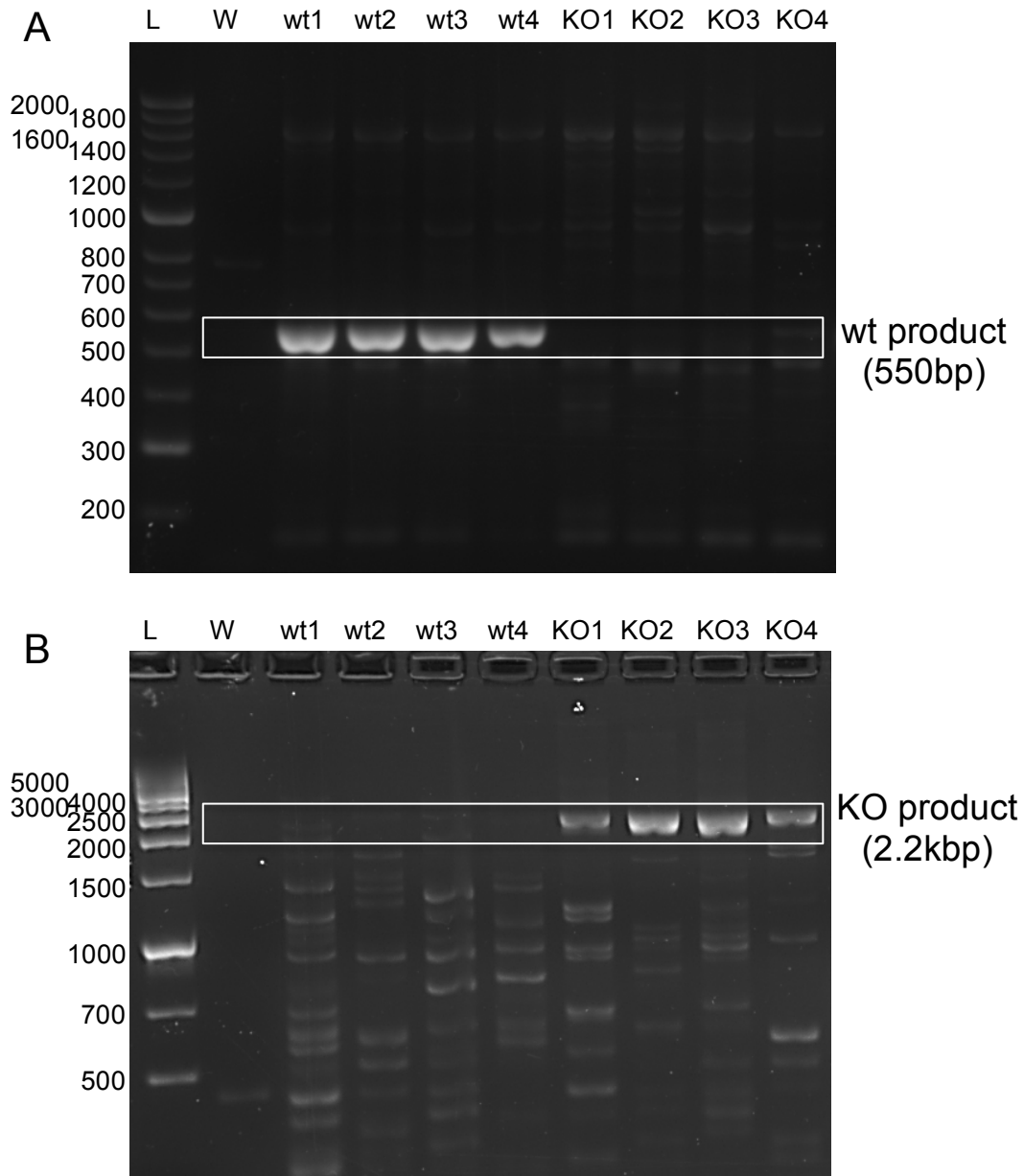
Figure 3.13 Verification of genomic DNA in samples from wild-type and AC5/6 KO mice

The presence of the wild-type (wt) or knock-out (KO) AC6 allele in DNA extracted from the tail-tips of mice used for experiments in Figure 3.12 was assessed by PCR amplification using specific primers for either the wt or KO allele. The DNA samples were stained, and run by electrophoresis across an agarose gel, alongside a DNA ladder (L) of known basepair (bp) sizes, and a water control (W). The stain was visualised under UV light and the gel was photographed. [A] shows the results of amplification in the presence of the wt primers, which gave an expected product of 550bp. [B] shows the results of amplification in the presence of the KO primers, which gave an expected product of 2.2kbp.

3.4.7 Effect of K⁺ channel blockers on β-agonists inhibition

Previously, the $BK_{Ca,}$ (but not IK, SK or K_{ATP}) potassium channel has been implicated with a key role in the blockade of depolarisation by fenoterol (Freund-Michel *et al.*, 2010). Here, we examined the effect of blockers of these four types of K^+ channel on β-receptor agonist inhibition of capsaicin-induced depolarisation of guinea pig vagus nerve. The BK_{Ca} blocker paxilline (1μM) significantly reduced the inhibitory effect of the LABA compounds, olodaterol and formoterol, as well as that of β_1- and β_3-receptor agonists (*Fig. 3.14a*). Olodaterol was still significantly effective in the presence of blockers of the other K^+ channels (*Fig. 3.14b*). This data suggests that opening of BK_{Ca} channels plays a role in the inhibitory effects of β-agonists observed in the vagus nerve.
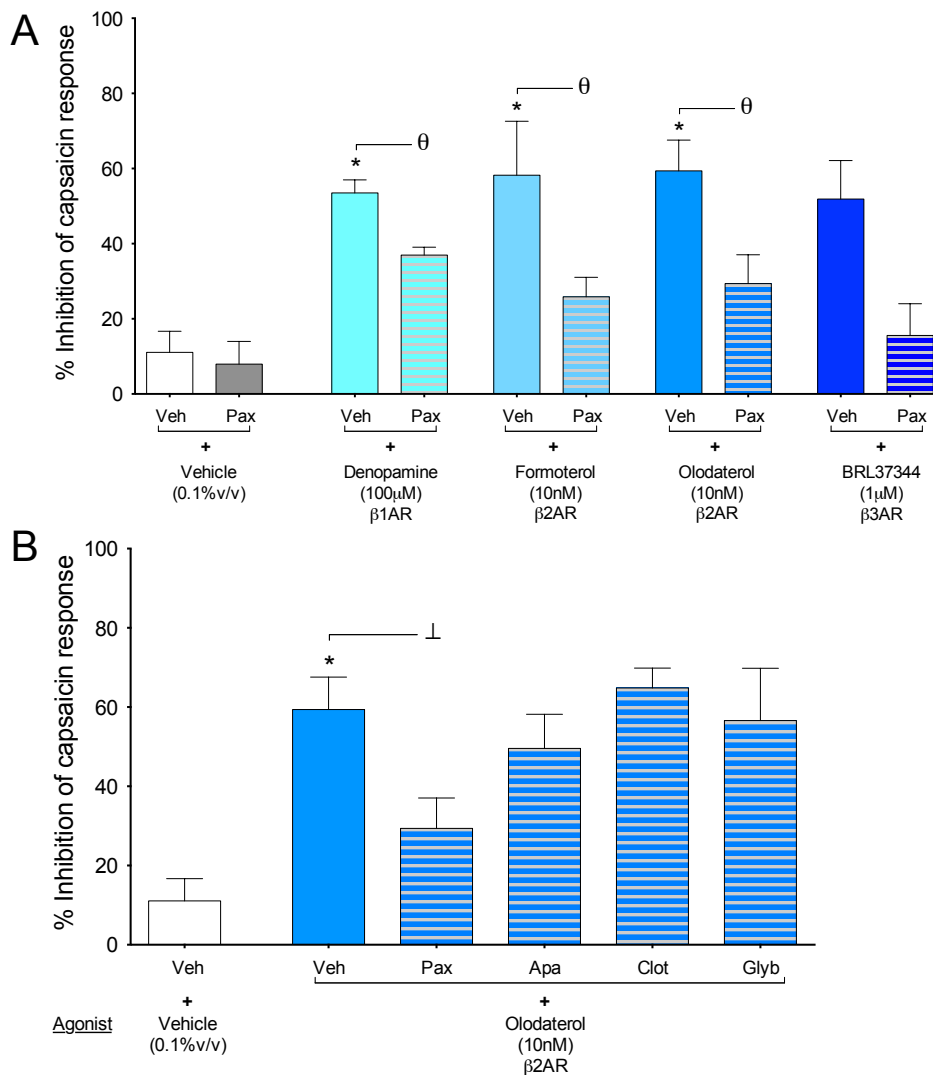
Figure 3.14 Effect of K$^+$ channel blockers on β-agonist inhibition of capsaicin-induced depolarisation of guinea pig vagus nerve

[A] The effect of paxilline (BK$_{Ca}$ blocker; 1μM; pre-incubated for 10min) on vehicle (0.1% DMSO), denopamine (β$_1$-adrenoreceptor agonist; 100μM; 10min), formoterol (β$_2$-adrenoreceptor agonist; 10nM; 20min), olodaterol (β$_2$-adrenoreceptor agonist; 10nM; 20min) or BRL37344 (β$_3$-adrenoreceptor agonist; 1μM; 10min) inhibition of capsaicin-induced (1μM; 2min) depolarisation of guinea pig vagus nerve. **[B]** The effect of vehicle (0.1% DMSO), paxilline (1μM), clotrimazole (IK$_{Ca}$ blocker; 10μM), apamin (SK$_{Ca}$ blocker; 1μM) or glybenclamide (K$_{ATP}$ blocker; 10μM), pre-incubated for 10min, on olodaterol (10nM; 20min) inhibition of capsaicin-induced (1μM; 2min) depolarisation of guinea pig vagus nerve. Data displayed as mean %inhibition of initial capsaicin stimulations (internal control) ±SEM, n=3-6, where * p<0.05 as determined by paired students t test compared to internal control, θ p<0.05 as determined Mann-Whitney U test compared to vehicle control, and ⊥ p<0.05 as determined by Kruskal-Wallis with Dunn's post-test compared to vehicle control.

3.5 Summary/Discussion

The aim of this chapter was to examine the effect of two clinically relevant LABAs on sensory nerve depolarisation and cough, and further to investigate the mechanism of action and signaling pathways mediating any anti-tussive action of β-adrenergic receptor agonists in sensory nerves.

The LABA compounds formoterol and olodaterol were evaluated in a guinea pig model of capsaicin-induced cough. At concentrations reported to be bronchoprotective against acetylcholine-induced pulmonary resistance in an *in vivo* model of bronchoconstriction (Bouyssou *et al.*, 2010), both olodaterol and formoterol were effective at inhibiting capsaicin-induced coughs and also the firing of single airway terminating C-fibres. This finding suggests that these compounds are likely to be useful as anti-tussives within the therapeutic range for their intended bronchodilator effects. What is more, both LABA compounds inhibited capsaicin-evoked firing of airway-terminating C-fibres, which are thought to play an important role in the dysregulation of the normal cough reflex in respiratory diseases such as asthma and COPD (Coleridge & Coleridge, 1984). As well as inhibiting capsaicin-evoked firing of airway C-fibres, the LABA compounds also had an inhibitory effect on capsaicin-induced calcium influx in isolated airway-terminating jugular ganglia neurons, further suggesting that β_2-receptor agonists exert their anti-tussive activity by inhibiting sensory nerve depolarisation, which is independent of their bronchodilator activity. This finding is important, as it has been suggested previously that β_2-adrenergic receptor agonists may inhibit cough via their (non-neuronal) bronchodilator effect (Pounsford *et al.*, 1985; Mulrennan *et al.*, 2004).

It was also determined that both of the LABA compounds (olodaterol and formoterol), as well as agonists of β_1- and β_3-adrenergic receptors, were effective at inhibiting capsaicin-induced vagus nerve depolarisation *in vitro*. These agonists also inhibited other tussive stimuli, such as acrolein (TRPA1), and endogenous tussive stimuli such as PGE_2 and BK, indicating a general inhibitory activity on sensory nerves rather than a direct effect on TRPV1 activation.

The LABA compounds are reported to cause maximal relaxation of *in vitro* smooth muscle preparations at approximately 10nM (Bouyssou *et al.*, 2010), which is the same concentration at which they caused significant inhibition of vagus nerve depolarisation in the isolated vagus nerve preparation. This further suggests that the LABA compounds would be likely to have an anti-tussive effect at the same concentrations as those used for bronchodilation.

Having established that the LABA compounds were effective at inhibiting *in vivo* cough, and that these compounds, as well as the β_1- and β_3-adrenergic receptor agonists, inhibit depolarisation of vagal sensory nerves, the receptors and signalling pathways activated by the agonists were examined. This is the first time that an inhibitory effect on sensory nerve activation has been shown by β_1- and β_3-adrenergic receptor agonism. Indeed, it has been reported that β_3-adrenergic receptor agonism potentiates capsaicin-induced depolarisation of rat vagal chemosensitive neurons (Gu *et al.*, 2006). Due to this apparent contradiction, it was important to assess the specificity of the agonist tools being used. Initially pharmacological tools were used in the isolated vagal preparation to show that the inhibitory effect of the agonists could be blocked by a single selected concentration of selective antagonists of the β_1-, β_2- and β_3-adrenergic receptors. Furthermore it was clearly shown that the β_2- and β_3-adrenergic receptor agonists had no effect in mice lacking the respective receptors, and that all three agonists were just as effective in the non-respective knock-out and wild-type mice.

Having thus confirmed that agonists were acting on the β_1-, β_2- and β_3-adrenergic receptors to inhibit sensory nerve depolarisation, key experiments were repeated in human vagus nerve tissue, showing that the findings of an inhibitory effect of β_1-, β_2- and β_3-adrenergic receptor agonism on sensory nerve depolarisation and therefore cough may be reasonably expected to translate to humans in the clinic.

Much research has been conducted into the signalling of β_2-adrenergic receptors in airway smooth muscle relaxation, with the classical view being that β_2-adrenergic receptors couple to $G_{\alpha s}$, inducing adenylyl cyclase activation, causing an increase in cAMP, which activates PKA to cause downstream opening of potassium channels (Cook *et al.*, 1993; Johnson, 1998). However, it has been suggested that, in vagal sensory nerves, β_2-adrenergic receptor agonists cause downstream activation of PKG rather than PKA (Freund-Michel *et al.*, 2010). For this thesis however, the involvement of PKA/G was not investigated, rather certain critical steps in the classical β_2-adrenergic receptor agonists pathway were examined – namely adenylyl cyclase and potassium channel involvement.

Using genetic tools available in the mouse, it was shown that adenylyl cyclase sub-type 6 is the predominant form of AC expressed in mouse vagal ganglia, and further, in mice adenylyl cyclase 5/6 KO mouse vagus nerve tissue, the β-adrenergic receptor agonists did not inhibit sensory nerve depolarisation. What is more, an activator of adenylyl cyclase (forskolin), which mimicked the inhibitory effect of β-adrenergic receptor agonists

in wild-type vagus nerve tissue also had no effect in AC5/6 KO mouse tissue, demonstrating the critical involvement of AC in this process.

Finally the involvement of potassium channels was examined, with the result that only the BK_{Ca} channel blocker paxilline was effective at partially reversing the inhibitory effect on sensory nerves. This pharmacological assessment confirmed the involvement of the BK_{Ca} channel in the β-receptor effect on sensory nerves, however further work with a wider range of concentrations and/or mice lacking the BK_{Ca} channel would be required to conclusively demonstrate a role for this channel. However, a mechanism of action involving hyper-polarisation of sensory nerves via opening of potassium channels (reducing cellular excitability) concurs with the finding that β-receptor agonists have a general anti-tussive effect on multiple stimuli, rather than an effect on a specific 'upstream' calcium channel such as TRPV1. Indeed, it has previously been shown that an opener of BK_{Ca} channels (NS1619) has similar effects to β-receptor agonists; both inhibiting vagus nerve firing to a range of tussive agents and inhibiting CA-evoked cough (Fox *et al.*, 1997).

These findings, taken together, indicate that β-receptor agonists, and specifically the LABA compounds olodaterol and formoterol, may be useful as general and clinically safe anti-tussives.

4. Theophylline

4.1 Introduction

The methylxanthine theobromine has previously been shown to inhibit cough and sensory nerve activation in both pre-clinical and in clinical models (Usmani, 2004). This anti-tussive activity was suggested to be a property unique to theobromine, and not a function of its methylxanthine structure (Usmani, 2004). However recently it has been demonstrated that theophylline can also inhibit citric acid evoked cough in guinea pigs (Mokry & Nosalova, 2011), suggesting that methylxanthines as a drug class may have anti-tussive activity.

Theophylline is widely known as a bronchodilator, which has been used for the treatment of respiratory diseases such as asthma and COPD for several decades, and yet its mechanism of action in this role has not been fully elucidated. Theophylline is a weak and non-specific inhibitor of phosphodiesterases (PDEs), causing cAMP and cGMP accumulation at higher concentrations, and this property is frequently associated with its bronchodilator activity (Rabe *et al.*, 1995; Niewoehner *et al.*, 2002). It is thought that this cAMP accumulation in airway smooth muscle initiates a signaling cascade which results in opening of large-conductance potassium channels to cause relaxation of smooth muscle (Miura *et al.*, 1992b; Ise *et al.*, 2003; Liu *et al.*, 2003; Wu *et al.*, 2004; Xin *et al.*, 2012). The signaling mechanisms by which theophylline, or methylxanthines more generally, exert their anti-tussive effects have not yet been investigated.

The aim of this chapter was to study the effect of theophylline on sensory nerve depolarisation and cough to a range of different stimuli, to gauge its effectiveness as a general anti-tussive. The secondary aim of this chapter was to examine the mechanism of action of theophylline in sensory nerves and neurons.

In order to fulfill these aims, the ability of theophylline to inhibit both citric acid- and capsaicin-evoked cough, as well as capsaicin-evoked *in vivo* sensory nerve firing and *in vitro* intracellular calcium movement in airway neurons was examined. Furthermore, the isolated vagus nerve preparation was used to examine the mechanism of action of theophylline, as a more pharmacologically amenable and cost-effective assay system. Using this system, the effect of theophylline was examined on depolarisation induced by a range of exogenous and endogenous tussive stimuli. Pharmacological blockers of potassium channels were used in the isolated vagus preparation to examine the role of potassium channels in the effects of theophylline. Furthermore, this preparation allowed the use of human donor vagal tissue to show that the effects observed in animal models may translate to the clinic.

Finally, the nature of the interaction between theophylline and specific potassium channels highlighted by experiments in the isolated vagus perparation was examined using whole cell perforated and inside-out single channel patch clamp techniques on primary guinea pig jugular ganglia neurons.

4.2 Methods

4.2.1 Determining the effect of theophylline on capsaicin- and CA-evoked cough in the conscious guinea pig

In order to examine the effect of theophylline, male Dunkin Hartley guinea pigs were administered vehicle (0.5%w/v methylcellulose, 0.2%v/v tween80 in PBS), or theophylline (0.3-100mg.kg^{-1}, i.p.) 1h prior to challenge with tussive agent. For cough recordings, a guinea pig was placed into a Perspex chamber attached to a microphone where coughs could be observed and recorded as described previously (*Methods 2.2*). A baseline recording was taken for 5min, and then capsaicin (60μM) was administered by aerosol for 5min, during which time coughs were counted and Penh recorded, as well as for a further 5min post-exposure, using the Buxco Cough Analyser. Alternatively, citric acid (300mM) was administered by aerosol for 10min, during which time coughs were counted and Penh recorded using the Buxco Cough Analyser. The route of administration was selected from Mokry & Nosalova (2011), and the timings for assessment of cough response following treatment was selected from Freund-Michel *et al*. (2010).

4.2.2 Determining the effect of theophylline on guinea pig airway-terminating C-fibre firing

The general premise of this technique was to assess the effect of theophylline on the firing activity of a single afferent airway-terminating C-fibre in response to capsaicin. To that end, surgery and setup was performed as described in *Methods 2.6* to allow assessment of action potentials generated by capsaicin aerosol in single afferent C-fibres of anaethetised and artificially ventilated guinea pigs. After surgery and setup, the animals were allowed to stabilise for at least 30min. After a control baseline recording of at least 2min, capsaicin (100μM) was administered by aerosol for 15s and changes in fibre activity, intra-tracheal pressure and blood pressure were continuously recorded until baseline or a steady state was re-established. After 10min, the capsaicin aerosol (15s, 100μM) was repeated. After a further 10min vehicle (0.1%v/v DMSO in PBS) or theophylline (100mg.kg^{-1}) were administered *i.v.*. A final capsaicin aerosol (15s, 100μM) was administered 20min after vehicle/drug administration. In addition, in order to further separate the action of theophylline on sensory nerve firing from its known bronchodilator activity in an *in vivo* setting, the experiments above were repeated, except with PGE$_2$ as the challenge agent instead of capsaicin. The protocol described above was therefore modified by evoking nerve firing with aerosolised PGE$_2$ (100μg.ml^{-1}, 1min) instead of aerosolised capsaicin. In addition, an application of aerosolised capsaicin was used prior at the start of the experiment to aid identification of a C-fibre.

4.2.3 Determining the effect of theophylline on capsaicin-induced responses in isolated airway jugular ganglia neurons

Male Dunkin-Hartley guinea pigs were euthanised, jugular ganglia were removed, and individual jugular ganglia neurons were isolated as described in *Methods 2.4*. The jugular and nodose ganglia contain the majority of the cell bodies for airway terminating vagal sensory neurons. Previous data in our group indicated that a greater proportion of jugular ganglia neurons respond to capsaicin (Grace *et al*., 2012). For this reason only jugular neurons were used to examine the inhibitory effects of theophylline.

As described previously (*Methods 2.4.1*) guinea pigs had been *i.n.* dosed with DiI, a retrograde tracer dye, and airway-terminating neurons isolated from these animals were identified by the DiI present in their plasma membranes. Isolated neurons were incubated (37°C, 5%CO_2) overnight in complete F12 media (containing penicillin and streptavidin), in order to allow adherence to poly-D-lysine- and laminin-coated fluorodishes. Immediately prior to imaging experiments, cells were incubated with the calcium sensitive dye Fura-2AM.

To test the effect of theophylline on the release of calcium in the ganglia, cells were acquired using a confocal epi-fluorescence microscope, and sensory neurons were identified by their body characteristics (20-30µM diameter) and light diffraction in phase contrast, whilst DiI emission was used to identify airway-terminating neurons. The fluorodish was connected to a pressurised perfusion system that allowed rapid change of the bath solutions as per the following protocol:

An initial control response to application of K_{50} solution (50mM hyper-potassium solution containing; 50mM KCl; 91.4mM NaCl; 1mM $MgCl_2$; 1.8mM $CaCl_2$; 0.33mM NaH_2PO_4; 10mM D-Glucose; 10mM HEPES; pH adjusted with NaOH to 7.4) was used to assess cell viability, and this response was used to normalise all subsequent responses within each cell. After perfusion with ECS to remove the K50 solution and allow recovery of calcium and voltage dye emissions back to baseline, cells were then stimulated twice with capsaicin (1µm) as control responses followed by a wash with ECS to recover baseline levels. The cells were then incubated with vehicle (0.1% DMSO in ECS), or theophylline (0.01, 0.1, 1 or 1µM) for 10min, and then re-stimulated with capsaicin in the presence of test compounds. ECS was then perfused for 10min to remove capsaicin and theophylline, following which a final capsaicin stimulation and K_{50} application were used to confirm that capsaicin responses recovered once the drug was removed, and that the cell(s) remained viable.

4.2.4 Determining the effect of theophylline on isolated vagus nerve depolarisation

The following experimental protocol was used to examine the effects of theophylline on isolated vagus nerve depolarisation induced by tussive stimuli. Following sacrifice of the guinea pig and dissection of the vagus nerve as outlined previously (*Methods 2.3*), the isolated nerve was allowed to equilibrate for 10min in the grease-gap chamber, following which capsaicin (1µM) was perfused for 2min, causing depolarisation. The nerve was then 'washed' (perfusion of KH alone) until the signal returned to baseline. Two reproducible responses to capsaicin were recorded in this way, following which the vagus nerve was incubated with vehicle (0.1% DMSO) or theophylline (0.1-100µM) for 10min, according to a standard protocol for examining efficacy in this system (Freund-Michel *et al.*, 2010). The vagus was then re-challenged with capsaicin, in the presence of test compound. Following a 'wash' period of 10min, a final stimulation of capsaicin was used to determine tissue viability. On each piece of nerve tissue, only one concentration of any vehicle or drug was examined.

A single sub-maximal concentration of theophylline was selected for evaluation against, acrolein (300µM), PGE_2 (10µM), or bradykinin (3µM). These compounds were used in place of capsaicin as described above.

4.2.5 Determining the role of K^+ channels in the action of theophylline on vagus nerve depolarisation

The role of potassium channels in the action of theophylline on vagus nerves was investigated by examining the effect of the K^+ channel blockers paxilline, clotrimazole, apamin or glibenclamide on inhibition of capsaicin by theophylline. The channel blockers were evaluated against a single sub-maximal concentration of theophylline on guinea pig vagus nerve. The protocol used here was modified from that described in <u>*Section 4.2.4*</u>, as follows: two control responses to capsaicin were generated, and then vehicle (0.1% v/v DMSO), paxilline (1µM), apamin (1µM), glibenclamide (10µM) or clotrimazole (10µM) were incubated for 10min alone, and then co-incubated for 10min with vehicle or theophylline (single sub-maximal concentration). The nerve was then stimulated with capsaicin in the presence of both vehicle/channel blocker and vehicle/theophylline, followed by a 'wash' period of 10min, and a final stimulation with capsaicin alone. Concentrations of potsassium channel blockers were selected from Freund-Michel *et al.* (2010).

4.2.6 Determining the mechanism of action of theophylline on K⁺ channels in guinea pig airway neurons

The role of potassium channels in the action of theophylline on sensory nerves, and the nature of this interaction, was investigated using whole cell perforated, and also inside-out single channel type patch clamping techniques. Primary guinea pig jugular ganglia were isolated and allowed to adhere to laminin and poly-D-lysine coated petri dishes as described in *Methods 2.4*. Isolated neurons were incubated (37°C, 5%CO$_2$) overnight in complete F12 media (containing penicillin and streptavidin), in order to allow adherence to poly-D-lysine- and laminin-coated fluorodishes.

Ganglia cells were acquired using a bright field microscope, and sensory neurons were identified by their body characteristics (20-30μM diameter) and light diffraction in phase contrast. The fluorodish was connected to a pressurised perfusion system that allowed rapid change of the bath solutions. Prior to experiments isolated neurons were bathed in ECS, and all experiments were performed in this media except where otherwise stated.

For whole cell patch clamp recording, a 2-3MΩ micropipette (Borosilicate glass, Warner Instruments, UK), back filled with 325μg.ml^{-1} amphotericin-B, was sealed to the cell of interest using a 3-6gΩ seal. The cell membrane inside the micropipette was then perforated by the amphotericin B diffusing to the pipette tip within 20min. Potassium currents were elicited using 800ms depolarizing square pulses at 10mV increments between -100mV and 120mV with a holding potential of -70mV. Current amplitude was measured as the average of the current value during the last 50ms of the pulse. Immediately prior to each recording, ECS was replaced with a modified extracellular solution (mECS, in mM: KCl 5.4, Choline-Cl 143.5, MgCl$_2$ 1, NaH$_2$PO$_4$ 0.33, D-Glucose 10, HEPES 10. pH 7.4 using KOH at 37°C, 321.71mOsm.L^{-1}) supplemented with tetrodotoxin (300 nM), nifedipine (1 μM), paxilline (1 μM) and glibenclamide (10 μM), to block the opening of sodium and calcium channels, as well as potassium channels apart from those of interest. The perfusion was reverted back to ECS between series of voltage-step recordings. Clotrimazole- and apamin-sensitive currents were calculated by the current difference between control recordings in mECS and currents recorded during perfusion of clotrimazole (10 μM) or apamin (1 μM) in mECS.

For excised inside-out recording, a 5-8MΩ micropipette (Borosilicate glass, Warner instruments, UK) was sealed to the cell of interest using a seal of at least 10gΩ. The cell membrane patch held inside the micropipette was then ripped from the rest of the cell to leave an intact membrane sealed to the micropipette tip separated from the intracellular machinery. Potassium currents were recorded in iso-potassium conditions, using an

intra-cellular solution (ICS, in mM; K-Gluconate 120, KCl 30, HEPES 10 and EGTA 10; pH adjusted to 7.2 using KOH, 330mOsm.L^{-1}) in the bath, and modified ICS (ICS + Sucrose 5mM, 335 mOsm.L^{-1}) in the micropipette, supplemented with tetrodotoxin (300nM), nifedipine (1µM), clotrimazole (1µM), paxilline (1µM) and glibenclamide (10µM) to block the opening of sodium and calcium channels, as well as potassium channels, apart from those of interest. Clotrimazole- and apamin-sensitive currents were then assessed in the absence or presence of theophylline in ICS (500nM free Ca^{2+}) and low calcium ICS (20nM free Ca^{2+}). Free calcium concentrations of 20nM and 500nM were achieved by adding to the bath soultion calcium amounts calculated using the Stanford University software "Ca- EGTA Calculator v1.3"

4.3 Anti-tussive potential of theophylline

4.3.1 Theophylline effect on naïve guinea pig cough

The effect of theophylline on numbers of cough evoked by capsaicin or citric acid was examined. Conscious guinea pigs were treated with vehicle or theophylline (0.3-100mg.kg^{-1} i.p.), and 1h later were challenged with an aerosol of capsaicin (60μM) or citric acid (300mM) to evoke cough, during which, and for 5 minutes post-aerosol, the number of coughs evoked was recorded. Theophylline at 30 and 100mg.kg^{-1} significantly attenuated the cough response to capsaicin, whereas coughs evoked by citric acid were also inhibited only at 100mg.kg^{-1} (*Fig. 4.1*).
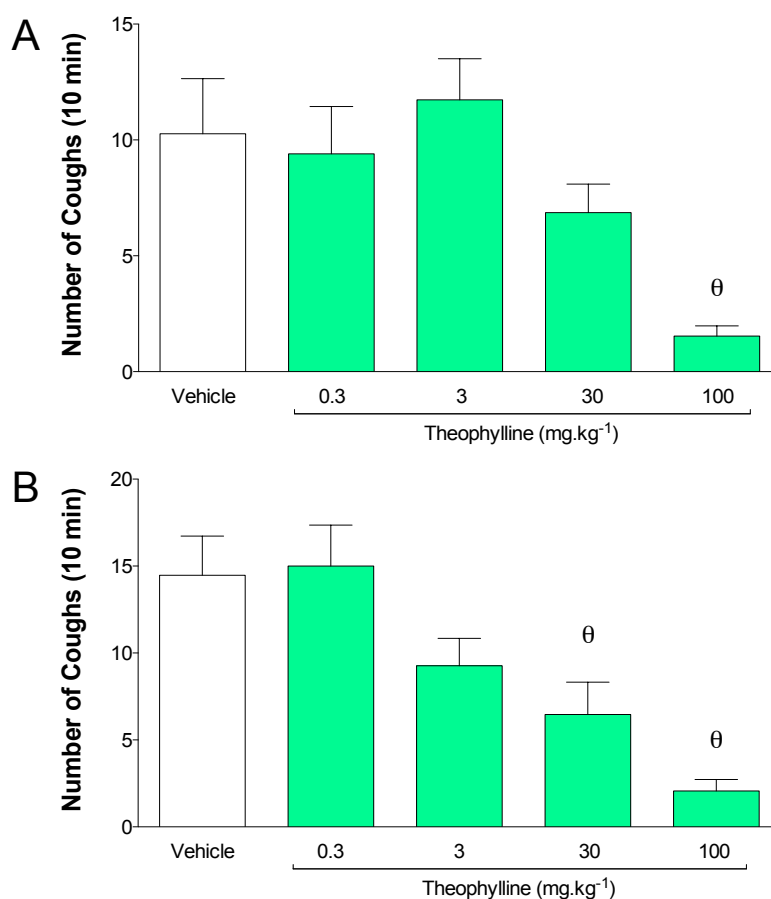
Figure 4.1 Dose response of theophylline effect on guinea pig capsaicin-or citric acid-evoked cough
*Guinea pigs were pre-treated with vehicle (0.5%w/v methylcellulose, 0.2%v/v tween80 in PBS) or theophylline (100mg.kg^{-1}), administered i.p., 1h prior to **[A]** 5min aerosol of capsaicin (60μM) after which number of coughs was recorded for a 10min period (including 5min of aerosol plus 5min post-aerosol), or, **[B]** 10min aerosol of citric acid (0.3M) during which number of coughs was recorded. Data displayed as mean±SEM, n=12-15, θ p<0.05 as determined by Kruskal-Wallis with Dunn's post-test, comparing all columns to vehicle control.*

4.3.2 Theophylline effect on guinea pig airway-terminating C-fibre firing

The effect of theophylline on capsaicin-evoked firing of airway-terminating, single vagal afferent chemosensitive C-fibres and capsaicin-evoked bronchoconstriction was examined, as shown in the example trace below (*Fig. 4.2a*). At the same dose (100mg.kg^{-1} *i.v.*) that inhibits the *in vivo* cough response, theophylline significantly reduced the peak firing rate (13.2±5.9 to 3.7±1.4 imp.s^{-1}) and number of action potentials (210.3±44.3 to 37.7±20.2 impulses) evoked by intra-tracheal instillation of a capsaicin (100µM) aerosol (*Fig. 4.2b&c*). Baseline activity was recorded immediately prior to capsaicin aerosol, and there was little spontaneous activity in these fibres (data not shown). Theophylline also reduced capsaicin-evoked bronchoconstriction (*Fig. 4.2d*).
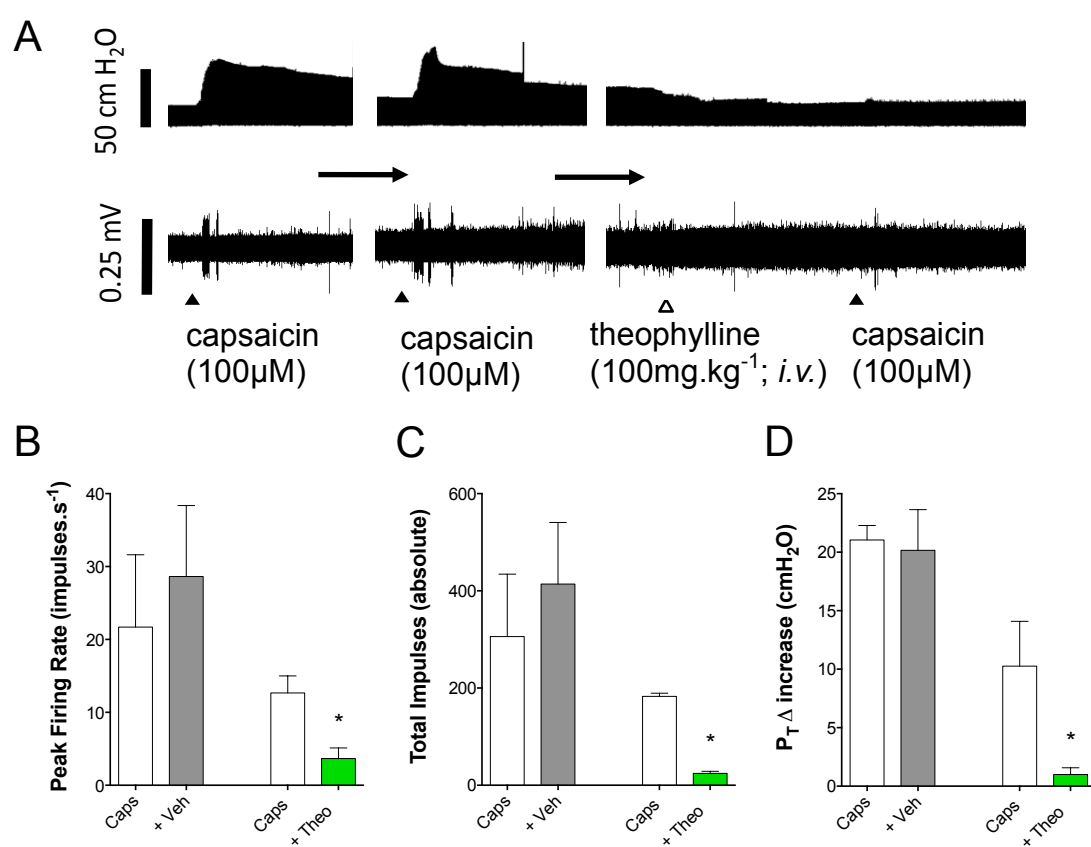
Figure 4.2 Theophylline inhibition of capsaicin-evoked action potentials and bronchoconstriction in airway-terminating chemosensitive C-fibres
*[A] Example trace showing the effect of theophylline on capsaicin-evoked action potential firing of an airway-terminating, chemosensitive C-fibre. Lower panel shows action potential firing of the C-fibre, whilst upper panel shows tracheal pressure on same time-scale, with arrows indicating time of drug applications. [B-D] All animals were firstly exposed to aerosolised capsaicin (15s, 100µM) alone, twice consecutively (internal control), and vehicle (0.5%w/v methylcellulose, 0.2%v/v tween80 in PBS) or theophylline (100mg.kg^{-1}) was administered i.p. 1h prior to subsequent capsaicin application. Single C-fibre afferent nerve responses were assessed by recording [B] firing rate and [C] total impulses. Capsaicin evoked bronchoconstriction [D] was also assessed. Data displayed as mean±SEM, n=3 where * p<0.05 as determined by paired students t-test compared to respective internal control (caps).*

Whilst it can be seen in the example trace (*Fig. 4.2*) above that capsaicin evoked firing prior to the bronchoconstriction (increase in tracheal pressure), it is unclear that the inhibitory effect of theophylline on C-fibres firing in this preparation is separate from its bronchodilator activity. Similarly to capsaicin, PGE_2 evokes firing of C-fibres, but unlike capsaicin it does not cause bronchoconstriction. Theophylline was therefore evaluated against this tussive agent to determine if it was directly inhibiting C-fibre firing independently of any bronchodilator activity. Indeed, theophylline significantly reduced the peak firing rate (16.4±4.7 to 4.3±2.3 imp.s^{-1}; *Fig. 4.3a&b*) and number of action potentials (180.3±23.6 to 20.4±5.8 impulses; *Fig. 4.3c&d*) evoked by PGE_2 aerosol (100μg.ml^{-1}, 1min). PGE_2 did not, however, cause any bronchoconstriction (*4.3e&f*).
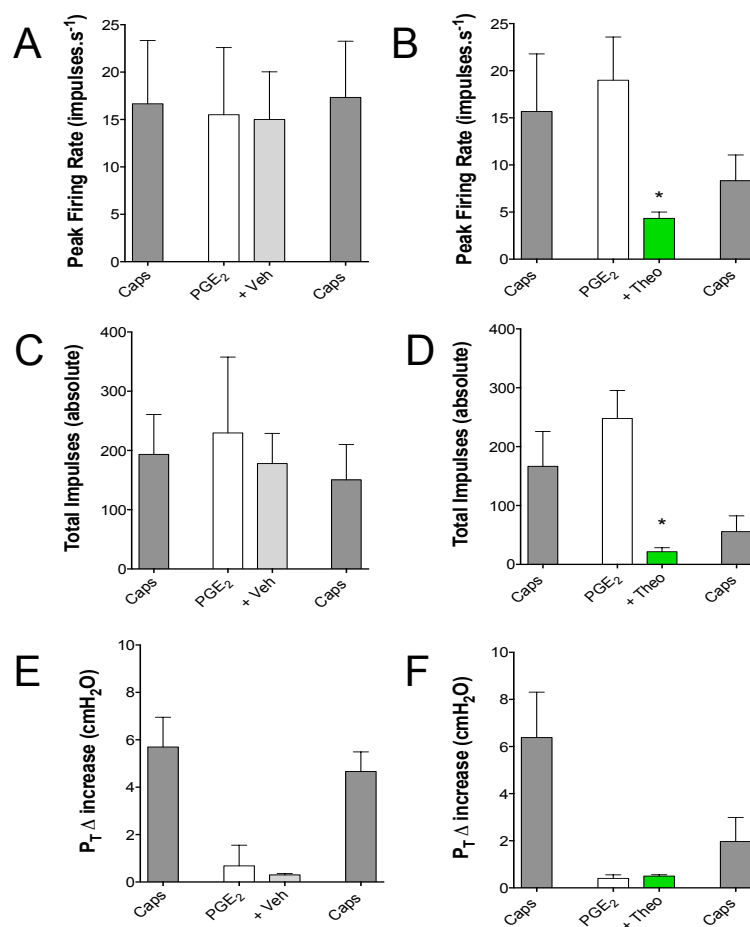
Figure 4.3 Theophylline inhibition of PGE₂-evoked action potentials in airway-terminating chemosensitive C-fibres
All animals were firstly exposed to aerosolised capsaicin (cap; 15s, 100μM) to identify chemosensitive fibres. For experiments animals were exposed to aerosolised PGE₂ (100μg.ml⁻¹, 1min) alone, twice consecutively (internal control), and then vehicle (0.5%w/v methylcellulose, 0.2%v/v tween80 in PBS) or theophylline (100mg.kg⁻¹) was administered 1h prior to subsequent administration of PGE₂. Single afferent C-fibre responses were assessed by monitoring [A&B] firing rate, and [C&D] total impulses. [E&F] Bronchoconstriction was also assessed. Data displayed as mean±SEM, n=3 where * p<0.05 as determined by paired students t-test compared to respective internal control (PGE₂).

4.3.3 Theophylline effect on guinea pig airway isolated neurons

The effect of theophylline on capsaicin-induced intracellular calcium levels was examined in single chemo-sensitive airway-terminating neurons of the jugular ganglia. Theophylline significantly inhibited the capsaicin-induced increase in intracellular calcium levels at all concentrations used, in a dose-dependent manner (*Fig. 4.4*).
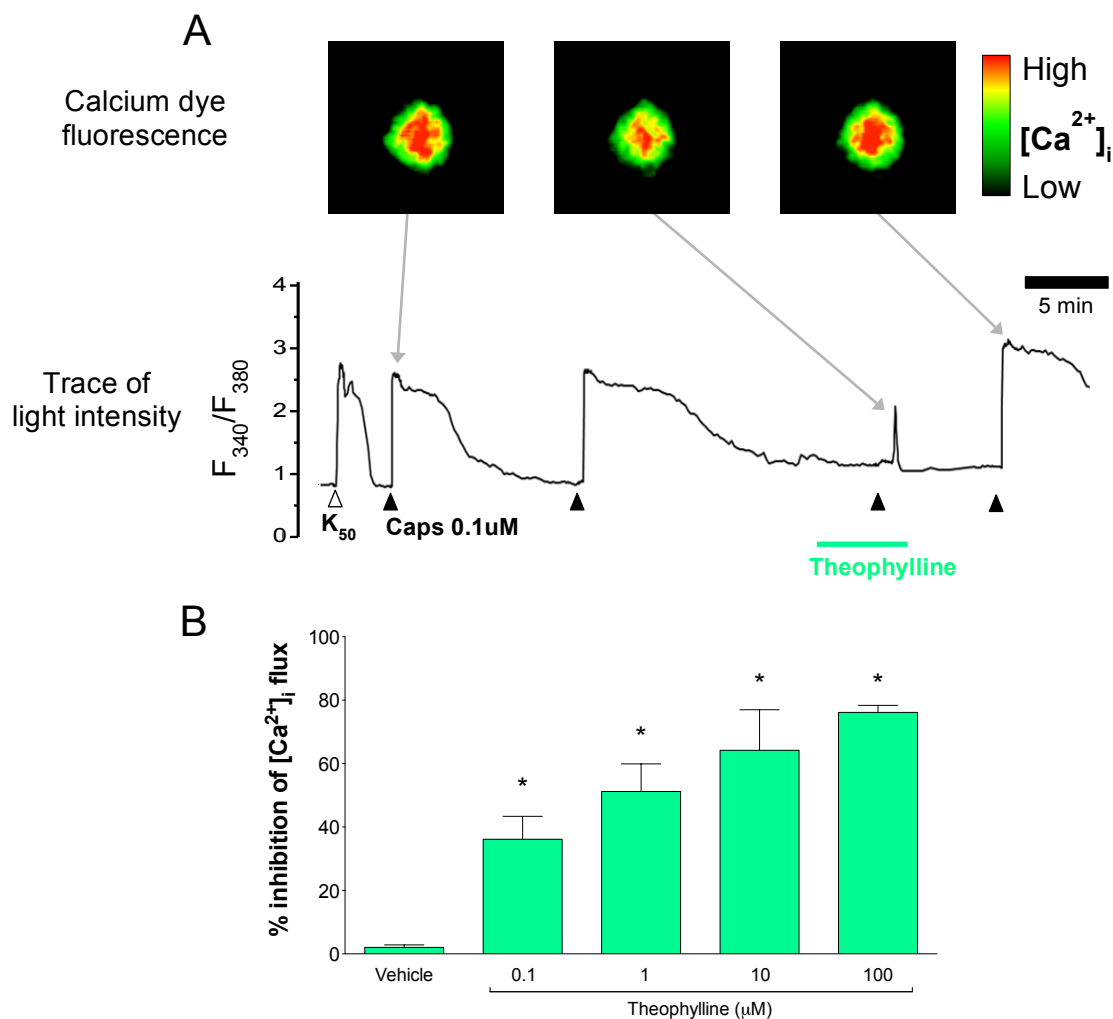
Figure 4.4 Theophylline inhibition of capsaicin-induced calcium influx in jugular airway-terminating neurons
*[A] Representative trace of theophylline effect on capsaicin-induced intracellular calcium levels. Open triangles indicate K_{50} (50mM KCl in ECS solution, 15s) application, closed triangles indicate capsaicin (caps; 0.1µM, 30s) application, line indicates theophylline (1nM, 5min) incubation. [B] Summary graph shows the effect of theophylline (0.1-100µM) on capsaicin(0.1µM)-induced increases in intracellular calcium. Data displayed as mean %inhibition of initial capsaicin stimulations (internal control) ±SEM, n=9-20 cells from 3-4 guinea pigs, * p<0.05 as determined by paired students t-test compared to respective internal control.*

4.4 Mechanism of action of theophylline

4.4.1 Theophylline effect on guinea pig vagus

The effect of theophylline on capsaicin-induced depolarisation of guinea pig isolated vagus nerves was examined. Theophylline (0.1-100µM) caused a concentration-dependent inhibition of capsaicin-induced sensory nerve depolarisation, which was significant at concentrations of 1µM or higher (*Fig. 4.5*).
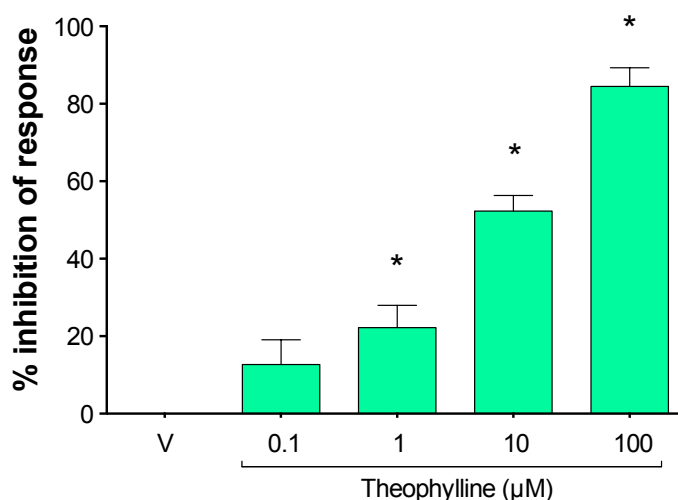
Figure 4.5 Non-cumulative concentration-response of theophylline effect on capsaicin depolarisation of guinea pig isolated vagus nerve
*Isolated guinea pig vagus nerve was incubated with vehicle or theophylline (0.1-100µM) to examine the effect on subsequent capsaicin depolarisation. Data displayed as mean %inhibition of initial capsaicin stimulations (internal control) ±SEM, n=8-18, * p<0.05 as determined by paired students t-test compared to respective internal control.*

4.4.2 Theophylline effect on alternative tussive stimuli

A sub-maximal concentration of theophylline, which was effective at inhibiting capsaicin-induced vagus nerve depolarisation, was evaluated for its ability to inhibit depolarisation induced by sub-maximal concentrations of a range of alternative tussive stimuli – acrolein (300µM), a TRPA1 agonist, and the endogenous tussive stimuli PGE_2 (10µM) and bradykinin (3µM). Theophylline was effective at inhibiting all of these alternative tussive stimuli by approximately 55-80% at the single concentration of 10µM used (*Fig. 4.6*), suggesting a general mechanism of action on sensory nerve depolarisation rather than activity at the capsaicin receptor TRPV1.
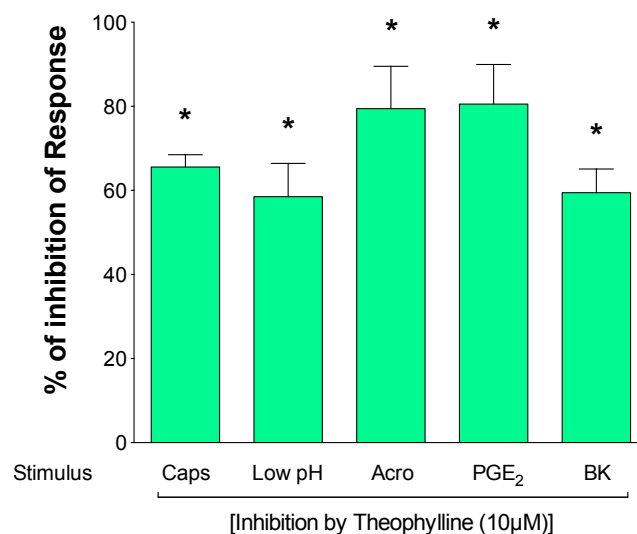
Figure 4.6 Effect of theophylline on tussive stimuli-induced depolarisation of guinea pig isolated vagus nerve
*Isolated guinea pig vagus nerve was incubated with theophylline (10µM) to examine the effect on subsequent depolarisations induced by either capsaicin (1µM), acrolein (300µM), PGE$_2$ (10µM) or BK (3µM). Data displayed as mean %inhibition of initial stimulations (internal control) ±SEM, n=3-4, * $p<0.05$ as determined by paired students t-test compared to respective internal control.*

4.4.3 Role of K$^+$ channels in theophylline inhibition on guinea pig vagus

The effect of blockers of BK$_{Ca}$, IK$_{Ca}$, SK$_{Ca}$, K$_{ATP}$ type potassium channels (paxilline, clotrimazole, apamin and glibenclamide respectively) on theophylline inhibition of capsaicin-induced depolarisation of guinea pig vagus nerve was examined. The SK$_{Ca}$ blocker apamin (1µM) significantly reduced the effect of theophylline on capsaicin-induced depolarisation (*Fig. 4.7*), indicating the SK$_{Ca}$ channel is involved in the inhibitory effects of theophylline on guinea pig vagus nerve. Whilst theophylline was still effective in the presence of blockers of the other K$^+$ channels, in the case of the IK$_{Ca}$ blocker clotrimazole (10µM), there did appear to be a small reduction in the effect of theophylline, although this did not reach significance. This data suggests that opening of potassium channels, and specifically SK$_{Ca}$ type potassium channels, plays a role in the inhibitory effects of theophylline on sensory nerve depolarisation.
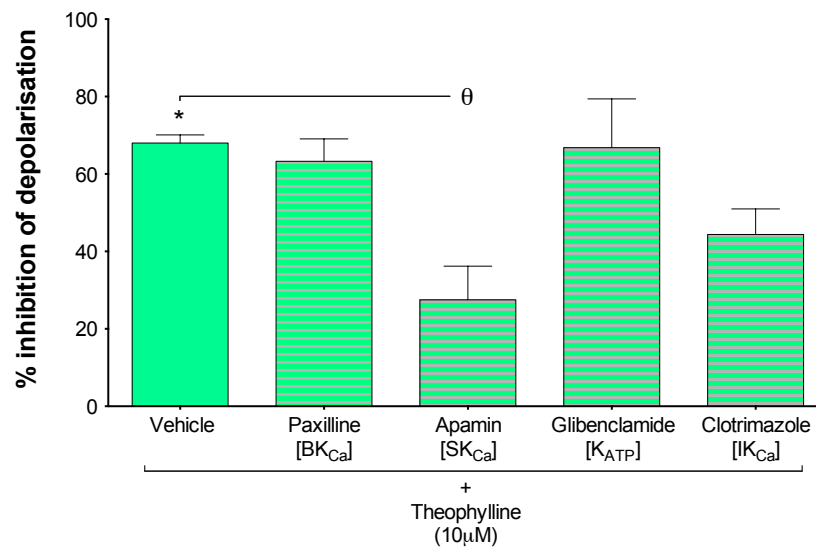
Figure 4.7 Effect of K⁺ channel blockers on theophylline inhibition of capsaicin-induced vagus nerve depolarisation
*The effect of vehicle (0.1% DMSO), paxilline (BK$_{Ca}$ blocker; 1μM), apamin (SK$_{Ca}$ blocker; 1μM), glibenclamide (K$_{ATP}$ blocker; 10μM) or clotrimazole (IK$_{Ca}$ blocker; 10μM) pre-incubated for 10min, on theophylline (10μM) inhibition of capsaicin-induced (1μM; 2min) depolarisation of guinea pig vagus nerve. Data displayed as mean %inhibition of initial capsaicin stimulations (internal control) ±SEM, n=3-6, where * p<0.05 as determined by paired students t test compared to internal control, and θ p<0.05 as determined Mann-Whitney U test compared to vehicle control.*

4.4.4 Theophylline effect on human vagus

Key experiments performed on guinea pig vagus nerve were repeated on human vagus nerve tissue, in order to determine whether the anti-tussive effects observed in guinea pig would be likely to translate to humans. The effect of a single concentration of theophylline on capsaicin-induced depolarisation was examined in the presence of, and following pre-incubation (10min) with, vehicle, apamin or clotrimazole. Similarly to the results observed in guinea pig vagus nerve tissue, theophylline was effective at inhibiting capsaicin-induced depolarisation of human sensory nerves (*Fig. 4.8*). Again, similarly to the results obtained in guinea pig vagus, apamin significantly reduced the inhibitory effect of theophylline, whereas clotrimazole (IK$_{Ca}$) did not have a significant effect, demonstrating that at least the SK$_{Ca}$ channel plays a role in the effect of theophylline on human vagus nerve.
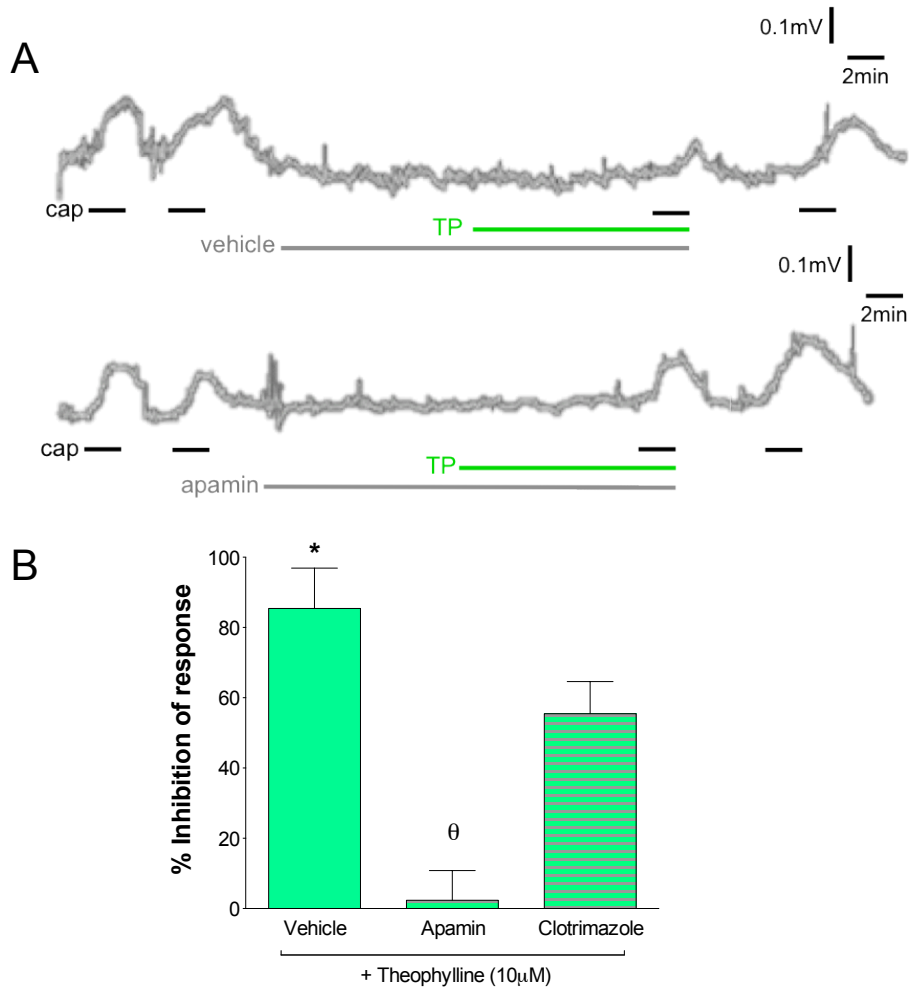
Figure 4.8 Effect of theophylline ± K⁺ channel blockers on depolarisation of human vagus nerve

[A] Representative traces of theophylline effect on capsaicin (cap) depolarisation of human vagus nerves, in presence of vehicle (upper; 0.1% DMSO) OR apamin (lower; 1μM). [B] The effect of vehicle (0.1% DMSO), apamin (SK_{Ca} blocker; 1μM), or clotrimazole (IK_{Ca} blocker; 10μM), pre-incubated for 10min, on theophylline (10μM) inhibition of capsaicin-induced (1μM; 2min) depolarisation of human vagus nerve. Data displayed as mean %inhibition of initial capsaicin stimulations (internal control) ±SEM, n=3-4, * p<0.05 as determined by paired students t-test compared to respective internal control, and θ p<0.05 as determined Mann-Whitney U test compared to vehicle control.

4.4.5 Effect of theophylline on K$^+$ currents

The effect of theophylline on resting membrane potential was examined using patch clamp on primary jugular neurons. In a perforated patch experiment, which preserves the intracellular content and $[Ca^{2+}]_i$, theophylline caused a significant hyperpolarisation of the cell membrane (*Fig. 4.9 panel a*). The effect of theophylline on SK_{Ca} and IK_{Ca} currents was then investigated. In the presence of theophylline, both apamin- and clotrimazole-sensitive potassium currents over a series of voltage steps were increased, as shown in the representative traces in *Figure 4.9*, *panels b & c*, respectively. The apamin- and clotrimazole-sensitive currents evoked by the voltage steps were then normalised to the size of the cell they were recorded from to give the current density. Overall theophylline increased both apamin- and clotrimazole-sensitive potassium current density, indicating that theophylline increases opening of both SK_{Ca} and IK_{Ca} channels, respectively. The effect on the apamin-sensitive (SK_{Ca}) current (*Fig. 4.9d*) reached significance at lower voltages (-30mV, $p<0.05$) than that of the clotrimazole-sensitive (IK_{Ca}) current (*Fig. 4.9e*: 60mV, $p<0.05$), indicating that theophylline is likely to have a greater effect on SK_{Ca} at voltages closer to the resting membrane potential of the cell (-70mV).

The single-channel, or inside-out patch clamp technique was then used to investigate whether theophylline acts directly on SK_{Ca}, independent of second messengers. This technique allows examination of the activity of a small number of channels on a patch of membrane separated from the rest of the cell. *Figure 4.10a* shows an example trace of the current through the SK_{Ca} channels present on the cell membrane patch in the absence and presence of theophylline, from which the frequency and number of SK_{Ca} channels in an open state was determined. As can be seen in the summary amplitude frequency histograms theophylline increased the channel open probability of SK_{Ca} channels by 62±7% at -100 mV voltage and in the presence of 500nM free calcium (*Fig. 4.10c*). In addition, the theophylline effect appeared to be calcium-dependent as when $[Ca^{2+}]_i$ is reduced to 20nM (the calcium concentration at which the open probability falls near to zero in the control) theophylline failed to induce any further channel opening (*Fig. 4.10b*).
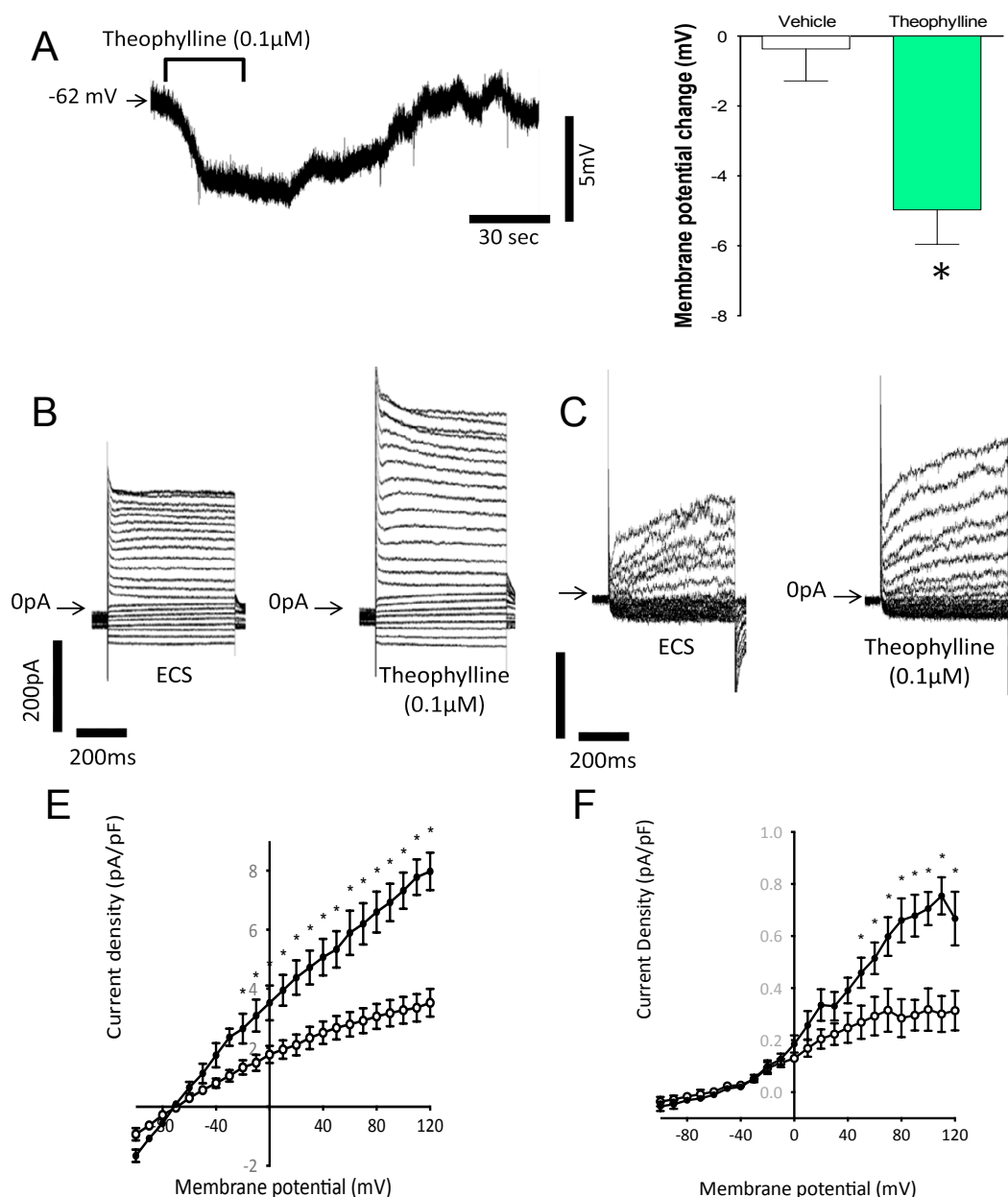
Figure 4.9 Theophylline effect on K+ channels in whole cell perforated patch clamped guinea pig jugular ganglia neurons
[A] *Representative trace (left) and summary graph (right, n=4) of theophylline effect on membrane resting voltage. * p<0.05 as determined by Mann-Whitney U test. Example trace of **[B]** apamin(1μM)-sensitive current, and **[C]** clotrimazole(1μM)-sensitive current elicited by square depolarising pulses in normal conditions (ECS; left graph) and after incubation with theophylline (0.1 μM; right graph). **[D]** & **[E]** summary graphs of current density-voltage relationship for apamin and clotrimazole-sensitive currents, respectively; open circles=normal conditions (ECS), closed circles=in presence of theophylline (0.1μM), data displayed as mean±SEM, n=4, * p<0.05 as determined by two-way paired ANOVA with Bonferroni's post-hoc test.*
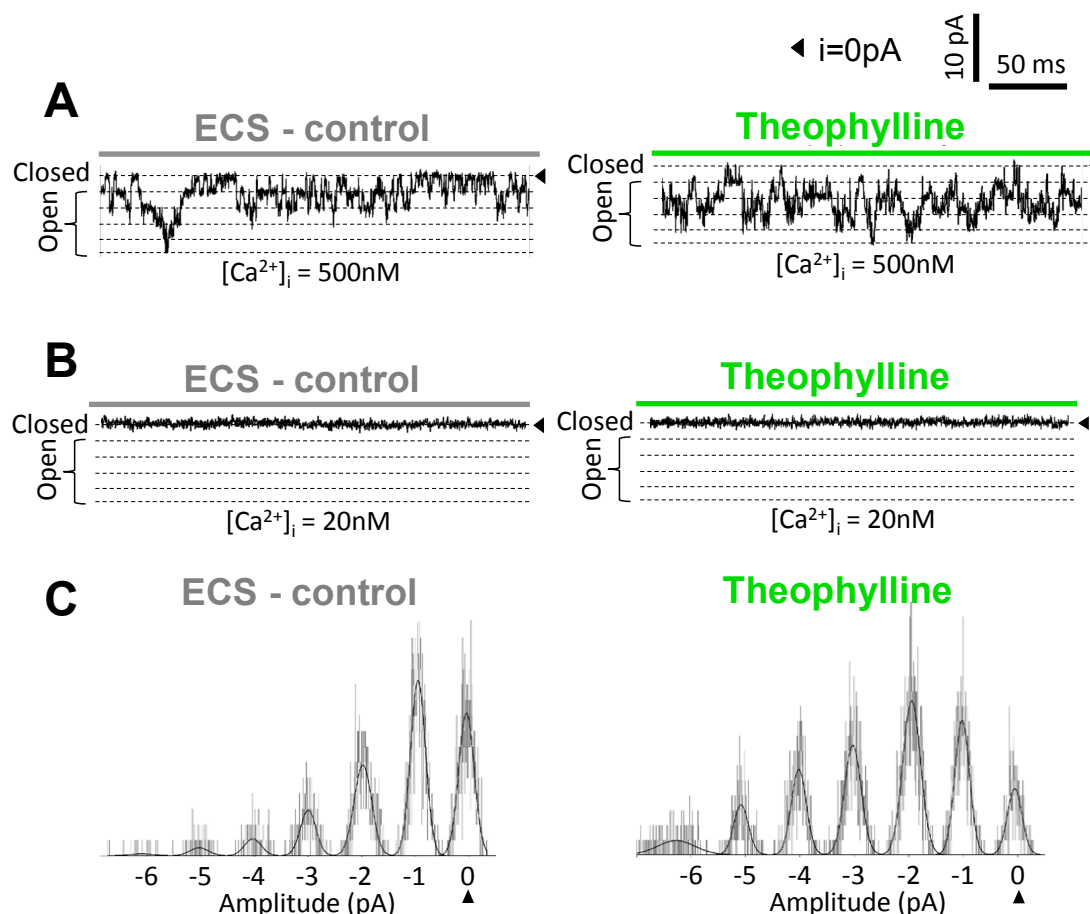
Figure 4.10 Theophylline effect on K+ channels in patch clamped isolated membrane from guinea pig jugular ganglia neurons
[A] Traces of a single channel recorded from the same patch of membrane in the presence of the vehicle control (left) and in the presence of 0.1µM theophylline (right), acquired at a voltage of -100mV in ECS (500nM free-calcium). [B] Traces acquired as in A, except in a low free-calcium (20nM) solution. Channel closed state (i=0 pA) is indicated by black triangles. The different open states recorded from this patch are indicated with a bracket on the left and identified by a dotted line. Current and time scales are indicated top right. [C] Normalised amplitude histograms of the single channel traces recorded over 30s for 500nM free calcium from the patch displayed in A. Histograms were fitted using a Gaussian function. The current level corresponding to the closed state of channel (0pA) is indicated by a black triangle. Amplitude scale is indicated below the histograms on the X-axis.

4.5 Summary/Discussion

The aim of this chapter was to study the effect of theophylline on sensory nerve depolarisation and cough. The anti-tussive activity of theophylline was demonstrated in conscious guinea pig models of evoked cough to both capsaicin and citric acid challenge, which are commonly used tussive stimuli in objective clinical cough studies (Laude *et al.*, 1993). Theophylline significantly inhibited both citric acid and capsaicin-evoked cough, although the dose (100mg.kg^{-1}) required to significantly inhibit evoked cough here was greater than the reported 10mg.kg^{-1} reported in a similar study by Mokry & Nosalova (2011). It should be noted that in the latter study, despite a higher concentration of citric acid being nebulized (0.6M instead of 0.3M), the challenge period was only 2 minutes, compared to the challenge period of 5 minutes used here. This is reflected in the different numbers of coughs evoked in control groups of the two studies, with approximately 4-6 coughs evoked by a 2 minute aerosol, and 14.47±2.26 coughs evoked by the 5 minute aerosol in the current study. Another difference between these studies is the use of a repeated-measures study design in the study performed by Mokry & Nosalova, enabling the use of a paired students t-test, which increased the statistical power to detect smaller differences between control and treatment challenges. In this study it did appear that there was some anti-tussive effect of theophylline on CA cough at 3 and 30mg.kg^{-1}, although this effect was not statistically significant with the methodology used here.

The same dose of theophylline that was effective at inhibiting cough (100mg.kg^{-1}) was also effective at inhibiting capsaicin-evoked *in vivo* firing of airway C-fibres, which are thought to play a role in the dysregulation of the cough reflex in respiratory disease (Coleridge & Coleridge, 1984). In the same system, theophylline also inhibited capsaicin-evoked bronchoconstriction as assessed by measuring pulmonary pressure, indicating that theophylline is effective at inhibiting cough and bronchoconstriction at this dose. In order to determine whether the inhibition of nerve firing by theophylline in this *in vivo* setting was distinct from its bronchodilator activity, the effect of theophylline on PGE$_2$-evoked firing was examined. Similarly to capsaicin, PGE$_2$ evokes firing of C-fibres (via TRPV1 and TRPA1; Grace *et al.*, 2012) but unlike capsaicin, PGE$_2$ does not cause bronchoconstriction. The inhibition by theophylline of PGE$_2$-evoked C-fibre firing shows that theophylline has a direct inhibitory effect *in vivo* which is independent of its bronchodilator activity. Additionally, the inhibition by theophylline of capsaicin-induced calcium influx in isolated jugular-origin, airway-terminating neurons shows that theophylline also has a direct inhibitory effect directly on sensory neurons in an *in vitro* setting.

A concentration-dependent inhibitory effect of theophylline was observed on capsaicin-induced depolarisation of guinea pig isolated vagus nerve, and a sub-maximal concentration of theophylline inhibited depolarisation induced by acrolein, PGE_2 and bradykinin. Inhibition of this range of agonists, comprising exogenous and endogenous activators of at least the TRPV1 and TRPA1 ion channels (Grace *et al.*, 2012), suggests that theophylline exerts a general anti-excitatory effect on sensory nerve depolarisation, rather than being a specific antagonist of a particular ion channel such as TRPV1. This is further confirmed by the inhibition by theophylline of both capsaicin- and citric acid-evoked cough, as capsaicin causes cough via TRPV1 (Grace *et al.*, 2012), whereas citric acid causes cough partially through TRPV1 and partially through an as yet unknown alternative mechanism (Lalloo *et al.*, 1995; Kollarik & Undem, 2004; Canning, 2006b).

Having thus confirmed that theophylline has a general inhibitory directly on sensory nerve depolarisation, the isolated vagus nerve system was further used to examine its mechanism of action. Currently the mechanism of action of theophylline, even in it's traditional role as a bronchodilator, is not entirely clear. Bronchodilation by theophylline is often attributed to its activity as a weak, non-specific phosphodiesterase (PDE) inhibitor, as frequently bronchodilation by theophylline in airway smooth muscle is associated with cAMP accumulation (Rabe *et al.*, 1995; Niewoehner *et al.*, 2002; Hansel *et al.*, 2004). Theophylline has greatest activity on PDE4, with a reported IC_{50} of approximately 400µM (Schudt *et al.*, 1991), yet in the data presented here theophylline significantly inhibited capsaicin-induced vagus nerve depolarisation at concentrations of 1µM and above, with an approximate IC_{50} of approximately 2µM. That theophylline exerts its inhibitory effects on sensory nerve depolarisation at such concentrations, well below its reported IC_{50} for PDE inhibition, indicates that the effect of theophylline on depolarisation of isolated vagus nerve is extremely unlikely to be due to its activity as a PDE inhibitor.

Aside from PDE inhibition, another well-known property of theophylline is it's potent antagonism of adenosine receptors (Pauwels & Joos, 1995). In the context of isolated vagus depolarisation, adenosine receptor antagonism seems an unlikely mechanism of action for theophylline, as, in vagus nerve tissue isolated from the normal endogenous systems/mediators, there should not be adenosine receptor agonists present. Indeed, in the same isolated systems used here, it has been shown that capsaicin-induced sensory nerve and neuron responses are completely blocked by a specific TRPV1 antagonist (JNJ17203212) (Grace *et al.*, 2012). Furthermore, capsaicin-evoked cough *in vivo* is also completely blocked by the same specific TRPV1 antagonist (Grace *et al.*, 2012), and therefore antagonism of the adenosine receptor is not a feasible mechanism for inhibition of capsaicin-evoked cough by theophylline.

There is however, some evidence that methylxanthines cause relaxation of airway smooth muscle via an activity on potassium channels, with several studies finding bronchodilation is partially reversed by blockers of large-conductance calcium-activated potassium channels (BK$_{Ca}$) (Miura *et al.*, 1992a; Ise *et al.*, 2003; Liu *et al.*, 2003; Wu *et al.*, 2004; Xin *et al.*, 2012). Additionally, a study by Wu *et al.* (2004) found that small-conductance calcium activated potassium channels (SK$_{Ca}$) and ATP-sensitive potassium channels (K$_{ATP}$) play a role in theophylline bronchorelaxation. In primary neurons, albeit not afferent peripheral neurons, methylxanthines have been suggested to affect potassium currents and potassium permeability with a requirement for calcium to be present, suggesting a role for calcium activated potassium channels (Munakata & Akaike, 1993; Simasko & Yan, 1993). Additionally, it has also been demonstrated that theophylline can activate calcium activated intermediate-conductance (IK$_{Ca}$) type potassium channels in a heterologous HEK293 cell line which stably expresses the IK$_{Ca}$ channel (Schrøder *et al.*, 2000).

The use of pharmacological blockers of these types of potassium channels in guinea pig vagus nerve revealed that SK$_{Ca}$, and to a lesser extent IK$_{Ca}$, channels are involved in the inhibitory effect of theophylline on guinea pig isolated vagus nerve. What is more, key experiments were repeated with similar results in human vagus nerve, demonstrating firstly that the anti-tussive effect of theophylline is likely to translate to the clinic, and secondly that the mechanism of theophylline inhibition of human sensory nerve depolarisation is likely to be the same as in guinea pig.

Experiments with isolated patch clamped jugular ganglia neurons confirmed that theophylline opens IK$_{Ca}$ and SK$_{Ca}$ channels, causing a hyperpolarisation of the cell that would explain its inhibitory action on sensory nerves. Using an inside-out patch clamp technique, it was shown that there was a requirement for calcium to be present for theophylline to open IK$_{Ca}$ and SK$_{Ca}$ channels. However, theophylline did not cause an increase in calcium (at the concentrations used here) in primary guinea pig airway jugular neurons, as determined by calcium imaging. What is more, the increased current activity of IK$_{Ca}$ and SK$_{Ca}$ channels was maintained when intracellular calcium influx was inhibited in whole cell patch clamp of the same cell type. These findings are in agreement with Schrøder et al. (2000) that the effect of theophylline on potassium channels is independent of an increase in intracellular calcium. The reduced activity of theophylline on IK$_{Ca}$ compared to SK$_{Ca}$ channels, and the lack of an effect on BK$_{Ca}$, could be a function of the differing calcium-sensitivity and voltage-dependence of these channels. IK$_{Ca}$ open probability is poorly dependent on voltage and is largely regulated by [Ca^{2+}]$_i$ with an opening concentration threshold that can vary over a wide range of

concentrations depending on the conditions whereas BK_{Ca} open probability is voltage dependent and needs high $[Ca^{2+}]_i$ (Cui *et al.*, 1997; Dai *et al.*, 2009). SK_{Ca} on the other hand is highly sensitive to calcium and voltage-insensitive. This gives SK_{Ca} the ability to open at low $[Ca^{2+}]_i$, independently from the membrane potential, and furthermore it has been shown to be important in controlling neuronal excitability and firing rate along axons (Weatherall *et al.*, 2010). The higher threshold of calcium-induced activation for IK_{Ca} gating, and the need for both increased membrane potential and $[Ca^{2+}]_i$ to open BK_{Ca} would suggest that the resting $[Ca^{2+}]_i$ under these conditions would not be sufficient for theophylline to open these channels. However, the possible activation of IK_{Ca} and/or BK_{Ca} by theophylline, when the sensory neurons are activated, $[Ca^{2+}]_i$ raised and the membrane depolarised, also remains a possibility.

Hyperpolarisation of sensory neurons by theophylline, via opening IK_{Ca} and SK_{Ca} channels, would explain its mode of action as a general inhibitor of excitatory stimuli in vagus nerves. What is more, its effectiveness at inhibiting human vagus sensory nerve depolarisation indicates that theophylline may be useful as a general anti-tussive in the clinic. In patients theophylline is generally administered orally, and levels in plasma are monitored to maintain a concentration of 10-20µg.ml^{-1}, which is considered to be the therapeutic window in which efficacy is achieved without excessive side-effects (Barnes, 2005). Theophylline inhibits capsaicin-induced depolarisation of human isolated vagus nerve by approx. 80% at 10µM, whereas in human airway smooth muscle, theophylline is reported to inhibit acetylcholine and capsaicin-induced bronchoconstriction with IC_{50} values of 150µM and 100µM (Guillot *et al.*, 1984; Manzini *et al.*, 1987; Finney *et al.*, 2006). This comparison of *in vitro* potency would suggest that theophylline could be expected to inhibit cough at or below plasma concentrations used to achieve bronchodilation. However, it has been highlighted that theophylline is actually a relatively poor bronchodilator, as, assuming 60% protein binding, plasma levels of 375µM, or 67µg.ml^{-1} would be required for 50% of the maximum bronchodilator effect of theophylline *in vivo* (Barnes *et al.*, 1998). If the same theoretical calculation (assuming 60% protein binding as before) were to be performed using the *in vitro* data generated here, then given the approximate IC_{50} of 2µM in guinea pig vagus nerve, a plasma concentration of only 5µM, or 0.9µg.ml^{-1} of theophylline would be required for anti-tussive effects. Of course the only way to truly determine the efficacy of theophylline to inhibit cough at therapeutic doses would be to perform a suitably powered, double-blind crossover study with objective cough counting as a primary end-point. The anti-tussive properties of theophylline presented here argue for such a clinical study, as, given that theophylline is a known quantity in terms of clinical use/approval, re-labelling/re-

purposing of theophylline for use as a cough therapy would be quicker and cheaper than developing and gaining clinical approval for an entirely novel class of compounds.

5. FAAH inhibitors

5.1 Introduction

Agonists of cannabinoid receptors, for example the archetypal non-selective Δ^9THC (the active constituent of marijuana/*Cannabis Sativa*), have been investigated as potential therapies for pain, migraine, glaucoma, and hypertension (Axelrod & Felder, 1998). Two types of cannabinoid receptor (CB) with differing expression patterns have so far been identified, with CB_1 receptors highly expressed within the CNS, and CB_2 receptors highly expressed in non-neuronal peripheral tissues (Regard *et al.*, 2008). However this view is overly simplistic, as CB_1 receptors are also expressed in the periphery (Kress & Kuner, 2009), and CB_2 receptors are also expressed in the CNS (Storr *et al.*, 2002; Duncan *et al.*, 2008) and the peripheral nervous system (Griffin *et al.*, 1997; Atwood & Mackie, 2010). Despite the relatively high expression of CB_2 receptors in the lung (http://pdsp.med.unc.edu/ShaunCell/family.php, Regard *et al.*, 2008), cannabinoids are relatively unexplored as respiratory therapeutics. Recently however, non-specific cannabinoid agonists and CB_2 agonists were shown to inhibit CA-evoked cough and sensory nerve depolarisation to a range of agonists including capsaicin and PGE_2 via the CB_2, but not the CB_1 receptor, indicating that the CB_2 receptor may prove the better target for anti-tussive therapies (Patel *et al.*, 2003; Belvisi *et al.*, 2009). Selective agonism of CB_2 should also avoid the side-effects of non-specific cannabinoid agonists, which can cause drowsiness, anxiety and panic via their activity on CB_1 receptors, due to their predominant expression in the CNS (Moreira *et al.*, 2009).

The integral membrane protein Fatty Acid Amide Hydrolase (FAAH) is the primary catabolic enzyme responsible for the breakdown of the endocannabinoid fatty acid amide (FAA) N-arachidonoyl ethanolamine (anandamide, AEA), as well as other related FAA signaling lipids such as palmitoylethanolamine (PEA), N-oleoylethanolamide (OEA) and linoleoyl ethanolamide (LEA). Pharmacological inhibition or deletion of FAAH has been shown to be anti-hyperalgesic and anti-nociceptive in pre-clinical models of pain and inflammation and is associated with a concomitant elevation in levels of FAAs (Kinsey *et al.*, 2009; Schlosburg *et al.*, 2009; Meyers *et al.*, 2011). FAAs are endogenously produced and cause activation of the cannabinoid CB_1 and CB_2 receptors (Mechoulam *et al.*, 1998), and are also known to activate non-cannabinoid receptors such as TRPV1, protease proliferator-activated receptors (PPAR) and opioid receptors (LoVerme *et al.*, 2005a; Chang *et al.*, 2006; Ahn *et al.*, 2009). The effects of cannabinoid agonists are known to be mediated by various potassium channels (Felder *et al.*, 1995). In particular, although in non-neuronal cell types, the effects of AEA have been found to be sensitive to a blocker of SK_{Ca} type potassium channels (Welch *et al.*, 1995b), as well as general blockade of all Ca^{2+}-activated potassium channels, including large (BK_{Ca}), intermediate IK_{Ca}), and small-conductance (SK_{Ca}) potassium channels (Yang *et al.*, 2007).

The aim of this chapter was to study the effect of a novel FAAH inhibitor (FAAHi), PF04862853 (Meyers *et al.*, 2011; Huggins *et al.*, 2012), on sensory nerve depolarisation and cough to gauge its effectiveness as an anti-tussive. Furthermore, the secondary aim of this chapter was to examine the mechanism of action of FAAHi inhibition in sensory nerves and neurons.

In order to fulfill these aims, the ability of PF04862853 to inhibit citric acid-evoked cough was examined. In addition, the effect of *in vivo* administration of PF04862853 on plasma levels of the FAAs AEA, PEA, OEA, and LEA was examined.

The isolated vagus nerve preparation was used to examine the mechanism of action of PF04862853, as a more pharmacologically amenable and cost-effective assay system. Using this system, the effect of PF04862853, was examined on low pH- and capsaicin-induced depolarisation of guinea pig. The ability of PEA to inhibit low pH-induced calcium responses in guinea pig isolated vagal neurons was examined in order to determine the effect of a FAA specifically on airway-terminating neurons. Selective antagonists of CB_1 and CB_2 receptors were then used to determine the cannabinoid receptor subtype involved in FAAHi/FAA activity on sensory nerve. PEA was then selected in order to further evaluate the role of potassium channels in its activity on isolated vagus nerve depolarisation using pharmacological blockers of potassium channels. Finally, key experiments were repeated using vagus nerve from human donor tissues to show that the effects observed in animal models would translate to the clinic.

5.2 Methods

5.2.1 Determining the effect of a FAAH inhibitor on CA-induced cough and FAA plasma levels in the conscious guinea pig

Cough experiments and plasma analysis of fatty acid amides described in this section were performed by researchers at Pfizer (Sandwich, UK).

In order to examine the effect of FAAH inhibition, vehicle (0.5%w/v methylcellulose, 0.1%v/v tween80), or PF04862853 (1mg.kg^{-1}) were administered p.o. to male Dunkin Hartley guinea pigs 4h prior to challenge with tussive agent. For cough recordings, a guinea pig was placed into a Perspex chamber attached to a microphone where coughs could be observed and recorded, as described previously (*Methods 2.2*). A baseline recording was taken for 5min, and then citric acid (0.3M) was administered by aerosol for 10min, during which time coughs were counted, as well as for a further 5min post-exposure, using the Buxco Cough Analyser. Blood samples were taken from the saphenous vein immediately following cough challenge and euthanisation. The timing for these assessments was based on a preliminary time-course study performed by researchers at Pfizer (Sandwich, UK) of plasma levels of PF04862853 showing peak exposure at 4h following p.o. administration (data not shown).

Levels of the FAAs AEA, PEA, OEA, and LEA in blood plasma were assessed by liquid chromatography mass-spectroscopy (LC-MS) as follows. Blood samples were centrifuged and whole plasma was decanted and stored at –20°C until assay. Plasma (100µl) was mixed with internal standard (30µl) in 50% MeOH (AEA, PEA, OEA and LEA), 1.5M Tris (pH 10, 100µL), and methyl *t*-butyl ether (700µL). The mixture was vigorously mixed in a shaker (45min) and then centrifuged (200 x *g*, 15min). The resulting supernatant was dried under a stream of N_2 gas (room temperature), and re-suspended in Diluton buffer (100µL). Lipid measurements were performed by LC-MS using an Agilent (1100) instrument. Solvent A was composed of 95:5 v/v H_2O:MeOH, solvent B was composed of 65:35:5 v/v/v isopropanol:MeOH:H_2O, and 0.1% formic acid was added to each solvent to assist in ion formation. The flow rate for each run started at 0.1 ml.min^{-1} with 0% B. At 5min, the solvent was immediately changed to 60% B with a flow rate of 0.4ml.min^{-1} and increased linearly to 100% B over 10 min. This was followed by an isocratic gradient of 100% B for 5 min at 0.5ml.min^{-1} before equilibrating for 3 min at 0% B at 0.5 ml.min^{-1}. The following parameters were used to measure the indicated metabolites (precursor ion, product ion, collision, energy in V): AEA (348, 62, 35), PEA (300, 62, 35), OEA (326, 62, 35), and LEA (324, 62, 35), and PF04862853 (392, 143, 55). Analysis was performed with an electrospray ionization (ESI) source. The capillary was set to 4kV and the fragmentor was set to 100V. The drying gas temperature was

$350°C$, the drying gas flow rate was $11\ l.min^{-1}$, and the nebulizer pressure was 35psi. The LC-MS/MS system consisted of Shimadzu SCL-10A VP pumps, CTC-PAL auto sampler, and Sciex API 4000 mass spectrometer. The LC separation conditions consisted of a 5-min gradient from 0 to 80% (v/v) MeOH in water with 0.1% formic acid at $0.2ml.min^{-1}$ on a Phenomenex Gemini C6 Phenyl column (3µm, 2 x 50mm). Comparing the mass ion peak areas with those of the corresponding deuterated standards allowed quantification of AEA, PEA, OEA and LEA levels.

5.2.2 Determining the effect of FAA on isolated vagus nerve depolarisation

The following experimental protocol was used to examine the effects of the FAAs AEA, PEA, OEA and LEA on isolated vagus nerve depolarisation induced by tussive stimuli. Following sacrifice of the guinea pig and dissection of the vagus nerve as outlined previously (*Methods 2.3*), the isolated nerve was allowed to equilibrate for 10min in the grease-gap chamber, following which capsaicin (1µM) or low pH KH solution (pH 5) was perfused for 2min, causing depolarisation. The nerve was then 'washed' (perfusion of KH alone) until the signal returned to baseline. Two reproducible responses to the same tussive stimulus were recorded in this way, following which the vagus nerve was incubated with vehicle (0.1% DMSO), PF04862853 (0.1-100µM), PEA (0.001-1µM), AEA (0.1µM), OEA (0.1µM), or LEA (0.1µM) for 10min, according to a standard protocol for examining efficacy in this system (Freund-Michel *et al.*, 2010). The vagus was then re-challenged with the initial tussive stimulus in the presence of test compound. Following a 'wash' period of 10min, a final stimulation of the tussive stimulus was used to determine tissue viability. On each piece of nerve tissue, only one concentration of any vehicle or drug was examined.

Key experiments were repeated on human vagus nerve tissue dissected from whole lungs unsuitable for transplant, as outlined in *Methods 2.3.1*.

5.2.3 Determining the effect of PEA on low pH-induced responses in isolated airway jugular ganglia neurons

Male Dunkin Hartley guinea pigs were euthanised, jugular ganglia were removed, and individual jugular ganglia neurons were isolated as described previously (*Methods 2.4*). The jugular and nodose ganglia contain the majority of the cell bodies for airway terminating vagal neurons. Previous data in our group indicated that a greater proportion of jugular ganglia neurons respond to capsaicin (Grace *et al.*, 2012). For this reason only jugular neurons were used to examine the inhibitory effects of PEA.

As described previously (*Methods 2.4.1*) guinea pigs had been *i.n.* dosed with DiI, a retrograde tracer dye, and airway-terminating neurons isolated from these animals were identified by the DiI present in their plasma membranes. Isolated neurons were incubated (37°C, 5%CO_2) overnight in complete F12 media (containing penicillin and streptavidin), in order to allow adherence to poly-D-lysine- and laminin-coated fluorodishes. Immediately prior to imaging experiments, cells were incubated with the calcium sensitive dye Fura-2AM.

To test the effect of PEA on the release of calcium in the ganglia, cells were acquired using a confocal epi-fluorescence microscope, and sensory neurons were identified by their body characteristics (20-30μM diameter) and light diffraction in phase contrast, whilst DiI emission was used to identify airway-terminating neurons. The fluorodish was connected to a pressurised perfusion system that allowed rapid change of the bath solutions as per the following protocol:

An initial control response to application of K_{50} solution (50mM hyper-potassium solution containing; 50mM KCl; 91.4mM NaCl; 1mM $MgCl_2$; 1.8mM $CaCl_2$; 0.33mM NaH_2PO_4; 10mM D-Glucose; 10mM HEPES; pH adjusted with NaOH to 7.4) was used to assess cell viability, and this response was used to normalise all subsequent responses within each cell. After perfusion with ECS to remove the K_{50} solution and allow recovery of calcium and voltage dye emissions back to baseline, cells were then stimulated twice with low pH ECS (pH 6) as control responses followed by a wash with ECS to recover baseline levels. The cells were then incubated with vehicle (0.1% DMSO in ECS), or PEA (1nM) for 5min, and then re-stimulated with low pH ECS in the presence of test compounds. ECS was then perfused to washout drugs for at least 10min, or until signal returned to baseline, following which final low pH, and then K_{50} applications were used to confirm that low pH responses recovered once the drug was removed, and that the cell(s) remained viable.

5.2.4 Determining the role of cannabinoid receptors and K^+ channels in the action of FAAH inhibitor and FAA on vagus nerve depolarisation

The role of cannabinoid receptors in the action of the FAAs PEA, AEA, OEA and LEA on vagus nerves was investigated using selective antagonists of cannabinoid receptors types 1 and 2 (CB_1 - SR141716A; CB_2 - SR144528). The role of potassium channels was similarly examined using the K^+ channel blockers paxilline, clotrimazole, apamin and glibenclamide. The antagonists and channel blockers were evaluated against a single sub-maximal concentration of PEA, AEA, OEA or LEA on guinea pig vagus nerve. The protocol used here was modified from that described in *Section 5.2.3*, as follows: two

control responses to capsaicin (1µM) or low pH KH solution (pH 5) were generated, and then vehicle (0.1% v/v DMSO), SR141716A (0.01µM), SR144528 (0.01µM), paxilline (1µM), apamin (1µM), glibenclamide (10µM) or clotrimazole (10µM) were incubated for 10min alone, and then co-incubated for 10min with vehicle (0.1% v/v DMSO), PEA (0.1µM), AEA (0.1µM), OEA (0.1µM) or LEA (0.1µM). The nerve was then stimulated with tussive stimulus in the presence of both vehicle/CB antagonist/channel blocker and vehicle/FAA, followed by a 'wash' period of 10min, and a final stimulation with tussive stimulus alone. The concentration of the CB antagonists used here (0.01µM) is similar to that used previously (Belvisi *et al.*, 2009), and were close to the pA2 values for the CB_1 (SR141716A; 7.9nM) and CB_2 (SR144528; 6.3nM) antagonists (Rinaldi-Carmona *et al.*, 1994; 1998). The concentrations of potassium channel blockers were selected from Freund-Michel *et al.* (2010).

Key experiments were repeated on human vagus nerve tissue dissected from whole lungs unsuitable for transplant, as outlined in *Methods 2.3.1*.

5.3 Anti-tussive potential of FAAHi

5.3.1 FAAHi effect on naïve guinea pig cough and plasma FAA levels

The effect of the FAAH inhibitor PF04862853 on numbers of cough evoked by capsaicin or citric acid was examined. Conscious guinea pigs were treated with vehicle or PF04862853 (1mg.kg^{-1} p.o.), and 4h later were challenged with an aerosol of citric acid (0.3mM) for 10min to evoke cough, during which, and for 5 minutes post-aerosol, the number of coughs evoked was recorded. PF04862853 at 1mg.kg^{-1} significantly reduced the number of coughs evoked to 8.40±2.24, from 16.10±2.84 in the vehicle control group (*Fig. 5.1a*).
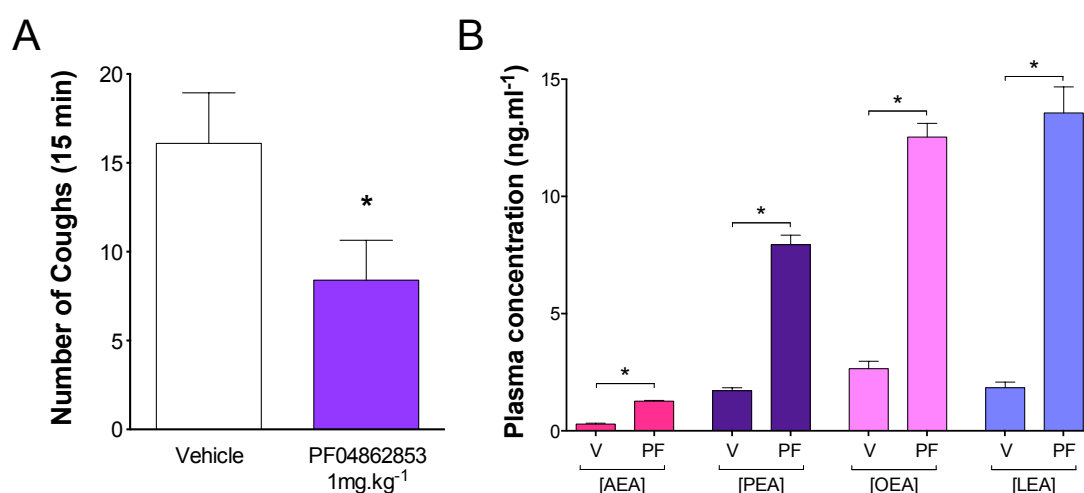
Figure 5.1 Effect of PF04862853 on citric acid-evoked cough and plasma FAA levels in guinea pigs
*Guinea pigs were pre-treated with vehicle (0.5%w/v methylcellulose, 0.2%v/v tween80 in PBS) or PF04862853 (1mg.kg^{-1} i.p.) 4h prior to **[A]** recording of coughs evoked over 15min by 5min aerosol of citric acid (0.3M) (including 5min recording during aerosol plus 10min post-aerosol), following which **[B]** blood samples were taken (i.v.) to determine plasma levels of AEA, PEA, OEA and LEA. Data displayed as mean±SEM, n=8, * p<0.05 as determined by students t-test compared to respective controls.*

Plasma levels of AEA, PEA, OEA and LEA were also elevated variously by between ~6-9 fold in the PF04862853-treated animals at this efficacious dose (*Fig. 5.1b*). These data indicate that inhibition of FAAH enzyme activity and sustained elevation of AEA, PEA, OEA and LEA are associated with the antitussive activity of PF04862853 in the guinea-pig cough model.

5.4 Mechanism of action of FAAHi

5.4.1 FAA effect on guinea pig vagus

As PF04862853 was shown to increase FAA levels *in vivo*, the effect of a selected FAA, palmitoylethanolamide (PEA), on low pH- and capsaicin-induced depolarisation of guinea pig isolated vagus nerves was examined to determine if it had similar inhibitory activity. The selected concentration range for PEA in this assay was based on the plasma concentration (10ng.ml^{-1}, or ~0.03µM) reached 4h after PF04862853 administration *in vivo* (*Section 5.3.1*). PEA (0.001-1µM) caused a concentration-dependent inhibition of both low pH- and capsaicin-induced sensory nerve depolarisation, which reached significance at concentrations of 0.01µM and higher (*Fig. 5.2*).
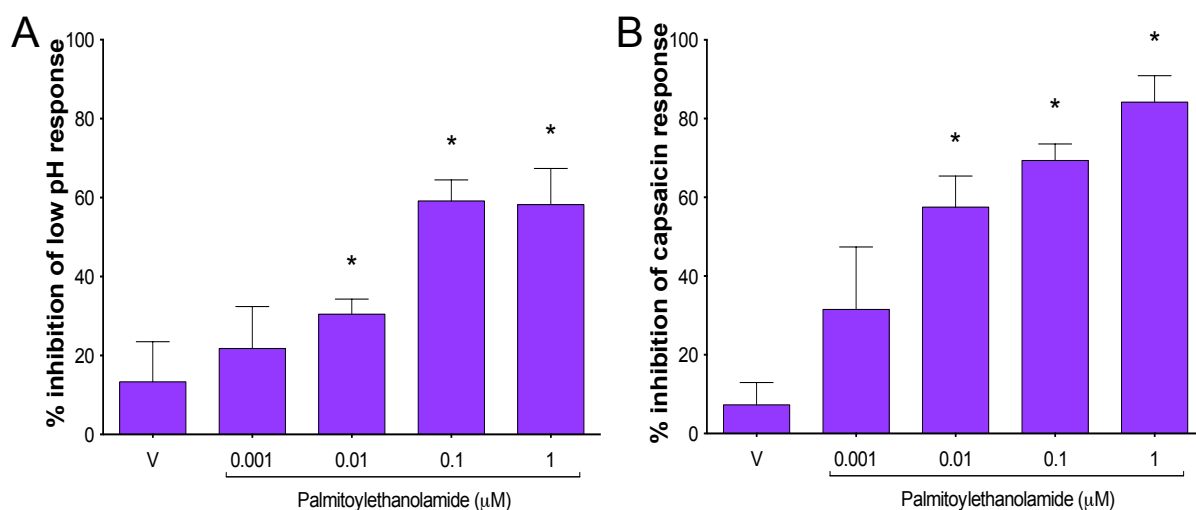
Figure 5.2 Non-cumulative concentration-response of PEA effect on low pH- or capsaicin-depolarisation of guinea pig isolated vagus nerve
*Isolated guinea pig vagus nerve was incubated with either vehicle (0.1% DMSO) or PEA (Palmitoylethanolamide; 0.001-1µM) to examine the effect on subsequent **[A]** low pH(5) or **[B]** capsaicin induced depolarisation. Data displayed as mean %inhibition of initial low pH(5) or capsaicin stimulations (internal controls) ±SEM, n=3-4, * p<0.05 as determined by paired students t-test compared to respective internal control.*

5.4.2 FAA effect on guinea pig airway isolated neurons

The effect of PEA on low pH-induced intracellular calcium flux was examined in single chemo-sensitive airway-terminating neurons of the jugular ganglia. PEA (1nM) significantly inhibited the increase in intracellular calcium levels caused by low pH (6) solution (*Fig. 5.3*).
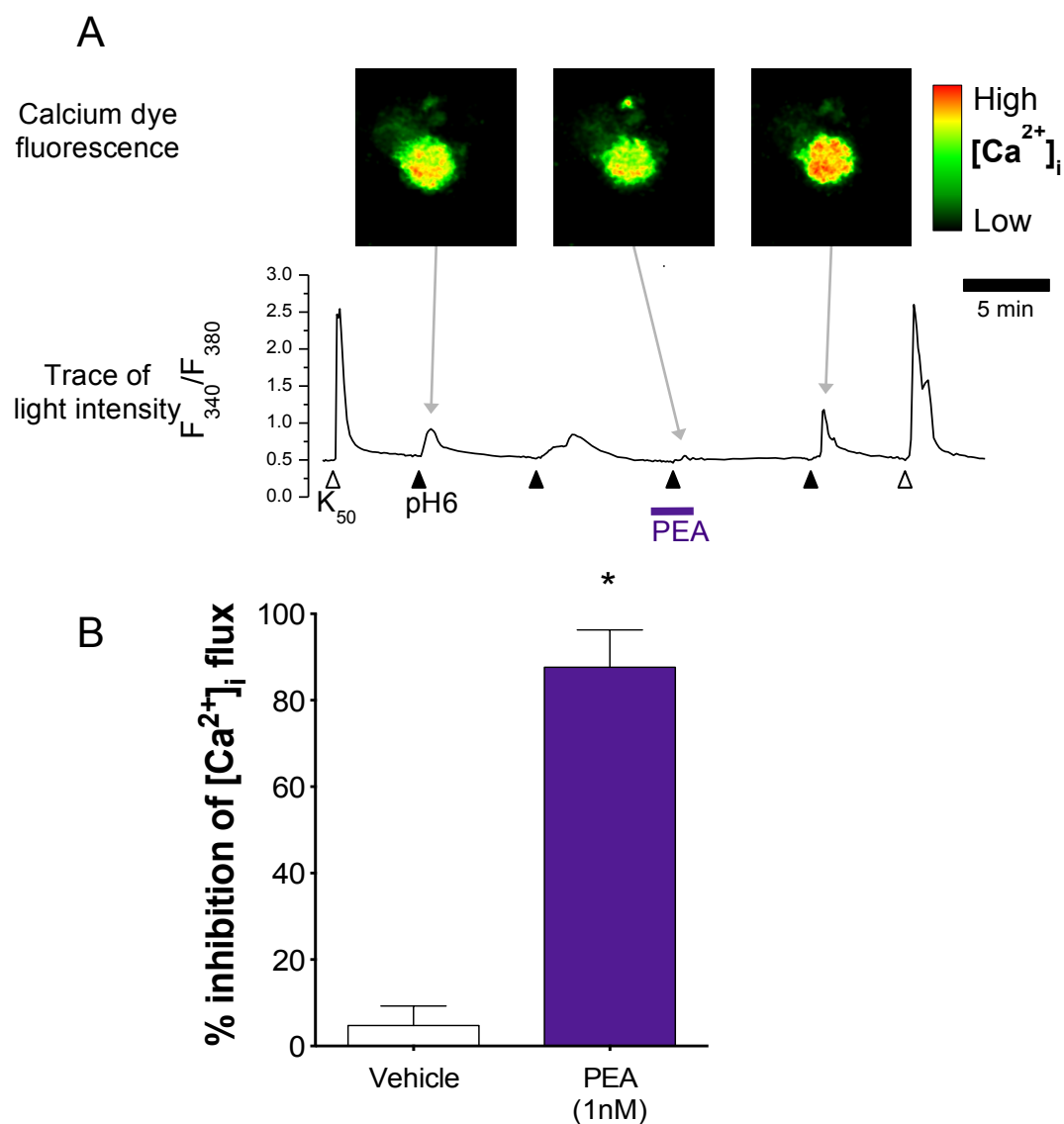
Figure 5.3 Effect of PEA on low pH-induced intracellular calcium flux in jugular airway-terminating neurons
*[A] Representative trace of PEA effect on low pH(6)-induced intracellular calcium levels. Open triangles indicate K_{50} (50mM KCl in ECS solution, 15s) application, closed triangles indicate pH6 (0.1μM, 30s) application, line indicates PEA (1nM, 2min) incubation. Images above trace show false-coloured representation of calcium fluorescence at time-points indicated by grey arrows. [B] Summary graph shows the effect of vehicle (0.1% DMSO) or PEA (1nM) on low pH-induced increases in intracellular calcium. Data displayed as mean %inhibition of initial low pH stimulations (internal control) ±SEM, n=5-14 cells from 3-5 guinea pigs, * p<0.05 as determined by paired students t-test compared to internal control.*

5.4.3 Effect of CB antagonists on FAA inhibition of vagus nerve depolarisation

PF04862853 increased plasma levels of FAAs (PEA, AEA, OEA and LEA) *in vivo*, and PF04862853 inhibited guinea pig vagus nerve depolarisation. One of these elevated FAAs, PEA, was shown to also concentration-dependently inhibit guinea pig vagus nerve depolarisation (*Section 5.4.1*). Therefore the effect of PEA (0.1µM) on low pH- and capsaicin-induced depolarisation was examined in the presence of vehicle (0.1% DMSO) or the CB$_2$-receptor selective antagonist (SR144528; 0.01µM) in order to determine if this FAA also inhibited vagal nerve depolarisation through the same receptor as the FAAHi.
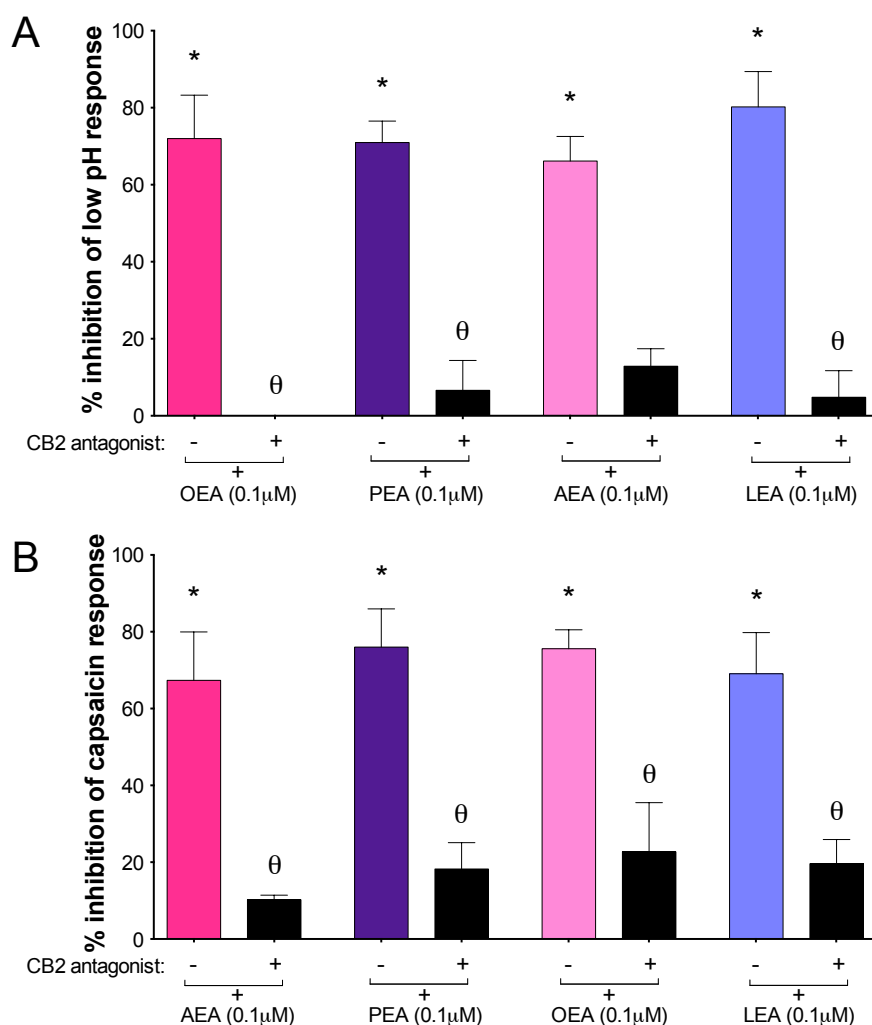
Figure 5.4 Effect of a CB$_2$ selective antagonist on PEA inhibition of guinea pig vagus nerve depolarisation
The effect of vehicle (0.1% DMSO) or CB$_2$ antagonist (SR144528; 0.01µM), pre-incubated for 10min, on PEA, AEA, OEA or LEA (all 0.1µM; 10min) inhibition of [A] low pH (pH5; 2min) or [B] capsaicin (1µM; 2min) depolarisation of guinea pig vagus nerve. Data displayed as mean %inhibition of initial stimulations (internal controls) ±SEM, n=4, * p<0.05 as determined by paired students t-test to internal control, θ p<0.05 as determined by Mann-Whitney U test to relevant vehicle control group.

Furthermore, the effect of same concentration (0.1µM) of the related FAAs OEA, AEA and LEA (which were also elevated by PF04862853 *in vivo*) on low pH- and capsaicin-induced depolarisation was examined in the presence of vehicle or CB_2-receptor selective antagonist (SR144528; 0.01µM). Similar to PEA, OEA, AEA, and LEA all inhibited both low pH- and capsaicin-induced depolarisation of guinea pig vagus nerve, an effect that was blocked by the CB_2-receptor antagonist SR144528 (*Fig. 5.4*).

5.4.4 Effect of K$^+$ channel blockers on FAA inhibition

The effect of blockers of BK_{Ca}, IK_{Ca}, SK_{Ca}, and K_{ATP} type potassium channels (paxilline, clotrimazole, apamin and glibenclamide respectively) on PEA inhibition of low pH- and capsaicin-induced depolarisation of guinea pig vagus nerve was examined. The SK_{Ca} blocker apamin (1µM) significantly reduced the effect of PEA on both low pH- and capsaicin-induced depolarisation, whereas the other K^+ channel blockers, at the concentrations tested here, did not have a significant effect on PEA-mediated inhibition (*Fig. 5.5*). This data suggests that opening of potassium channels, and specifically SK_{Ca} type potassium channels, plays a role in the inhibitory effects of PEA on sensory nerve depolarisation.
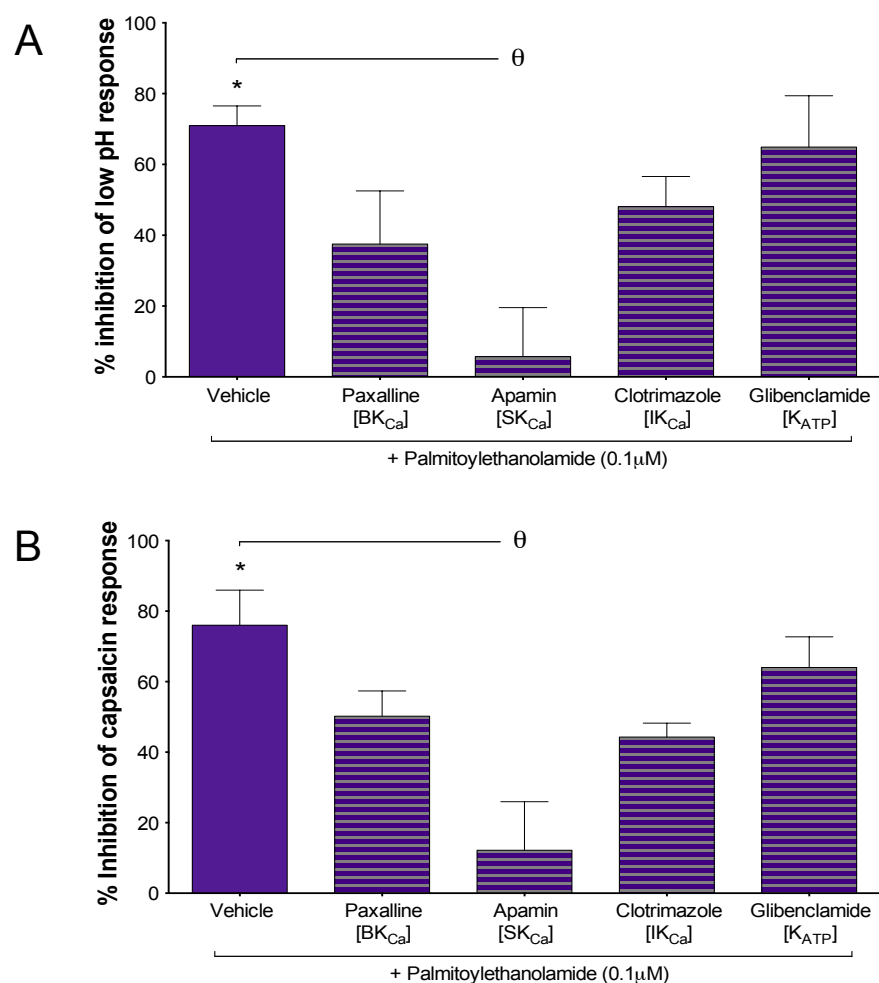
Figure 5.5 Effect of K^+ channel blockers on PEA inhibition of guinea pig vagus nerve depolarisation

*The effect of vehicle (0.1% DMSO), paxilline (BK_{Ca} blocker; 1µM), apamin (SK_{Ca} blocker; 1µM), clotrimazole (IK_{Ca} blocker; 10µM) or glibenclamide (K_{ATP} blocker; 10µM), pre-incubated for 10min, on PEA (0.1µM; 10min) inhibition of **[A]** low pH (pH5) or **[B]** capsaicin (1µM; 2min) depolarisation of guinea pig vagus nerve. Data displayed as mean %inhibition of initial low pH or capsaicin stimulations (internal control) ±SEM, n=4-5, where * p<0.05 as determined by paired students t test compared to internal control, and θ p<0.05 as determined Mann-Whitney U test compared to vehicle control.*

5.4.5 FAA effect in human vagus

Key experiments with FAAs performed on guinea pig vagus nerve were repeated on human vagus nerve tissue, in order to determine whether the anti-tussive effects observed in guinea pig would be likely to translate to humans. The effect of a single concentration of PEA on low pH-induced depolarisation was examined in the presence of, and following pre-incubation with, vehicle, CB_1 or CB_2 antagonist, or SK_{Ca} channel blocker (drugs, timings and concentrations as per *Sections 5.4.3 & 5.4.4*). In concordance with the results observed in guinea pig vagus nerve tissue, PEA (0.1µM) was effective at inhibiting low pH-induced depolarisation of human sensory nerves (*Fig. 5.6*). Furthermore, the inhibitory effect of PEA was significantly reversed by both the CB_2 antagonist and apamin, whereas the CB_1 receptor antagonist had no effect.
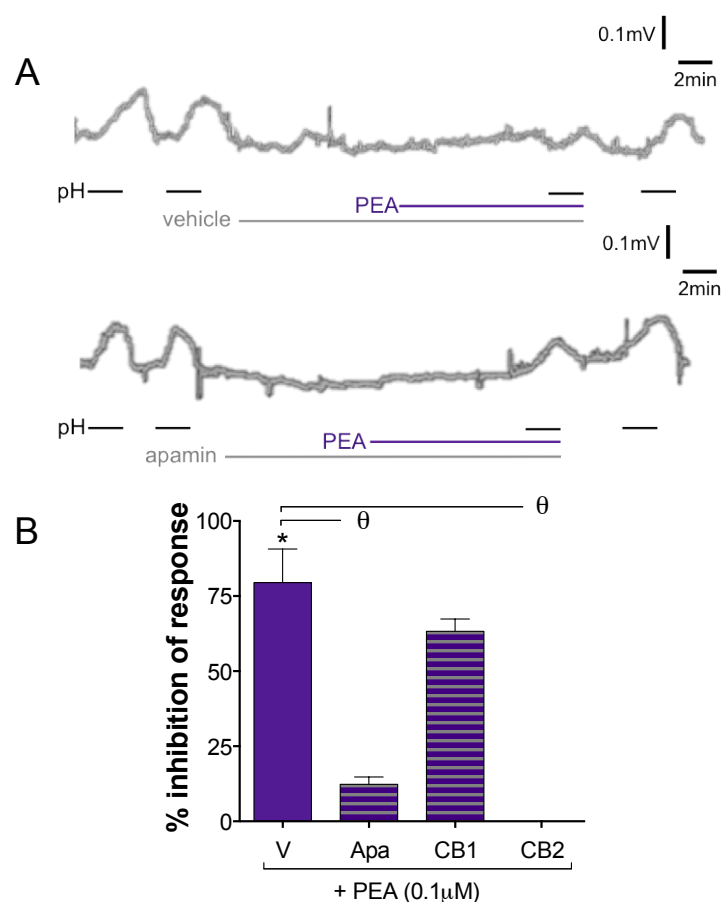
Figure 5.6 Effect of PEA ± apamin or CB_1/$_2$ antagonists on depolarisation of human vagus nerve
*[A] Representative traces of PEA effect on pH 5 induced (pH) depolarisation of human vagus nerves, in presence of vehicle (upper; 0.1% DMSO) OR apamin (lower; 1µM). [B] The effect of vehicle (0.1% DMSO), apamin (SK_{Ca} blocker; 1µM), CB_1 antagonist (SR141716A; 0.01µM) or CB_2 antagonist (SR144528; 0.01µM), pre-incubated for 10min, on PEA (1nM; 10min) inhibition of low pH-induced (pH5; 2min) depolarisation of human vagus nerve. Data displayed as mean %inhibition of initial low pH stimulations (internal control) ±SEM, n=3, * p<0.05 as determined by paired students t-test compared to respective internal control, and θ p<0.05 as determined by Mann-Whitney U test compared to vehicle control.*

5.5 Summary/Discussion

The aim of this chapter was to study the effect of the novel FAAHi, PF04862853, on sensory nerve depolarisation and cough. The anti-tussive activity of PF04862853 was demonstrated by its ability to reduce the numbers of CA-evoked coughs in a conscious guinea pig model. In this study (performed at Pfizer, Sandwich, UK), the effect of this FAAHi on plasma concentrations of the FAAs PEA, AEA, OEA and LEA was examined in the same animals used for cough evaluation. Plasma concentrations of all of the FAAs examined were elevated at the same time-point (4h) that the inhibition of cough was demonstrated. PF048642853 is a recently developed compound, which is reported to be potent and selective for the FAAH enzyme (Meyers *et al.*, 2011). In addition this compound displayed analgesic effects *in vivo* in a model of inflammatory pain (Meyers *et al.*, 2011). The elevation of FAAs reported here following the administration of PF04862853 is consistent with previous studies demonstrating increased levels of the same FAAs caused by the same compound at similar doses to those that inhibited cough in the guinea pig (as shown here) (Meyers *et al.*, 2011). It was additionally shown in the same study that the same doses of PF04862853 almost completely abolished the activity of the FAAH enzyme in the rat (Meyers *et al.*, 2011).

The endogenous FAAs AEA, PEA and OEA have each been implicated in the regulation of feeding and nociception (Mechoulam *et al.*, 1998; Suardíaz *et al.*, 2007). However, aside from being implicated with some anti-inflammatory activity, little is known about LEA and its potential role in nociceptive fibres (Ishida *et al.*, 2013). In order to examine this phenomenon further, one of the elevated FAAs, PEA, was also evaluated in the same preparation. Exogenous PEA applied to the isolated vagus nerve also concentration-dependently inhibited low pH- and capsaicin-induced depolarisation, and furthermore, AEA, OEA and LEA at 0.1µM each had a similar inhibitory effect. The effectiveness of the FAAs against both of these stimuli suggests a general anti-excitatory effect on sensory nerve depolarisation, as although capsaicin is known to cause depolarisation through TRPV1 alone, low pH activates vagal afferents not just via TRPV1 but also via additional mechanisms which have not yet been fully elucidated (Fox *et al.*, 1995; Kollarik & Undem, 2002). What is more, PEA (1nM) almost completely inhibited the $[Ca^{2+}]_i$ influx induced by low pH in guinea pig airway-terminating jugular neurons; further indicating that FAAs have a direct inhibitory effect on the cell type which is likely to mediate the cough reflex.

It is interesting to note that PEA caused significant inhibition of low pH- and capsaicin-induced vagus nerve depolarisation at concentrations of 0.01µM, with approximate IC_{50} values of 17 and 3nM respectively; these effective concentrations of exogenous PEA are

similar to the plasma levels of PEA (approximately 8ng.ml^{-1} or 25nM) reached following *in vivo* administration of PF04862853. It should be noted however that this does not imply that these concentrations of PEA would be reached at the vagus nerve termini *in vivo* (due to unknown plasma binding levels and distances of termini from blood vessels), more that the concentrations effective on vagus nerves are theoretically plausible in an *in vivo* setting.

The mechanism by which the FAAs inhibit depolarisation was then examined; firstly by determining the receptor(s) they may act on. Interestingly, whilst the FAAs are known as 'endocannabinoids', the receptor through which they act is not entirely clear. Of the FAAs examined here, AEA (anandamide) is the best examined, and the functions of AEA are mediated mostly by the CB_1 receptor (Palmer *et al.*, 2002), although it has also been shown to induce functional effects through the CB_2 receptor (Palmer *et al.*, 2002), PPAR (O'Sullivan, 2007), TRPV1 (Starowicz *et al.*, 2007), and TRPM8 (De Petrocellis *et al.*, 2007) in various tissues. Generally it appears that AEA has greatest functional affinity for the CB receptors, with reported IC_{50} values of 0.5μM and 1.9μM against CB_1 and CB_2 receptors, respectively, in functional assay systems (Felder *et al.*, 1995), compared to 10-30μM for PPARα (Sun *et al.*, 2007), 5-8μM for TRPV1 (Smart *et al.*, 2000; Ralevic *et al.*, 2001; Tognetto *et al.*, 2001; Oz, 2006), & 3μM for TRPM8 (De Petrocellis *et al.*, 2007). AEA has been shown to cause depolarisation of rat and guinea pig vagus nerves (Kagaya *et al.*, 2002; Weller *et al.*, 2011), $[Ca^{2+}]_i$ influx in sensory neurons (Jia *et al.*, 2002), and cough (Jia *et al.*, 2002) through the TRPV1 receptor. It should be noted of this apparently contradictory data that the stimulatory effects of AEA (anandamide) were observed at concentrations at least 100 times higher than those used here. No activation/depolarisation of vagus nerve or neurons was observed in any of the assay systems used here. Indeed, bearing in mind the reported efficacy of AEA at various receptors in cell- and tissue-based assays when considering the functional inhibition of depolarisation by AEA observed here at 0.1μM suggests that this effect is most likely mediated by cannabinoid receptors.

In contrast to AEA, PEA is thought to act predominantly via CB_2 receptors (Calignano *et al.*, 1998; Jaggar *et al.*, 1998; Calignano *et al.*, 2000; Farquhar-Smith & Rice, 2001; Conti *et al.*, 2002; Helyes *et al.*, 2003), and not via CB_1 receptors (Lambert & Di Marzo, 1999), despite one report of functional antagonism with the CB_1 antagonist SR141716A (Costa *et al.*, 2008). Intriguingly, despite one initial contrary report (Facci *et al.*, 1995), PEA has been shown not to bind directly to the CB_2 receptor (Griffin *et al.*, 2000), but rather is thought to be a 'functional agonist' of the CB_2 receptor, as many of the pharmacological (Calignano *et al.*, 1998; Jaggar *et al.*, 1998; Calignano *et al.*, 2000; Conti *et al.*, 2002), as

well as the analgesic (Calignano *et al.*, 1998; Farquhar-Smith & Rice, 2001; 2003; Helyes *et al.*, 2003) actions of PEA are inhibited by the specific CB_2 antagonist/inverse agonist SR144528. In addition to cannabinoid receptors, and similarly to AEA, PEA has also been shown to activate TRPV1 channels (Ambrosino *et al.*, 2013) and PPAR receptors (LoVerme *et al.*, 2005a; b; Costa *et al.*, 2008). However, PEA is reportedly more efficacious at CB_2 receptors, with an IC_{50} value of 0.015µM (Yoshihara *et al.*, 2005), compared to approximately 3µM at TRPV1 channels (Ambrosino *et al.*, 2013) and 3µM at PPAR receptors (LoVerme *et al.*, 2005a; b). In the data presented here, PEA appeared to reach a maximal effect on vagus depolarisation at 1µM, with approximate IC_{50} values in this system of 17nM and 3nM against low pH- and capsaicin-induced depolarisation, respectively.

Given that FAAs have only been reported to activate TRP channels and PPAR receptors at much higher concentrations than those used here, the effect of selective CB_1 and CB_2 receptor antagonists on inhibition by the FAAs was examined. Against both capsaicin- and low pH-induced depolarisation of guinea pig vagus nerve, the CB_2, but not the CB_1 specific antagonist reversed the inhibition caused by each of the applied FAAs. This data is in agreement with previous studies of exogenous cannabinoid agonists in this preparation, where it was demonstrated that the non-specific CB agonist CP55940, as well as the CB_2 selective agonists JWH133 and GW833972A each caused inhibition of vagus nerve depolarisation via the CB_2 receptor (Patel *et al.*, 2003; Belvisi *et al.*, 2009). The concentration of the antagonists used here (0.01µM) is similar to that used previously (Belvisi *et al.*, 2009), and was selected to be close to the pA2 values for the CB_1 (SR141716A; 7.9nM) and CB_2 (SR144528; 6.3nM) antagonists (Rinaldi-Carmona *et al.*, 1994; 1998). It therefore seems clear that, in this tissue type, and at these concentrations, AEA, PEA, OEA and LEA cause inhibition of induced depolarisation through the CB_2 receptor. Further investigation would be required to determine if the FAAs would cause depolarisation of sensory nerves at higher concentrations, as has been reported for AEA (Jia *et al.*, 2002; Kagaya *et al.*, 2002; Weller *et al.*, 2011). Moreover further investigation would be required to determine whether such high local and circulating plasma concentrations of FAAs could be caused by inhibition of FAAH, which might contraindicate their potential use as an anti-tussive. Of pertinence to the latter point, PF04862853 at the same concentration used here (1mg.kg^{-1}) was supra-maximal in causing complete inhibition of the FAAH enzyme (Meyers *et al.*, 2011), which resulted in plasma concentrations of only 3nM AEA, which would seem to preclude activity at receptors other than CB_2, at least in vagus nerve.

Whilst the FAAs examined here certainly appear to be 'functional agonists' of the CB_2 receptor (assuming SR144528 specificity), future work would be required to determine if PEA, OEA and LEA bind directly to CB_2 receptors. One hypothesis is that these FAAs have a so-called 'entourage effect', whereby they act as competitive substrates for FAAHi, indirectly allowing the elevation of AEA, which then binds to and activates cannabinoid receptors (Lambert & Di Marzo, 1999), although this theory has not been tested.

In order to further examine the mechanism of action of the FAAs, and thereby of FAAH inhibition, on sensory nerve depolarisation, the effect of pharmacological blockers of potassium channel blockers was profiled against the inhibitory effect of PEA. The effects of cannabinoid agonists are known to be mediated by various potassium channels (Felder et $al.$, 1995). In particular, although in non-neuronal cell types, the effects of AEA have been found to be sensitive to a blocker of SK_{Ca} type potassium channels (Welch et $al.$, 1995b), as well as general blockade of all Ca^{2+}-activated potassium channels, including large (BK_{Ca}), intermediate (IK_{Ca}), and small-conductance (SK_{Ca}) potassium channels (Yang et $al.$, 2007). In addition, the peripheral nociceptive effects of PEA were reversed using the ATP-activated potassium channel (K_{ATP}) channel blocker glibenclamide in a rat model of PGE_2 induced hyperalgesia (Romero & Duarte, 2012). The inhibitory effect of PEA on isolated guinea pig vagus nerve was therefore examined against blockers of the various calcium-activated potassium channels as well as ATP-activated potassium channels. PEA inhibition was found to be sensitive to apamin, suggesting that SK_{Ca} type potassium channels are involved in the CB_2-mediated FAA inhibition of depolarisation in guinea pig sensory nerves.

Key experiments were repeated in human isolated vagus nerve to examine if the mechanism of action of PF04862853 might be translatable to human tissue, and ergo the human cough reflex. Similar to guinea pig vagus nerve issue, PEA (0.1µM) caused a significant inhibition of low pH-induced human vagus nerve depolarisation. What is more, the CB_2 receptor antagonist and the SK_{Ca} channel blocker, but not the CB_1 receptor antagonist attenuated this inhibition. These results indicate that the functional effect of FAAH inhibition observed in animal models is likely to translate to the clinic, and that a similar mechanism of action in sensory nerve tissue may be expected.

One of the advantages of FAAH inhibition over the administration of exogenous cannabinoid agonists is the avoidance of the undesirable CNS effects associated with exogenous cannabinoid agonists (Moreira et $al.$, 2009). As demonstrated previously and in this study, the antinociceptive effect of FAAs such as PEA are peripherally mediated,

without the need for CNS involvement (Romero *et al.*, 2013). The FAAH inhibitors examined in clinical trials thus far, including the closely related compound PF04457845, have proven to be free of adverse effects (Huggins *et al.*, 2012; Li *et al.*, 2012). Indeed, the endogenous FAAs are reported to have far fewer adverse effects than exogenous cannabinoid agonists (Huggins *et al.*, 2012). In particular, PEA, given orally, appeared to have few adverse effects in a recent small clinical trial (Gagliano *et al.*, 2011), as well as in two larger trials in the former Czechslovakia during the 1970s (Masek *et al.*, 1974; Kahlich *et al.*, 1979). The latter trials even led to the clinical use of PEA for the treatment of acute respiratory diseases including influenza infections for a period. Although its use was discontinued, for reasons that are not clear, these reasons were apparently not related to adverse effects or the safety of the drug (LoVerme *et al.*, 2005b). There is also evidence that cannabinoid receptors, in particular CB_2 receptors, are inducible, and are up-regulated in tissues at the site of inflammation/pain, suggesting that levels of locally produced and systemic FAAs increased by inhibition of FAAH may be more effective at the site of inflammatory insult/injury (Ashton & Glass, 2007; Benito *et al.*, 2008; Pertwee, 2009; Miller & Devi, 2011).

In summary, inhibition of FAAH, thereby elevating endogenous levels of FAAs/endocannabinoids, would seem to be a promising strategy for the inhibition of cough. The FAAH inhibitor PF04862853 examined here was effective in a pre-clinical guinea pig model of evoked cough, apparently via a mechanism involving the elevation of local FAAs, causing inhibition of sensory nerve depolarisation via activation of CB_2 receptors, and downstream small-conductance potassium channels. A similar mechanism of action was demonstrated in human isolated vagus nerve. Future investigation would be required to determine whether these promising results would translate to an anti-tussive effect of FAAH inhibition on the *in vivo* cough reflex in humans.

6. Efficacy of anti-tussives in a disease model

6.1 Introduction

In the preceding three chapters the anti-tussive efficacy of three different classes of compounds has been investigated using *in vivo* and *ex vivo* models of cough and sensory nerve activation evoked by clinically relevant stimuli. The long acting β_2-adrenoreceptor agonists formoterol and olodaterol, as well as the methylxanthine theophylline, were each effective at inhibiting cough and sensory nerve activation/firing evoked by capsaicin challenge in naïve guinea pigs. Similarly, the novel fatty acid amide hydrolase inhibitor (FAAHi) PF04862853 was shown to inhibit coughs evoked by CA challenge in naïve guinea pigs, and the fatty acid amides, including PEA, levels of which were elevated by PF04862853, inhibited sensory nerve activation induced by a low pH stimulus.

Capsaicin and CA are frequently used as stimuli in animal models of cough to mimic their use as standard tussive stimuli in clinical studies. Both stimuli have been shown to evoke cough in a dose-dependent manner in both guinea pigs and in healthy humans (Laude *et al.*, 1993; Doherty *et al.*, 2000). It is generally accepted that in humans the 'normal' cough reflex sensitivity to these stimuli is modified in respiratory diseases where chronic persistent cough presents as a problematic symptom (Wong & Morice, 1999; Doherty *et al.*, 2000; Blanc *et al.*, 2009; McGarvey *et al.*, 2009). For example the threshold for cough evoked by capsaicin and citric acid is lowered in patients with acute respiratory infections (Empey *et al.*, 1976; O'Connell *et al.*, 1994; Vestbo *et al.*, 2013), asthma (Laude *et al.*, 1993; Doherty *et al.*, 2000) or COPD (Wong & Morice, 1999; Doherty *et al.*, 2000; Blanc *et al.*, 2009; McGarvey *et al.*, 2009). As the purpose of any anti-tussive medication is to inhibit cough in patients whom exhibit such an exaggerated cough response, examining the effect of novel therapeutics in animal models which display a similarly enhanced cough response may be considered a more pertinent indicator of efficacy compared to such investigations in naïve animals. As the spontaneous development of diseases such as COPD are rare in laboratory animals, mechanistic models of disease are used, whereby a challenge agent induces a similar phenotype, or aspects thereof, to that observed in the disease state.

The aim of this chapter was therefore to examine the efficacy of the anti-tussive compounds examined in the preceding chapters in a mechanistic disease model of enhanced cough and sensory nerve responses.

To evaluate the effectiveness of the compounds which have been shown in the preceding chapters to inhibit naïve capsaicin- and/or CA-evoked cough, a guinea pig model of cigarette smoke (CS)-enhanced capsaicin- and CA-evoked cough was selected.

CS exposure is considered to be a relevant challenge agent to drive this 'COPD-like' modification of the cough reflex, as cigarette smoking is the primary risk factor associated with the development of COPD (Empey *et al.*, 1976; O'Connell *et al.*, 1994; Vestbo *et al.*, 2013). In this model, guinea pigs are exposed to CS for 8 days, inducing an enhanced cough response to subsequent capsaicin or CA challenge. This increased sensitivity to tussive-stimulus challenge therefore mimics the lowered threshold to capsaicin or CA challenge observed in human smokers and COPD patients (Wong & Morice, 1999; Doherty *et al.*, 2000; Lewis *et al.*, 2007; Blanc *et al.*, 2009). What is more, it has previously been shown that vagus nerve depolarisation responses to capsaicin and low pH in tissues taken from CS-exposed guinea pigs are similarly enhanced compared to air-exposed comparator animals (Wortley *et al.*, 2011).

The two LABA compounds, formoterol and olodaterol, the methylxanthine theophylline, and the FAA PEA will be examined for their ability to inhibit the sensory nerve depolarisation observed following CS-exposure. Where possible, due to considerations of the costs involved in using *in vivo* models, the efficacious compounds were advanced to be examined for efficacy in the *in vivo* model of CS-enhanced capsaicin-evoked cough.

6.2 Methods

6.2.1 *In vivo* cigarette smoke exposures

Male Dunkin Hartley guinea pigs used for the purposes of this chapter were randomly assigned to be exposed to room air or CS for 1h periods, twice daily, for 8 days, as per the protocol described previously (*Methods 2.6*). 24h after the last exposure period, animals were either euthanised, and had vagus nerves dissected, or were used for the assessment of cough. The number of days exposure and time-point following exposure were selected from Lewis *et al.* (2007), where it was demonstrated that these parameters resulted in a significant and robust enhancement of capsaicin- and CA-evoked cough.

6.2.1 Evaluation of drug effectiveness against CS-enhanced capsaicin-induced isolated vagus nerve depolarisation

The aim of this protocol was to determine the effectiveness of the compounds examined in the previous chapters in a pre-clinical model of CS-enhanced capsaicin- or low pH-induced vagus nerve depolarisation. The protocol used here (described below) was the same as that used to examine agonist efficacy in vagus nerves from naïve guinea pigs. Sub-maximal concentrations of test compounds were selected from the preceding chapters as outlined in *Table 6.1* below. Drug vehicles were as indicated in the relevant chapter.

Test drug	Type	Concentration	Incubation time	Chapter
Olodaterol	LABA	10nM	20min	3
Theophylline	Methylxanthine	10µM	10min	4
PEA	Fatty Acid Amide	100nM	10min	5

Table 6.1 Details of compounds evaluated against CS-enhanced vagus nerve responses

Guinea pigs were sacrificed and vagus nerves were dissected as outlined previously (*Methods 2.3*) 24h after air/CS-exposure. The isolated nerve was allowed to equilibrate for 10min in the grease-gap chamber, following which capsaicin (1µM) or low pH KH solution (pH 5) was perfused for 2min, causing depolarisation. The nerve was then 'washed' (perfusion of KH alone) until the signal returned to baseline. Two reproducible responses to the same tussive stimulus were recorded in this way, following which the vagus nerve was incubated with test compound (for incubation time/concentration *see Table 6.1*). The vagus was then re-challenged with the initial tussive stimulus in the

presence of test compound. Following a 'wash' with KH solution alone for a period of 10min, a final stimulation of the tussive stimulus was used to determine tissue viability. On each piece of nerve tissue, only one concentration of any vehicle or drug was examined.

6.2.1 Evaluation of drug effectiveness against CS-enhanced capsaicin-evoked guinea pig cough

The aim of this protocol was to determine the effectiveness of the compounds examined in the previous chapters in a pre-clinical *in vivo* model of CS-enhanced capsaicin-evoked cough. The protocol used here (described below) was the same as that used to examine agonist efficacy against capsaicin-evoked cough in naïve guinea pigs. The only adaptation was to select a lower concentration of capsaicin (30µM) to be aerosolised as the challenge agent, in order to allow any CS-driven enhancement of the responses to be observed, and also to reduce the stress caused to the animals. Effective anti-tussive doses of test compounds were selected from the preceding chapters as outlined in *Table 6.2* below.

Test drug	Type	Dose	Route of administration	Chapter
Formoterol	LABA	3 µg.ml^{-1}	aerosol	3
Olodaterol	LABA	3µg.ml^{-1}	aerosol	3
Theophylline	Methyxanthine	100mg.kg^{-1}	intra-peritoneal	4

Table 6.2 Details of compounds evaluated against CS-enhanced cough responses

In order to examine the effect of the LABA compounds, 24h after the last exposure period guinea pigs were place in a Perspex chamber to receive vehicle (0.1% DMSO in PBS), olodaterol or formoterol (3µg.ml^{-1}), aerosolised for 10min (approx. volume 10ml), 50min prior to cough challenge. In order to examine the effect of the theophylline, 24h after the last exposure period guinea pigs were administered vehicle (0.5%w/v methylcellulose, 0.2%v/v tween80 in PBS) or theophylline 1h prior to cough challenge.

For cough recordings, a guinea pig was placed into a Perspex chamber attached to a microphone where coughs could be observed and recorded, as described previously (*Methods 2.2*). A baseline recording of was taken for 5min, and then capsaicin (30µM) or citric acid (0.3M) were administered by aerosol for 5min, during which time coughs were counted and Penh recorded, as well as for a further 5min post-exposure, using the Buxco Cough Analyser.

6.3 Effect of anti-tussives on CS-enhanced vagus responses

6.3.1 β-agonists effect on CS-enhanced vagus responses

Depolarisation induced by capsaicin was significantly increased in vagus nerves from guinea pigs exposed to CS for 8 days (0.41±0.05mV) compared to air-exposed comparators (0.23±0.03mV) (*Fig. 6.1a*). The novel LABA compound olodaterol significantly inhibited both the 'normal' and CS-enhanced capsaicin-induced sensory nerve depolarisation (*Fig. 6.1b*). There was no significant difference in the level of inhibition of depolarisation as determined by comparison of the percentage inhibition in the treatment groups by Mann-Whitney U test.
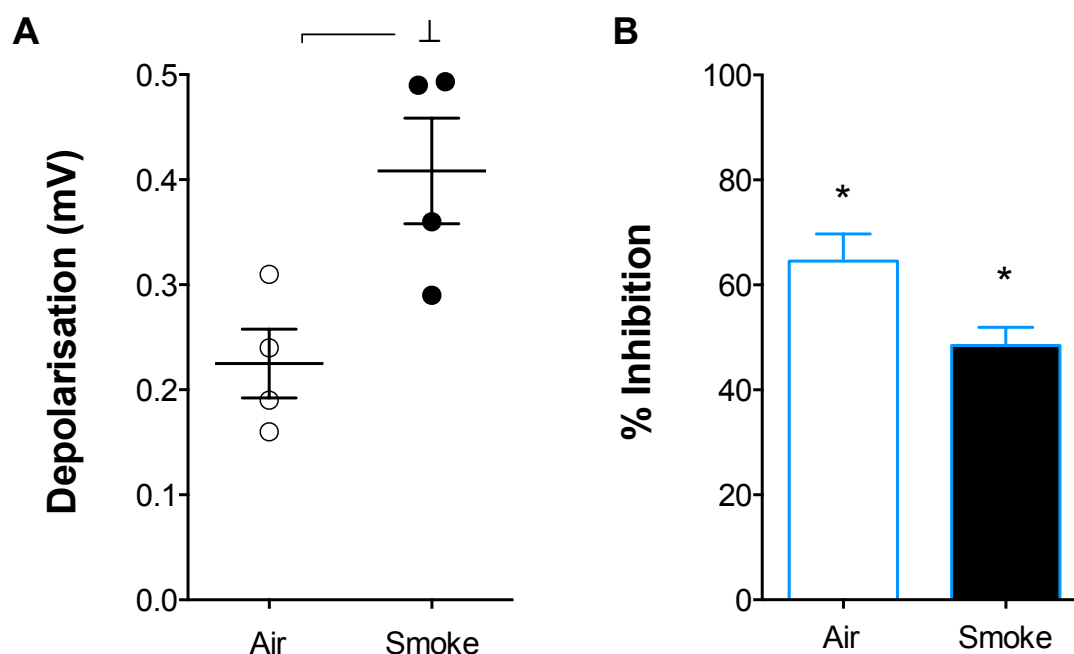
Figure 6.1 Effect of olodaterol on CS-enhanced capsaicin responses in guinea pig isolated vagus nerves
*[A] Magnitude of capsaicin-induced (1µM; 2min) depolarisation of vagus nerve tissue from air- and CS-exposed guinea pigs. [B] Effect of olodaterol (10nM; 20min) on capsaicin-induced depolarisation of vagus nerve tissue from air- or CS-exposed guinea pigs. Data displayed as mean±SEM, n=4, ⊥ p<0.05 as determined by unpaired students t-test * p<0.05 as determined by paired students t-test.*

6.3.2 Theophylline effect on CS-enhanced vagus responses

Depolarisation induced by capsaicin was significantly increased in vagus nerves from guinea pigs exposed to CS for 8 days (0.33±0.04mV) compared to air-exposed comparators (0.19±0.04mV) (*Fig. 6.2a*). The methylxanthine theophylline significantly inhibited both the 'normal' and CS-enhanced capsaicin-induced sensory nerve depolarisation (*Fig. 6.2b*). There was no significant difference in the level of inhibition of depolarisation as determined by comparison of the percentage inhibition in the treatment groups by Mann-Whitney U test.

Figure 6.2 Effect of theophylline on CS-enhanced capsaicin responses in guinea pig isolated vagus nerves
[A] Magnitude of capsaicin-induced (1µM; 2min) depolarisation of vagus nerve tissue from air- and CS-exposed guinea pigs. [B] Effect of theophylline (10µM; 10min) on capsaicin-induced depolarisation of vagus nerve tissue from air- or CS-exposed guinea pigs. Data displayed as mean±SEM, n=5, ⊥ p<0.05 as determined by unpaired students t-test * p<0.05 as determined by paired students t-test.

6.3.3 PEA effect on CS-enhanced vagus responses

Depolarisation induced by capsaicin was significantly increased in vagus nerves from guinea pigs exposed to CS for 8 days (0.20±0.02mV) compared to air-exposed comparators (0.10±0.02mV) (*Fig. 6.3a*). The fatty acid amide PEA significantly inhibited both the 'normal' and CS-enhanced low pH-induced sensory nerve depolarisation (*Fig. 6.3b*). There was no significant difference in the level of inhibition of depolarisation as determined by comparison of the percentage inhibition in the treatment groups by Mann-Whitney U test.
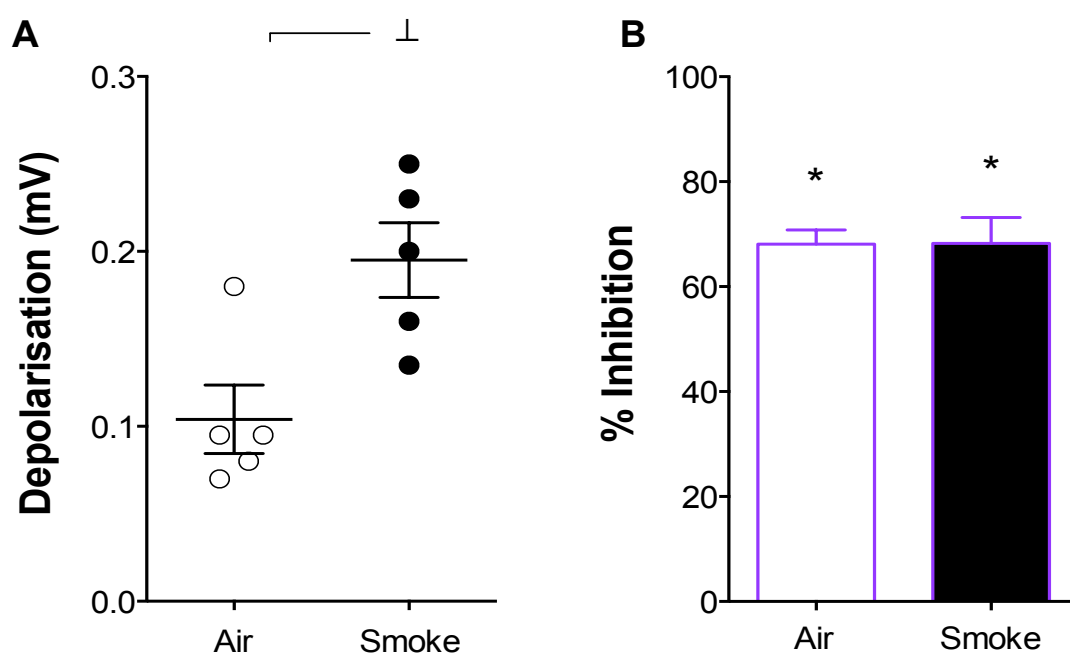
Figure 6.3 Effect of PEA on CS-enhanced low pH responses in guinea pig isolated vagus nerves
*[A] Magnitude of low pH-induced (pH5; 2min) depolarisation of vagus nerve tissue from air- and CS-exposed guinea pigs. [B] Effect of PEA (1nM; 10min) on low pH-induced depolarisation of vagus nerve tissue from air- or CS-exposed guinea pigs. Data displayed as mean±SEM, n=5, ⊥ p<0.05 as determined by unpaired students t-test * p<0.05 as determined by paired students t-test.*

6.4 Effect of anti-tussives on CS-enhanced cough responses

6.4.1 β-agonists effect on CS-enhanced cough responses

The number of coughs evoked by capsaicin was increased by approximately 100% in guinea pigs exposed to CS for 8 days (11.4±2.5 coughs; mean±SEM) compared to air-exposed comparators (6.0±1.3 coughs; mean±SEM) (*Fig. 6.4*). At the same dose that completely abolished capsaicin-evoked coughs in naïve animals (*Section 3.3.1*), the LABA compounds formoterol and olodaterol reduced the number of capsaicin-evoked coughs in CS-exposed guinea pigs to 1.8±1.2 and 0.3±0.2 coughs, respectively (*Fig. 6.4*).
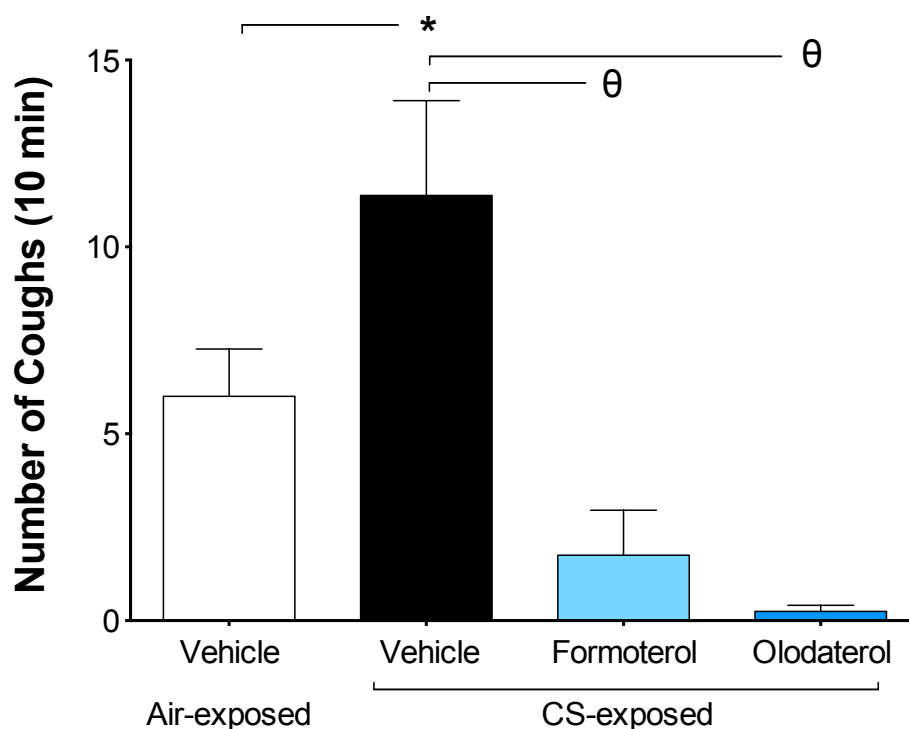
Figure 6.4 Effect of LABA compounds on CS-enhanced capsaicin-evoked guinea pig cough
Guinea pigs, which had been exposed to room air or CS, twice daily, for 8 days, were pre-treated with an aerosol of vehicle (0.1% DMSO in PBS), formoterol (3µg.ml^{-1}), or olodaterol (3µg.ml^{-1}) for 10min, 1h prior to challenge with an aerosol of capsaicin (30µM; 5min). Numbers of coughs were recorded for a 10min period, including 5min of aerosol plus 5min post-aerosol. Data displayed as mean±SEM, n=8, * p<0.05 as determined by Mann-Whitney U test compared to air/vehicle control, θ p<0.05 as determined by Kruskal-Wallis test with Dunn's post-hoc test, compared to CS/vehicle control.

6.4.2 Theophylline effect on CS-enhanced cough responses

The number of coughs evoked by capsaicin was increased by approximately 100% in guinea pigs exposed to CS for 8 days (12.0±1.9 coughs; mean±SEM) compared to air-exposed comparators (5.4±1.6 coughs; mean±SEM) (*Fig. 6.5*). At the same dose that significantly reduced capsaicin-evoked coughs in naïve animals (*Section 4.3.1*), theophylline reduced the number of capsaicin-evoked coughs in CS-exposed guinea pigs to 0.4±0.26 coughs (*Fig. 6.5*).
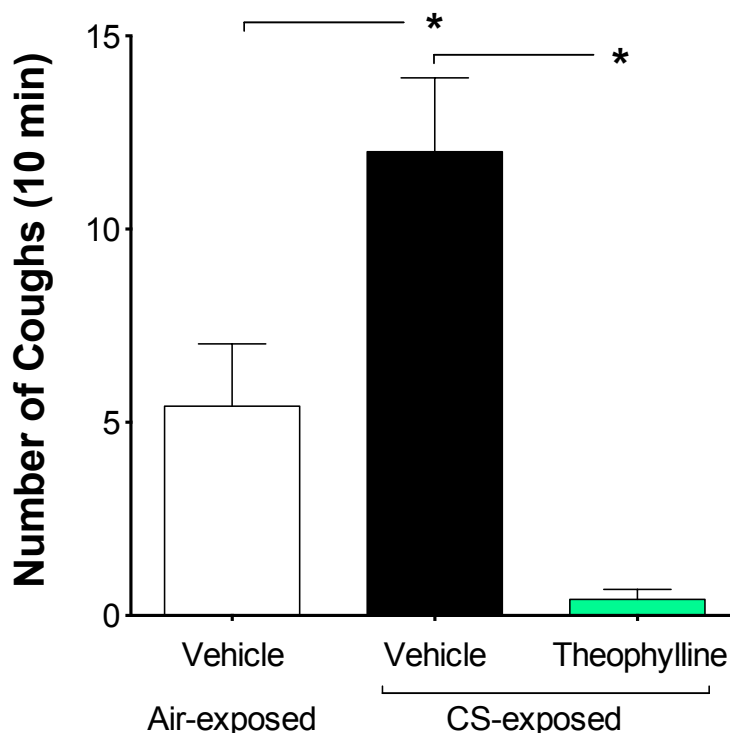
Figure 6.5 Effect of theophylline on CS-enhanced capsaicin-evoked guinea pig cough
Guinea pigs, which had been exposed to room air or CS, twice daily, for 8 days, were pre-treated with vehicle (0.5%w/v methylcellulose, 0.2%v/v tween80 in PBS) or theophylline (100mg.kg^{-1}), administered i.p., 1h prior to challenge with capsaicin aerosol (30µM; 5min). Numbers of coughs were recorded for a 10min period, including 5min of aerosol plus 5min post-aerosol. Data displayed as mean±SEM, n=12, * p<0.05 as determined by Mann-Whitney U test compared to air/vehicle control, θ p<0.05 as determined by Mann-Whitney U test compared to CS/vehicle control.

6.5 Summary/Discussion

The aim of this chapter was to examine the efficacy of the anti-tussive compounds, which have been shown (in the preceding chapters) to inhibit capsaicin- and/or CA-evoked cough in healthy animals, in a mechanistic disease model of CS-enhanced cough and sensory nerve responses.

It should be noted that the experimental data obtained in this chapter were obtained in a series of studies. In each set of experimental data the number of coughs and the magnitude of vagus nerve depolarisation responses evoked by capsaicin, CA or low pH was significantly increased in CS-exposed guinea pigs, compared to those exposed in parallel to room air. The reproducibility of these enhanced responses is indicative of the robustness of this mechanistic model in mimicking the enhanced cough responsiveness observed in smokers and COPD patients in clinical studies (Wong & Morice, 1999; Doherty *et al.*, 2000; Lewis *et al.*, 2007; Blanc *et al.*, 2009).

Each of the compounds was first examined for their ability to inhibit the enhanced tussive stimulus-induced sensory nerve depolarisation observed following CS-exposure. The recently developed β-agonist olodaterol, and the methyxanthine theophylline both significantly inhibited the CS-enhanced capsaicin-induced vagus nerve responses to a similar proportion to the inhibition observed in vagus nerves from air-exposed comparators. Likewise, the FAA PEA inhibited the CS-enhanced low pH-induced responses, again to a similar proportion as in air-exposed comparator tissues.

Following confirmation of the efficacy of these compounds in the *ex vivo* vagus nerve model of enhanced responsiveness, the effectiveness of the different classes of compound were examined in the *in vivo* model of CS-enhanced, capsaicin-evoked cough responses. Both of the LABA compounds, olodaterol and formoterol, inhibited the CS-enhanced cough response by greater than 90%. Furthermore, the methylxanthine theophylline also displayed significant inhibition of the CS-enhanced cough response. Unfortunately, due to the closure of the Sandwich UK Pfizer site and re-structuring of the company resulted in a lack of financial provision and adequate amounts of the compound with which to examine the effect of the FAAH inhibitor (PF04862853) in the CS-enhanced cough model at this time. The efficacy of PEA against CS-enhanced vagus nerve responses, as one of the downstream mediators of the anti-tussive effects of PF04862853, suggests that this FAAH inhibitor would be effective *in vivo* at inhibiting evoked cough in the CS-enhanced model, however future research would be required to affirm this supposition.

The data presented here shows that each of the classes of compound evaluated in this thesis can effectively inhibit the enhanced cough responses observed in the guinea pig CS-exposure model. As mentioned in the introduction to this chapter, examining the effect of a drug in an animal model which displays a disease-like phenotype is considered to be more indicative of the likely effectiveness in human disease than such investigations in naïve animals. However, as the mechanisms by which the cough response is altered in human disease have not been determined, the relevance of the model to the clinic may be questioned.

The primary consideration of a pre-clinical model of human disease, in order that it be useful and relevant, is that it displays an end-point resembling a relevant aspect of the clinical phenotype. It is difficult to compare objective clinical cough studies and objective animal cough studies directly, as they are typically not performed using the same methodology (*see Introduction 1.5.3*). Despite this, the lowered threshold concentration of capsaicin and CA required to evoke 5 coughs (C_5) in smokers and COPD patients (Wong & Morice, 1999; Doherty *et al.*, 2000; Lewis *et al.*, 2007; Blanc *et al.*, 2009), and the increased numbers of coughs evoked by a single concentration of the same stimuli in CS-exposed guinea pigs do both seem to indicate an increased sensitivity to the same tussive stimuli. This model was selected for its similar phenotype to the increased sensitivity to tussive stimuli observed in COPD patients, and in that specific sense, the model does appear to be clinically relevant.

It is worth mentioning here that whilst it is generally accepted that the cough reflex can be sensitised to agents such as capsaicin and CA in COPD patients, some studies do not find a modification of cough in COPD (Wong & Morice, 1999). When attempting to understand this discrepancy, it should be considered that COPD is a diagnostic label covering a wide range of disease manifestations; indeed the term 'COPD' was coined as an umbrella term for what used to be considered as separate obstructive lung diseases (Barnes, 2001). Chronic cough is only one symptom of COPD, and not all COPD patients exhibit chronic cough. Whether cough as a symptom is associated with a certain sub-population of COPD patients whom may have a common underlying cause is unclear. What is clear is that the heterogeneity of cough as a symptom in COPD may explain the disparity between the findings of various cough studies in COPD patients mentioned above. This heterogeneity of cough as a COPD symptom is reflected in the diagnostic criteria outlined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) (Vestbo *et al.*, 2013). To paraphrase the original document; "A clinical diagnosis of COPD should be considered in any patient who has… chronic cough… and a history of exposure to risk factors [including cigarette smoking]… [although] spirometry is required

to make the diagnosis in this clinical context… and conversely significant airflow limitation may develop without chronic cough and sputum production." It may be considered then that the CS-enhanced cough model is relevant to those COPD patients presenting with chronic cough who also have a lowered threshold/increased sensitivity to chemically (capsaicin/CA) evoked cough. The proportion of COPD chronic coughers whom fit into this category is unclear, as to date no large scale clinical studies have been performed evaluating the cough reflex sensitivity of COPD patients. However, clinical trials examining anti-tussive compounds would be likely to use objective cough monitoring initially, due to the advantages of such study design in determining efficacy. Furthermore it may be supposed that such clinical studies would seek to screen for chronic coughers who have a heightened sensitivity to capsaicin as part of their study design. It follows therefore that the CS-enhanced capsaicin-evoked cough model used here should be representative of the type of COPD patients (those with chronic cough and enhanced capsaicin sensitivity) who would be selected for clinical trials of novel anti-tussives.

Another key factor in the translatability of a disease model to the clinic is the clinical relevance of the challenge stimulus. Globally, the prevelant risk factor for developing COPD is cigarette smoking (Vestbo *et al.*, 2013). However, the development of COPD is not due to cigarette smoking alone, as whilst CS exposure is a prominent risk factor for COPD, only approximately 25% of smokers develop COPD (Løkke *et al.*, 2006). Genetic susceptibility, old age, exposure to other inhaled particles, asthma hyperreactivity and infections are some of the other risk factors indicated in COPD development (Vestbo, others). However, the interaction between these various factors in the development and progression of the disease is not currently fully understood, and therefore cigarette smoke exposure seems to be the most relevant and feasible challenge stimulus to use in a mechanistic animal model of COPD such as the one described in this chapter.

Given that the challenge stimulus used is relevant, and that the model represents a relevant phenotypic end-point, the remaining key question would be whether the mechanisms by which cough is enhanced in this model are the same as the mechanisms involved in the pathogenesis of chronic cough in COPD patients. Unfortunately the mechanisms underlying chronic cough in disease are currently not completely elucidated, and as such no definitive answer can be asserted to this question. What can be said, however, is that multiple clinical features and biomarkers associated with disease progression/severity of COPD are replicated in CS-exposure models in guinea pigs, as discussed more fully in the conclusion (*see Section 7.1.2*) to this thesis. These include structural changes such as fibrosis (Domínguez-Fandos *et al.*, 2012) and emphysema (Wright & Churg, 1990; Domínguez-Fandos *et al.*, 2012), as well as inflammatory cell

(predominantly neutrophilic) infiltration into the lungs (Domínguez-Fandos *et al.*, 2012). However it is generally considered that inflammatory mediators play an important role in the sensitisation of sensory nerve responses to capsaicin (Lee & Pisarri, 2001; Mazzone & McGovern, 2007). A number of mediators implicated with a role in sensory sensitisation, such as tumour necrosis factor (TNF)-α, interleukin-1β (IL-1β), interleukin-8 (IL-8), prostaglandin E2 (PGE_2) and neurokinins have been shown to be upregulated in CS-exposed guinea pigs (Kuo & Lu, 1995; Hong & Lee, 1996; Kwong *et al.*, 2001; Churg *et al.*, 2002; Wortley *et al.*, 2011; Luo *et al.*, 2012), again similarly to observations in human COPD patients (Keatings *et al.*, 1996; Chung, 2005; Miotto *et al.*, 2007; Chen *et al.*, 2008; Profita *et al.*, 2010; Vatrella *et al.*, 2010). As this model replicates many features of the human disease, investigating the mechanisms underlying enhanced cough in the CS-exposure model would be an interesting direction for future research, that might provide insights into the human condition (Bolser, 2004; Lewis *et al.*, 2007; Stevenson & Belvisi, 2008; Lee *et al.*, 2009).

In summary, both the long acting $β_2$-agonists, as well as theophylline were effective at inhibiting both the *in vivo* enhanced capsaicin cough and *ex vivo* enhanced isolated vagus capsaicin responses in CS-exposed guinea pigs. Whilst it is not possible to examine the FAAH inhibitor (PF04862853) *in vivo*, the FAA PEA, one of the downstream effectors of PF04862853 on vagus nerve, was effective at inhibiting the CS-enhanced isolated vagus low pH responses, hinting that PF04862853 would be effective in the *in vivo* enhanced cough model. As the CS-exposure model used appeared to replicate the disease condition of the cough reflex in COPD patients, these results suggest advancement to clinical research is warranted for all three classes of compound examined here, in order to confirm their usefulness as peripherally acting and efficacious anti-tussive therapies in man.

7 Conclusions

7.1 Summary of thesis

Excessive cough is one of the most common reasons that patients seek ambulatory care (Schappert & Rechtsteiner, 2011), and in a survey as much as 12% of the general population self-reported suffering with chronic cough and phlegm on most days for at least 3 months of the year (Cerveri *et al.*, 2003). Cough is a common symptom of upper respiratory tract infections, and in the majority of these cases cough resolves upon successful clearance of the infection, being considered an acute symptom where cough persists for less than 3 weeks (Pratter, 2006a). By contrast, chronic cough is defined as cough of a duration greater than 8 weeks, and the non-resolving, non-productive persistent cough which afflicts chronic cough patients can persist for many years (Dicpinigaitis, 2012). Chronic cough can be a distressing symptom with multiple physical and psycho-social consequences that can severely reduce sufferers quality of life (Irwin, 2006c), and this condition is commonly associated with PNDS, GERD, asthma and COPD (Pratter, 2006b). Whilst it has been suggested that successful diagnosis and treatment of PNDS and GERD may resolve cough (Irwin, 2006b), more recent evidence suggests, at least in the case of GERD, that the clinical picture may be less clear cut, as a recent Cochrane review of GERD treatments failed to find sufficient high quality evidence to support this claim (Chang *et al.*, 2011). Indeed, the view that acidic reflux is one of the major causes of chronic cough has been challenged by studies finding a lack of correlation between reflux events and cough in many chronic cough subjects, and also the lack of efficacy in appropriately designed clinical trials of acid suppression therapies (Smith & Houghton, 2013). Moreover, cough is often persistent in patients with chronic respiratory conditions, particularly in severe asthma and COPD, which are poorly controlled by standard treatments (Irwin & Curley, 1991). Therefore, where the underlying condition is not sufficiently controlled by medication, successful treatment fails to restore a normal cough reflex, or where a cause of cough cannot be identified (unexplained cough), direct cough suppressants are required.

Existing cough therapies, however, are either ineffective, or are associated with adverse effects (Yancy *et al.*, 2013). In general, centrally acting antitussives are the only moderately effective type of antitussive, but are commonly associated with adverse effects, including sedation and nausea (Bolser, 2006b). By contrast, peripherally acting antitussives are associated with far fewer adverse effects, but evidence for the efficacy of these therapies is less clear. For example, a meta-analysis of clinical studies of existing cough therapies found that moguisteine, a peripherally acting antitussive, provides a significant but highly variable improvement over placebo in terms of cough severity (assessed by subjective measures), but did not reduce cough frequency (Yancy *et al.*, 2013). Considering the ineffectiveness or risk of adverse effects associated with current

therapies, the high prevalence of chronic cough in the general population, and the impact of chronic cough on patients lives, new cough medications are urgently needed.

7.1.1 Summary of findings

This thesis therefore examined three classes of compound which have been implicated as potential antitussives; long-acting β_2-adrenergic receptor agonists (LABA), methylxanthines, and fatty acid amide hydrolase (FAAH) inhibitors. Short acting β_2-adrenergic receptor agonists (fenoterol, terbutaline) have previously been demonstrated to have a direct antitussive effect via an action on vagal sensory nerves; inhibiting capsaicin-induced cough in guinea pigs, and also depolarisation of guinea pig and human vagus nerve (Freund-Michel *et al.*, 2010). By contrast, LABA type bronchodilators are more commonly used clinically, but have not been investigated for any antitussive properties. The methylxanthine compound theophylline has been shown to inhibit capsaicin-evoked cough in subjects with angiotensin-converting enzyme inhibitor related cough (Cazzola *et al.*, 1993), but this potential has not been further investigated, despite another methylxanthine, theobromine, being shown to inhibit evoked cough and sensory nerve activation in both pre-clinical and human subjects (Usmani, 2004). In small clinical trials, FAAH inhibitors have been shown to causes elevation of endocannibinoid fatty acid amides (Li *et al.*, 2012). Whilst cannabinoid receptor agonists generally are associated with multiple adverse effects, a recent clinical trial found the FAAH inhibitor PF-04457845 was not associated with adverse effects in healthy volunteers. It therefore appears that increasing the levels of endogenously produced fatty acid amides is comparatively safer than exogenously administering cannabinoids (Li *et al.*, 2012). Cannabinoid receptor agonists have been shown to exert an antitussive effect via the CB_2 receptor on vagal afferent sensory nerves (Belvisi *et al.*, 2009).

To bring a newly discovered compound through lead testing and clinical trials can take around a decade and US$1 billion (DiMasi *et al.*, 2003). Therefore the compounds selected for evaluation in this thesis include the LABA formoterol, as well as the methylxanthine theophylline, both of which are clinically approved and indicated for use as bronchodilators in patients with asthma and COPD. The repurposing, or 'repositioning' of these existing bronchodilators as antitussives is an attractive prospect, given that repurposing of existing drugs can bring benefits to patients faster, and reduce the costs and failure risk to pharmaceutical companies compared to the full drug development cycle for a new compound (Novac, 2013). In addition, olodaterol, a novel LABA which has entered phase III clinical trials for use as a bronchodilator (http://www.clinicaltrials.gov/ct2/results?term=olodaterol&phase=2; accessed 20.08.13),

and PF04862853, a FAAH inhibitor in pre-clinical testing (Meyers *et al.*, 2011), were also evaluated for their antitussive potential.

Initially, it was shown that formoterol, olodaterol, theophylline, and PF04862853 were each able to inhibit either capsaicin-, CA-evoked cough, or both, in conscious guinea pigs. Once efficacy had been confirmed in this *in vivo* model, appropriate lines of investigation were undertaken for each compound in order to examine their activity on sensory nerve activation by tussive agents, and their mechanism of action in this context.

Formoterol, olodaterol and theophylline inhibited the capsaicin-evoked firing of C-fibres *in vivo*, demonstrating that these compounds are effective at inhibiting activation of the fibres thought to mediate the cough response to capsaicin (*see Introduction 1.2*; Canning, 2011). Theophylline was also shown to inhibit PGE_2-evoked firing of C-fibres *in vivo*, demonstrating an antitussive mechanism of action for theophylline independent of its bronchodilator activity. Furthermore, theophylline, as well as formoterol and olodaterol, were shown to inhibit capsaicin-induced calcium flux in isolated airway-terminating jugular neurons; demonstrating that each of these compounds can directly inhibit activation of the airway neurons likely involved in the cough reflex, independent of their bronchodilator activity. The FAAH inhibitor PF04862853 was shown to cause elevation of plasma levels of the fatty acid amides PEA, AEA, OEA and LEA. PEA was demonstrated to inhibit low pH-induced calcium flux in isolated airway-terminating jugular neurons – suggesting that the FAAs elevated by PF04862853 administration may exert an antitussive activity via an action on the sensory nerves involved in evoking cough.

The mechanism of action on sensory nerves of β-adrenergic receptor agonists, theophylline and the endocannibinoids elevated by PF04862853 (PEA, AEA, OEA and LEA) was then investigated, as summarised below (*Fig. 7.1, 7.2, and 7.3 respectively*).

Using selective antagonists and $β_2$- and $β_3$-adrenergic receptor KO mouse vagus nerve tissue it was shown that formoterol and olodaterol exert their inhibitory effects on sensory nerves via the $β_2$-adrenergic receptor. A novel finding was that $β_1$- and $β_3$-adrenergic receptor agonists had a similar effect; whilst $β_1$-receptor agonists are unlikely to have utility as antitussives due to their adverse effects on the cardiovascular system, $β_3$-receptor agonists are not as abundant in cardiac tissue, and therefore may be an interesting target for future drug development. Downstream of the receptors, the inhibitory activity of these agonists was investigated. Previously, the inhibitory action of SABA on vagal sensory nerves has been shown be via elevation of cyclic AMP (cAMP), activating Protein Kinase G (PKG) and downstream large conductance calcium-activated
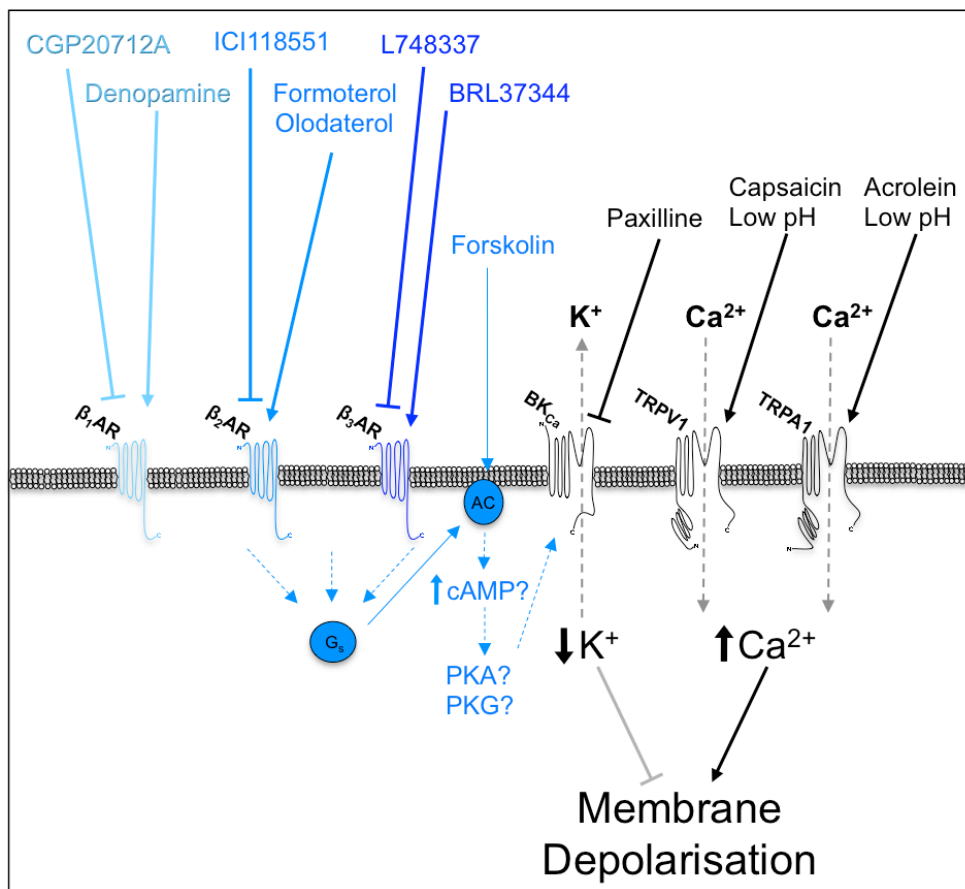
Figure 7.1 Schematic illustration of mechanism of action of LABA drugs
Simplified cartoon schematic of mechanism of action of formoterol, olodaterol (LABAs), denopamine (β₁-agonist) and BRL37344 (β₃-agonist) on sensory nerves. Denopamine, formoterol, olodaterol and BRL37344 were shown to cause inhibition of vagus nerve depolarisation through the β₁-, β₂- and β₃-adrenoreceptors (respectively), effects that were respectively blocked by the specific β₁-, β₂- and β₃-adrenoreceptor antagonists CGP20712A, ICI118551 and L748337. Downstream of the receptor, agonism of any of the β-adrenoreceptors caused opening of the BK$_{Ca}$ potassium channels, as their inhibitory effects were blocked by paxilline. It is thought that the influx of potassium reduces the excitability of the cell, thereby causing the observed functional inhibition of depolarisation by TRPV1 and TRPA1 agonists. The AC activator forskolin mimicked this functional inhibition of the same agonists, and the effect of the β-adrenoreceptor agonists was lost in AC KO mice, suggesting a G$_s$/AC/PKA or PKG pathway is involved in the opening of the BK$_{Ca}$ channel. Key - arrow=activating interaction, blocked line=inhibiting interaction, grey blocked line=functional inhibition, ?=unconfirmed pathway. Abbreviations; β₁AR/β₂AR/β₃AR; β-adrenergic receptors type 1/2/3, CGP20712A; β₁AR antagonist, ICI118551; β₂AR antagonist, L748337; β₃AR antagonist, AC; adenylyl cyclase, cAMP; cyclic adenosine monophosphate, PKA/G; protein kinase type A/G, BK$_{Ca}$; large-conductance calcium-activated potassium channel, TRPV1; transient receptor potential vanilloid type 1 receptor, TRPA1; TRP ankyrin type 1 receptor.

potassium channels (BK$_{Ca}$) (Freund-Michel *et al*., 2010). In this thesis it was demonstrated that AC activation plays a key role in the inhibitory activity of LABA and β₁- and β₃-adrenergic receptor agonists. Firstly forskolin, an activator of AC (the enzyme

regulating cAMP production) was shown to mimic the inhibitory activity of the β-adrenergic receptor agonists in mouse vagus nerve. Then, after observing that expression of AC6 was the highest of the 10 AC isoforms in mouse vagal ganglia, the inhibitory action of each of the β-adrenergic receptor agonists tested, as well as forskolin, was shown to be lost in AC5/6 KO mouse vagus tissue. Downstream of AC activation, a role for BK_{Ca}, but not of other calcium-activated potassium channels, was demonstrated in the inhibitory action of the β-adrenergic receptor agonists on guinea pig vagus nerve tissue. The activation of BK_{Ca} by β-adrenergic receptor agonists suggests a mechanism of action involving the efflux of potassium ions, causing hyperpolarisation of sensory neurons, and decreasing the general 'excitability' to future stimuli. Formoterol, olodaterol, and the $β_1$- and $β_3$-adrenergic receptor agonists were each shown to inhibit depolarisation evoked by exogenous and endogenous agonists of TRPV1 and TRPA1, a finding suggestive of such a general inhibition of sensory nerve activity. In addition, the LABA compounds and $β_1$-/$β_3$- agonists were shown to inhibit capsaicin-induced depolarisation of human vagus nerve tissue through their respective receptors, demonstrating the translational potential of this work to humans.

In contrast to the LABA compounds, theophylline is not thought to activate a metabotropic receptor, but is known to be a weak non-specific PDE inhibitor. Whilst PDE inhibitors have been shown to inhibit cough in guinea pigs (Mokry & Nosalova, 2011), in the current context this mechanism was considered unlikely due to the observed potency of theophylline on vagus nerves being far lower than its reported potency as a PDE inhibitor. Using pharmacological antagonists, the SK_{Ca} and IK_{Ca} channels were shown to be involved in the inhibitory action of theophylline on guinea pig vagus nerve depolarisation. Experiments with isolated patch clamped jugular ganglia neurons confirmed that theophylline opens SK_{Ca} and IK_{Ca} channels, causing a hyperpolarisation of the cell that would explain its inhibitory action on sensory nerves. Furthermore, theophylline increased the open potential of the SK_{Ca} channel in inside-out patch clamp experiments, demonstrating a mechanism of action independent of intracellular second messengers such as cAMP. The inhibitory activity of theophylline and the involvement of SK_{Ca} and IK_{Ca} was observed in human vagus nerve tissue, demonstrating the translational potential of this work to humans.
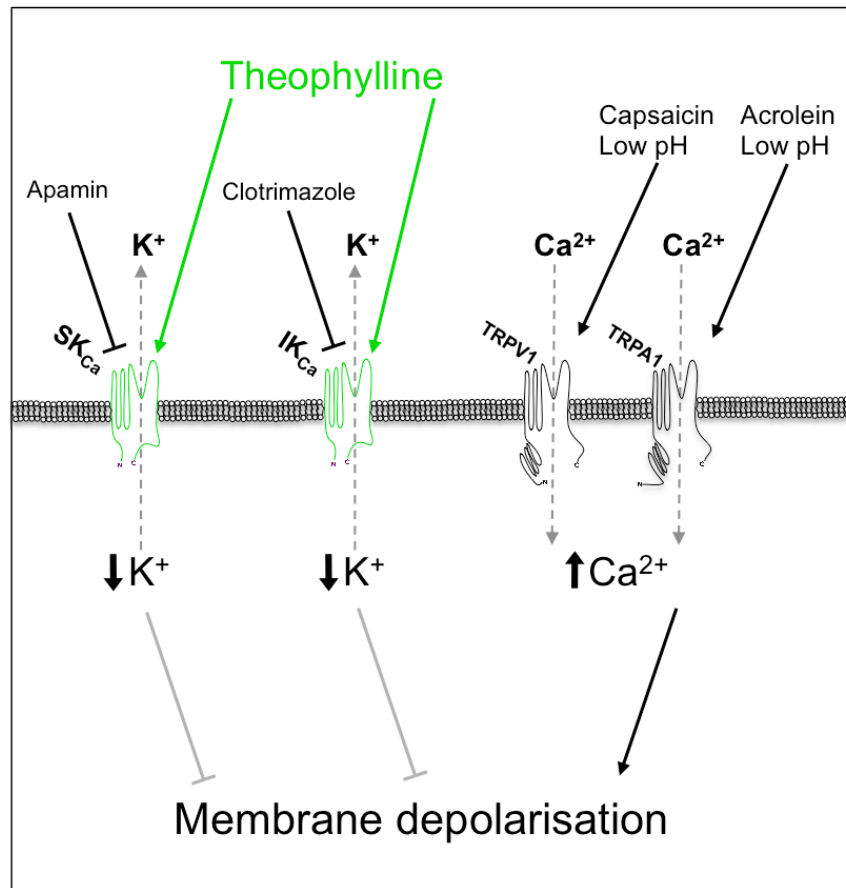
Figure 7.2 Schematic illustration of mechanism of action of theophylline
Simplified cartoon schematic of mechanism of action of theophylline (methylxanthine) on sensory nerves. Theophylline was shown to cause inhibition of vagus nerve depolarisation to a variety of tussive stimuli, including capsaicin, acrolein and low pH. This effect was shown to be via opening of SKCa and IKCa potassium channels, as the inhibitory effects of theophylline were reversed by blockers of these channels (apamin and coltrimazole). Using the inside-out patch clamp technique it was shown that theophylline directly opens these channels independently of intracellular second messengers. It was shown that the influx of potassium ions into the cell lowers the resting membrane potential voltage, and it is therefore thought that this reduces the excitability of vagal neurons, causing functional inhibition to a range of pro-tussive stimuli. Key - arrow=activating interaction, blocked line=inhibiting interaction, grey blocked line=functional inhibition. Abbreviations; SK_{Ca}; small-conductance calcium-activated potassium channel, IK_{Ca}; intermediate-conductance calcium-activated potassium channel, TRPV1; transient receptor potential vanilloid type 1 receptor, TRPA1; TRP ankyrin type 1 receptor.

Levels of the endogenous fatty acid amides PEA, AEA, OEA and LEA were shown to elevated by administration of PF04862853. Each of these FAAs was shown to exert inhibitory activity on capsaicin- and low pH-induced guinea pig vagus nerve depolarisation. As they are known to be endocannibinoids, the effect of CB_1 and CB_2 receptor antagonists was examined; showing that PEA-, AEA-, OEA- and LEA- mediated

inhibition of guinea pig vagus nerve depolarisation is via the CB_2, but not the CB_1 receptor. Whilst the intracellular signaling pathways downstream of CB_2 receptor activation have not been fully elucidated (Demuth & Molleman, 2006), cannabinoid receptor activation is known to cause downstream calcium-activated potassium channel
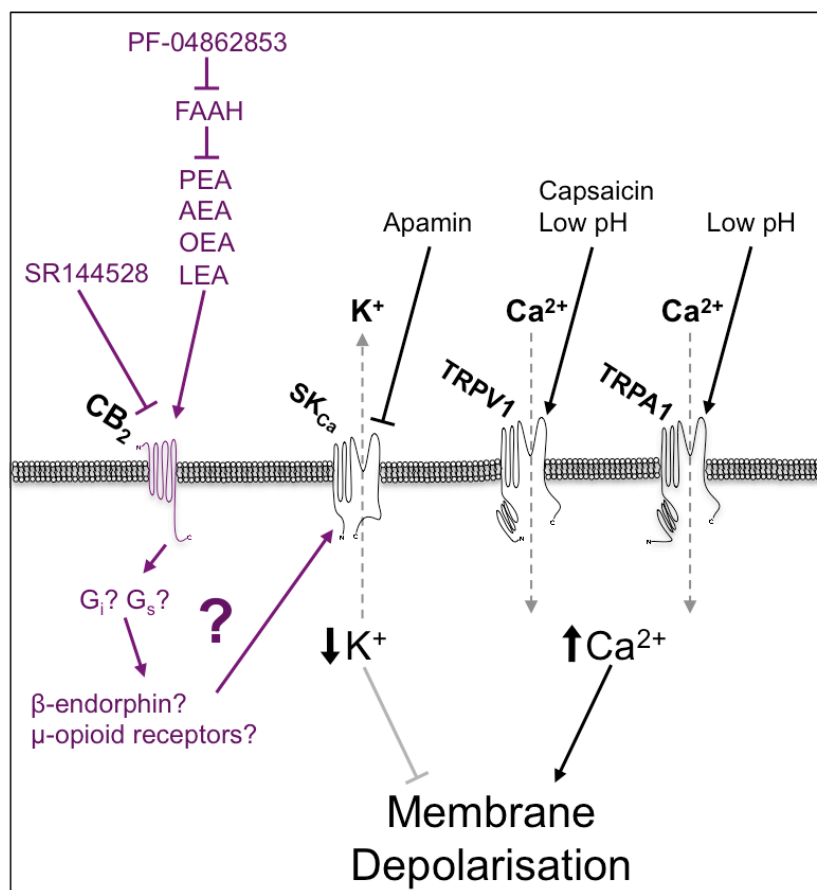
Figure 7.3 Illustration of mechanism of action of PF04862853
Simplified schematic of mechanism of action of PF04862853 (FAAHi), via fatty acid amides, on sensory nerves. PF-04862853 was shown to inhibit cough, and to elevate plasma levels of the endogenous FAAs PEA, AEA, OEA and LEA, an effect thought to be caused by inhibition of the FAAH enzyme. PEA, AEA, OEA and LEA were shown to inhibit vagus nerve depolarisation via the CB_2 receptor, an effect that was blocked by a specific CB_2 receptor antagonist. PEA was also shown to inhibit depolarisation via BKCa potassium channels, as the inhibitory effects of PEA were reversed by a blocker of this channel (apamin). It is thought that the influx of potassium reduces the excitability of the cell, thereby causing the observed functional inhibition of depolarisation by TRPV1 and TRPA1 agonists. The G protein receptor mediated pathway between CB2 activation and BKCa channel opening is unknown, and may be a G_i- or G_s-coupled cascade. In other systems it has been suggested that cannabinoid effects may be dependent on opioid pathway signaling. Key: arrow=activating interaction, blocked line=inhibiting interaction, grey blocked line=functional inhibition, ?=unconfirmed pathway. Abbreviations; FAAH; fatty acid amide hydrolase, PEA; palmitoylethanolamide, AEA; anandamide, OEA; oleoylethanolamide, LEA; linoleoylethanolamide, CB_2; cannabinoid type 2 receptor, SK_{Ca}; small-conductance calcium-activated potassium channel, TRPV1; transient receptor potential vanilloid type 1 receptor, TRPA1; TRP ankyrin type 1 receptor.

opening (Welch *et al.*, 1995a; Yang *et al.*, 2007). Using pharmacological antagonists, SK_{Ca} type potassium channels were implicated with a role in the inhibitory action of PEA on guinea pig vagus nerve depolarisation. Furthermore, PEA also inhibited low pH-induced depolarisation of human vagus nerve tissue, an effect reversed by both a CB_2 receptor antagonist or SK_{Ca} channel blocker, demonstrating that the mechanism of endocannibinoid action on sensory nerves may translate to humans.

Finally, as the purpose of any anti-tussive medication is to inhibit cough in patients whom exhibit such an exaggerated cough response, the effect of LABA, theophylline and PF04862853 were examined in an animal model of CS-enhanced cough and responses. Both LABA compounds, formoterol and olodaterol, as well as theophylline, were effective at inhibiting both the *in vivo* enhanced capsaicin cough and *ex vivo* enhanced, capsaicin-induced isolated vagus depolarisation observed in CS-exposed guinea pigs. Whilst it was not possible to examine the FAAH inhibitor (PF04862853) *in vivo*, the FAA PEA, one of the downstream effectors of PF04862853 on vagus nerve, was effective at inhibiting CS-enhanced, low pH-induced isolated vagus nerve depolarisation.

Importantly, both LABA compounds, as well as theophylline, were shown to inhibit capsaicin-evoked firing of C-fibres *in vivo*. Whilst the anti-tussive effects of LABAs have not been previously demonstrated, the LABA compound indacaterol has been shown to cause single fibre firing in a preparation similar to the one used in this thesis (Sladen *et al.*, 2009). However, it was not established whether this was an effect of the drug vehicle/delivery route, of indacaterol itself, or a drug effect common to the LABA class of drugs. Therefore the finding here that formoterol, as well as the novel LABA compound olodaterol, do not cause activation of airway single C-fibres suggests that LABA compounds as a class do not possess pro-tussive effects of themselves.

Collectively, these results suggest that each of the three classes of compound evaluated in this thesis possess efficacy as peripherally acting antitussives. Each of the compounds had a direct inhibitory effect on sensory nerve activation, mediated by distinct mechanisms, a common element of which is an effect on calcium-activated potassium channels.

7.1.2 Limitations of studies

The *in vivo* models of objective cough to capsaicin and CA were selected for use in this thesis due to the clinical relevance of these stimuli. In addition, it has been shown that

cough reflex sensitivity to both of these stimuli is similar between human subjects and guinea pigs (Laude *et al.*, 1993).

The examination of signaling pathways and activity of drugs on sensory nerve activation was also primarily completed in guinea pig and mouse tissue. Whilst the expression of various receptors and signaling proteins may differ between species, key experiments were repeated in human vagus nerve tissue, to demonstrate the effects observed in animal models translate to a relevant tissue.

Each of the techniques detailed in this thesis have different advantages and limitations. Therefore, where possible, the techniques used were selected to complement each other by mitigating these limitations. For example, the isolated vagus nerve recording technique allows relatively cost-effective pharmacological evaluation of sensory nerve activity in guinea pig, human and knock-out mouse tissues. However the technique cannot be used to examine the activity of only airway-terminating nerves. By contrast, the isolated neuron calcium imaging and single fibre nerve recording techniques do allow discrimination between airway and non-airway neurons/nerves. However, the single fibre technique is relatively labour-intensive and costly, as only one nerve fibre can be examined in a single animal. In addition, translational data cannot be obtained in either the isolated neuron calcium imaging or single fibre nerve recording techniques.

Whilst the above techniques were useful in demonstrating the antitussive efficacy of these compounds against the normal capsaicin-evoked cough reflex, in the clinic any antitussive medication would be expected to inhibit the exaggerated cough response observed in patients with chronic cough. The model of CS-enhanced cough and sensory nerve responses described in chapter 6 was therefore designed to mimic the enhanced sensitivity to capsaicin and CA observed in COPD patients with chronic cough (Wong & Morice, 1999; Doherty *et al.*, 2000; Blanc *et al.*, 2009). As cigarette smoking is the primary risk factor associated with the development of COPD, CS exposure was used as a relevant challenge agent to drive this 'COPD-like' modification of the cough reflex (Vestbo *et al.*, 2013). However, only approximately 25% of smokers develop COPD, indicating that other factors (genetic, alternate environmental exposures) likely play a role in the development of COPD (Løkke *et al.*, 2006; Vestbo *et al.*, 2013). As the model described here is one of only CS-exposure, and does not take into account such factors that may influence the susceptibility of individuals to COPD, the validity of the model may reasonably be questioned. Indeed, due to the shorter duration over which smoke exposures take place (8 days), it could be argued that the model employed here is more one of cigarette smoking than of COPD *per se*.

The exposure periods and duration of CS-exposures in the guinea pig model were selected following the validation studies previously completed, as described in Lewis *et al.* (2007). In the validation of this model, bronchoalveolar lavage (BAL) was taken from guinea pigs exposed to either room air or CS in parallel. The numbers of neutrophils in the BAL fluid were increased in CS-exposed guinea pigs (*see Appendix; Fig. A1*). Additionally, as described previously (*see Chapter 6*), enhanced cough and vagus nerve responses to capsaicin and low pH were observed in this model. The enhanced cough responses in these guinea pigs are suggestive of the enhanced sensitivity to the same tussive stimuli seen in chronic cough subjects with COPD (Wong & Morice, 1999; Doherty *et al.*, 2000; Blanc *et al.*, 2009). Furthermore, preliminary data obtained in the isolated vagus nerve preparation suggests that capsaicin and low pH evoked greater depolarisation in human smokers compared to non-smokers (*see Appendix; Fig. A2*). However, this data is limited due to the small population size, and the lack of information about the prevalence of cough in the sample subjects (*see Appendix; Table A1*). Beyond comparing these characteristics of the CS-exposure model to the clinical manifestations of chronic cough and COPD, it is not possible to affirmatively state that the mechanisms underlying the modification of the cough reflex are the same, as most of the hypotheses on mechanisms of modified human cough reflex come from animal studies.

However, investigating the mechanisms underlying enhanced cough in the CS- exposure model would be an interesting direction for future research, that might provide further insights into the human condition (Bolser, 2004; Lewis *et al.*, 2007; Stevenson & Belvisi, 2008; Lee *et al.*, 2009).

7.3 Future directions

In this section the potential direction of future research into the antitussive potential of the compounds examined in this thesis will be explored. Additionally, ideas for the future direction of research into the mechanisms of CS-enhanced cough will be considered.

7.3.1 Further studies examining antitussive activity & mechanisms

Formoterol, olodaterol, theophylline and PF04862853 have each been shown to inhibit either capsaicin- and/or CA-evoked cough. In addition, formoterol, olodaterol and theophylline have each been shown to inhibit capsaicin-evoked firing of C-fibres *in vivo*. Demonstrating that PF04862853 has a similar action on C-fibres would therefore likewise confirm that this compound inhibits activation of the fibres thought to mediate the cough response to capsaicin (Canning, 2011).

Further, the effect of repeated aerosol dosing of LABA compounds over time on their subsequent efficacy against cough could be investigated. β_2-adrenoreceptor agonists are known to cause agonist-induced desensitisation of the β_2-adrenoreceptor. Both SABA and LABA have been shown to induce desensitisation of the β_2-adrenoreceptor, leading to a clear loss of their bronchoprotection and bronchodilation function, as demonstrated in some studies (Newnham *et al.*, 1995; Aziz *et al.*, 1998; Giannini *et al.*, 2001; Tsagaraki *et al.*, 2006). Whilst it has been suggested that LABA cause more downregulation of the β_2-adrenoreceptor than SABA (January *et al.*, 1997; Clark *et al.*, 1999; Moore *et al.*, 2007), it has also been argued that the reverse may be true, as LABA achieve an equivalent functional efficacy with less receptor occupancy, which may reduce downregulation of the receptor at equi-effective doses relative to SABA (Charlton, 2009). Obviously, however, given the novelty of the findings presented here, the effect of long-term dosing of LABA on their anti-tussive efficacy has not been investigated. It would therefore be worthwhile to establish the possible effects any such desensitisation would have on the ability of the LABA compounds to inhibit cough.

It would also be interesting to examine the activity of each of the different classes of compound on other fibre types thought to be involved in the cough reflex. RARs and cough receptors have also been suggested to be key nerve fibres involved in regulating the cough reflex (Canning, 2004). This may provide more detailed information about the type of cough these drugs may be expected to inhibit. RARs and cough receptors are particularly implicated with a role in mediating cough evoked by mechanical stimuli. As discussed previously (*Introduction 1.3.4*), one hypothesis is that increased

responsiveness of nerve fibres to chemical stimuli is responsible for the unproductive cough observed in chronic cough subjects (Nasra & Belvisi, 2009). Therefore drugs that block chemically-evoked cough but not mechanically-evoked cough may inhibit the unproductive cough whilst leaving the defensive cough reflex intact. Should the drugs examined in this thesis have no effect on mechanically evoked RAR/cough receptor firing, they may thus prove to be ideal candidate antitussive compounds. If, on the other hand, they were to inhibit mechanical activation of airway afferent nerve fibres, an alternative clinical strategy would be titration of drug dosage to a level that alleviates the burden of excessive cough whilst leaving some level of defensive cough reflex intact.

Further to examining the effect of these drugs on mechanical pro-tussive stimuli, future work is required to further elucidate portions of the signalling pathways mediating inhibition of sensory nerve activation.

Several components of the signalling pathway downstream of β-adrenergic receptor activation remain to be investigated:

- Examine the activation of AC by β-adrenergic receptor agonists in guinea pig and human nerve tissues. Future studies may therefore examine the effect of AC activation on depolarisation, and also the effect of an AC inhibitor on the inhibitory activity of β-adrenergic receptor agonists in these tissues.

- Downstream of AC, the elevation of cAMP in response to LABA activation of the β_2-adrenergic receptor has not been confirmed. This could be achieved in guinea pig ganglia cells using a fluorescence energy resonance transfer (FRET) assay, using commercially available kits.

- Whilst it has been shown previously a PKG inhibitor blocks the activity of SABA, it has not been demonstrated that LABA and β_1- and β_3-adrenergic receptor agonists act in the same way.

- The effect of LABA in mouse vagus nerve tissue from BK_{Ca} knock-out mice ($Slo^{-/-}$) could be examined to confirm the role of the BK_{Ca} channel in LABA inhibition of capsaicin depolarisation.

- Furthermore, the effect of the BK_{Ca} blocker, paxilline, could be examined on the LABA inhibition of human vagus nerve depolarisation to confirm that the proposed mechanism translates to human subjects.

In addition to the above experiments to confirm and further probe elements of β-adrenergic receptor signaling in guinea pig ganglia, it would be interesting to repeat key experiments in human jugular ganglia neurons to confirm that the mechanism of action of

these compounds translates to human sensory nerves. Obtaining such cells would be invaluable as a translational research tool.

In contrast to β-adrenergic receptor, relatively few pathways remain to be elucidated for the mechanism of action of theophylline on sensory nerves. Inside-out patch clamping of guinea pig vagal ganglia demonstrated that theophylline acts on potassium channels independently of intracellular 2nd messenger activity. However, if human primary jugular ganglia cells could be obtained, it would be useful to confirm (using patch clamp techniques) that theophylline exerts the same effect on IK_{Ca} and SK_{Ca} channels in a relevant human cell type.

In guinea pigs, the FAAH inhibitor PF04862853 has been shown to cause elevation of plasma levels of the FAAs AEA, PEA, OEA and LEA. It was subsequently demonstrated that FAAs cause inhibition of sensory nerve depolarisation via CB_2 receptors and downstream IK_{Ca} channels. However, the intracellular signalling pathways between the receptor and ion channel have not yet been elucidated.

Initially, determining the type of G protein the CB_2 receptor is coupled to following activation by the FAAs may give an indication of the likely pathways involved in the downstream inhibition of capsaicin and low pH depolarisation (*Fig. 7.3*). Classically, cannabinoid receptors are thought to be coupled to G_i, which activate intracellular signalling cascades which result in the activation of potassium channels (Demuth & Molleman, 2006). If the inhibitory effects of PEA, AEA, OEA or LEA are blocked by the application of pertussis toxin, this may indicate a G_i mediated downstream effect. Alternatively, the inhibitory effects of the FAAs could be profiled in vagus tissue from $AC5/6^{-/-}$ mouse tissue, similarly to the methods described to examine the effects of the LABA compounds (*see Section 3.4.6*). However, whilst it has been shown that CB_2 can couple to G_s subunits (Demuth & Molleman, 2006), in the context of the findings of this thesis, a Gs-AC-cAMP-PKA pathway seems unlikely, given that the LABA compounds were shown to act via BK_{Ca}, whereas the inhibitory effect of the FAAs were found to act via SK_{Ca}.

Downstream of G protein coupling, it has been suggested that CB_2 receptor activation may stimulate the release of endogenous opioids, including β-endorphin (see Fig. 7.3; Ibrahim, 2005). Opiate-based drugs are some of the most efficacious antitussives, and whilst they are thought to act predominantly in the central nervous system, it is thought that activation of peripheral opioid receptors on sensory nerves may play a role in the inhibition of cough (Karlsson *et al.*, 1990; Adcock, 1991). Furthermore, it has been shown

that the antinociceptive effects induced by administration of AEA following FAAH inhibition can be blocked by opioid receptor antagonists (Haller *et al.*, 2008). It would therefore be interesting to examine the involvement of opioid receptors in the inhibitory effects of the FAAs on cough and sensory nerve responses.

Finally, it would also be interesting to examine the effect of combining LABA compounds and either theophylline or PF04862853 on sensory nerve depolarisation and cough. These drugs appear to act via the opening of different calcium-activated potassium channels, therefore may be expected to have additive antitussive effects. Clinically, administration of two antitussives that act via different pathways could provide more effective inhibition of cough than either alone, and may be beneficial in reducing any adverse effects.

7.3.2 Further studies examining CS-enhanced cough mechanisms

Investigating the mechanisms underlying enhanced cough in the CS- exposure model would be an interesting direction for future research. Indeed, some preliminary investigations have already been begun, using the CS-exposure model described previously (*see Chapter 6*) to examine the responses of isolated airway neurons to CS-exposure. This section will describe some of these initial findings, and suggest some future avenues of research.

Thus far, the capsaicin responsiveness of airway-terminating neurons isolated from the jugular and nodose ganglia of air- and CS-exposed guinea pigs has been examined. These investigations have shown that in CS-exposed animals, airway-terminating jugular neurons show an enhanced responsiveness to capsaicin, with the same concentration of capsaicin apparently causing a greater elevation of intracellular calcium influx (*see Appendix; Fig. A3a*). Additionally, whilst the majority of airway-terminating nodose neurons from air-exposed guinea pigs do not respond to capsaicin, in CS-exposed guinea pigs these cells become responsive to capsaicin (*see Appendix; Fig. A3b*).

Currently the mechanisms underlying these changes have not been elucidated, but these results are suggestive that two mechanisms may be involved in the CS-enhancement of the cough reflex evoked by capsaicin.

Firstly, the enhanced responsiveness of jugular origin neurons is suggestive of the 'sensitisation' of TRPV1 by inflammatory mediators, as has been previously described *in vitro* (*see Introduction 1.3.4*; Ma & Quirion, 2007; Petrus *et al.*, 2007; Zhang *et al.*, 2008b;

Adcock, 2009). This hypothesis could be examined by exposing primary guinea pig airway terminating jugular ganglia neurons to CS *in vitro*. Should it be possible to induce similar enhanced responsiveness to capsaicin *in vitro*, this would provide an ideal assay system to examine the mediators and pathways involved in such a process.

In addition, it is possible that a change in the phenotype of nodose-originating neurons is induced by CS-exposure, inducing the expression of proteins (such as TRPV1) that are normally only expressed by chemosensitive neurons. Such a phenotypic switch has been demonstrated in models of allergic airway inflammation, where nodose neurons have been shown to begin expressing TRPV1 and neuropeptides (SP, CGRP and NKA) which they do not express under normal conditions (Fischer *et al.*, 1996; Undem *et al.*, 1999; Myers *et al.*, 2002; Lieu *et al.*, 2012). Speculatively, this could indicate that mechanically sensitive Aδ-type fibres, such as RARs and cough receptors, which are normally non-chemosensitive, change phenotype following CS exposure. The first step in testing this hypothesis will be to examine the expression of TRPV1 in nodose neurons, using a single cell q-PCR technique as described by Lieu *et al.* (2012).

Additionally, it would be interesting to use the single fibre technique to examine whether the changes observed in the jugular and nodose cells *in vitro* equate to functional changes *in vivo*, by examining the responsiveness of C-fibres, and the sensitivity of mechanically sensitive fibres (RARs and cough receptors) to capsaicin. Previously, Bergren *et al.* (2001b) have demonstrated modulation of C-fibre and RAR activity in a similar preparation, although their model of enhanced responsiveness to capsaicin differs significantly from the model of CS-exposure used here, and is perhaps more a model of severe asthma than COPD. Guinea pigs were treated with either vehicle (saline) or ovalbumin (OVA), and then exposed to either air or CS for 120 days. The authors found that OVA/CS exposure enhanced the responsiveness of C-fibres to capsaicin, compared to all other treatment groups. Interestingly, no difference was observed in the responsiveness of C-fibres to capsaicin in saline/air-exposed animals compared to saline/CS-exposed animals. However, in a previous publication by the same author, using the same treatment protocol but with cough as an endpoint, capsaicin evoked a greater number of coughs in saline/CS-exposed animals compared to saline/air-exposed animals (Bergren, 2001a). This suggests that the CS-enhancement of capsaicin-evoked cough is due to an enhanced responsiveness of fibres other than C-fibres. Therefore, examining the responsiveness of Aδ-nociceptors, RARs, and cough receptors capsaicin may reveal the contribution of these fibres to the enhanced capsaicin-evoked cough observed following CS exposure, and potentially provide further insights into the

mechanisms by which the cough reflex is modified in chronic cough patients (Bolser, 2004; Lewis *et al.*, 2007; Stevenson & Belvisi, 2008; Lee *et al.*, 2009).

7.3.3 Concluding remarks

The data presented here shows that the related compound PF04862853 possesses antitussive efficacy in guinea pigs via elevation of endocannibinoids, which inhibit activation of airway sensory nerves via the CB_2 receptor. However, the signaling pathways activated by endocannibinoids downstream of the CB_2 receptor remain to be really investigated. Recent trials of the related FAAH inhibitor PF-04457845 have demonstrated the safety of this compound in humans (Huggins *et al.*, 2012; Li *et al.*, 2012). However, whilst inhibition of FAAH and elevation of endocannibinoids was demonstrated in these studies, PF-04457845 did not show analgesic efficacy against osteoarthritic knee pain. Therefore, whilst the data presented here would suggest that PF04862853 has potential as an antitussive, questions over the efficacy of FAAH inhibition as analgesics in pain would somewhat temper this optimistic statement.

LABAs and theophylline have previously been observed to have some antitussive activity, but the mechanism of this activity was either attributed to their bronchodilator activity (Pounsford *et al.*, 1985), or not elucidated (Cazzola *et al.*, 1993). Hopefully the data provided in this thesis goes some way further to demonstrating an antitussive effect for LABAs and theophylline via an action on sensory nerves, independent of their bronchodilator activity. The repositioning of existing therapies such as formoterol and theophylline could potentially be a relatively quick and cost-effective route to getting a safe and effective antitussive to the clinic. It would therefore be interesting to further determine the utility of these compounds as antitussives in humans by examining the effect of formoterol, olodaterol or theophylline on capsaicin- and PGE_2-evoked cough in a small clinical trial of healthy human volunteers.

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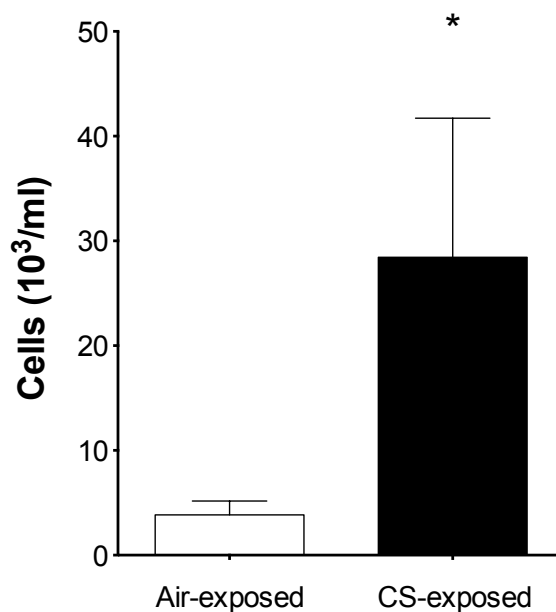
Appendix

Figure A1 Neutrophil levels in BAL fluid of air- or CS-exposed guinea pigs
*Graph shows the numbers of neutrophils in BAL fluid taken from guinea pigs exposed to either room air or CS for 1h periods, twice daily, for 8 days (see Methods 2.6). Bronchoalveolar lavage was recovered 24h after the last CS-exposure, and neutrophil numbers in the BAL were counted as described in McCluskie et al. (2004). Data displayed as mean±SEM, n=6, * p<0.05 as determined by Mann-Whitney U test.*
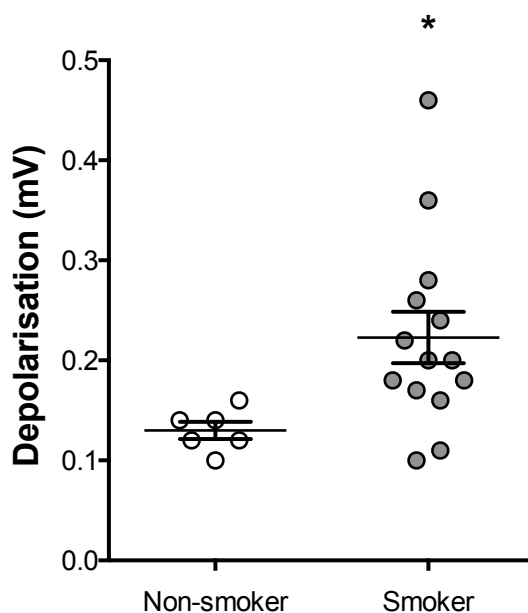

Figure A2 Capsaicin depolarisation of isolated vagus nerves from human non-smokers and smokers
*Magnitude of capsaicin-induced (1μM) depolarisation of vagus nerve tissue from human non-smoker or smoker subjects. The non-smoker and smoker subjects criteria (age/sex/smoking history) are given in Tables A1 and A2 respectively. Data displayed as mean±SEM, n=6-14, * p<0.05 as determined by Mann-Whitney U test.*

Age	Sex	COD	Smoking history	Other information
61	M	ICH/Stroke	None	Hypertension
45	F	Unknown	None	Normal lung function
58	F	Asphyxiation/Anoxia	None	-
60	F	Cerebovascular/Anoxia	None	-
48	M	Cerebovascular/Stroke	None	-
23	M	Asphyxiation/Anoxia	None	-
Mean Age	*M:F ratio*			
46.2	1:1			

Age	Sex	COD	Smoking history PPD	Years	Other information
72	F	Unknown	n/a	30	
58	F	ICH/Stroke	n/a	30	Hypertension
21	M	ICH/Stroke	0.5	40	Hypertension
28	M	Cardiovasc/Anoxia	n/a	13	-
54	M	Unknown	n/a	27	Cystic Fibrosis
51	M	ICH/Stroke	1.5	40	Alcohol abuse
43	M	Asphyxiation	1	15	Marijuana abuse
35	F	Head trauma	n/a	15	-
48	M	Head trauma	0.5	34	Crack cocaine abuse
51	F	ICH/CVA	1	37	Crack cocaine abuse
45	F	Stroke	1	27	Hypertension, marijuana abuse
45	M	ICH/Stroke	2	30	Marijuana abuse
39	F	Overdose/Anoxia	1	23	Asthmatic (unknown meds)
57	M	ICH/Stroke	1	20	Hypertension
Mean Age	*M:F ratio*				
46.2	8-6				

Table A1 Criteria for non-smoker and smokers subjects from whom isolated vagal tissue was obtained
*Table shows the age, sex, cause of death (COD), smoking history (packs per day [PPD] and years), and other relevant information for [**top**] smoker and [**bottom**] non-smoker subjects from whom vagal tissue was obtained for isolated vagus nerve depolarisation experiments presented in Figure A2.*
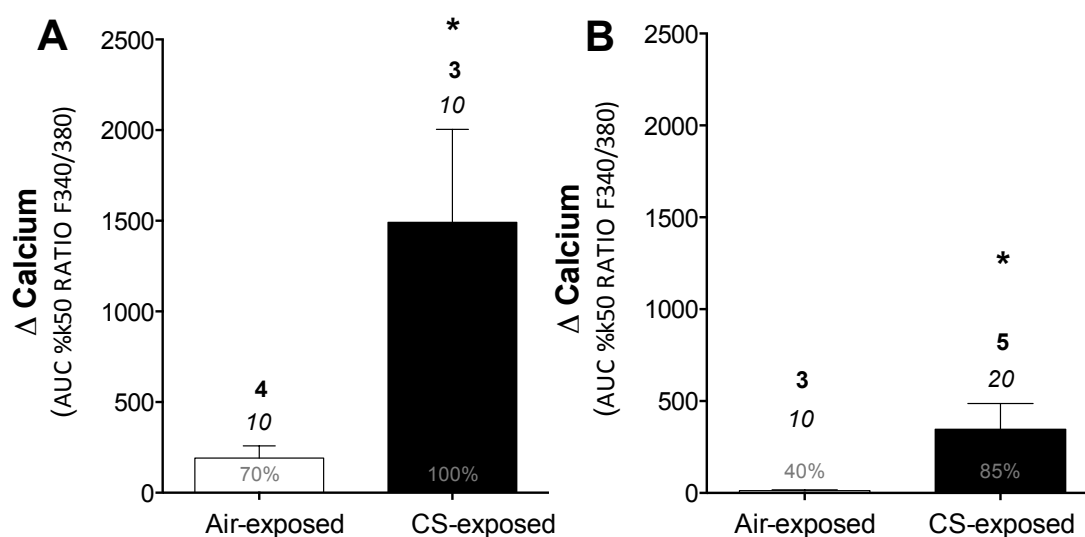
Figure A3 Effect of CS-exposures on capsaicin-induced intracellular calcium flux in jugular or nodose airway-terminating neurons
*Jugular and nodose neurons were isolated from guinea pigs (see Methods 2.4.2) that had been exposed to air or CS for 1h, twice daily, for 8 days (see Methods 2.6). Airway terminating neurons were identified by the presence of DiI stain, which had been administered 12 days before experiment end-point (see Methods 2.4.1). Capsaicin (1µM, 30s) was applied to either [A] jugular or [B] nodose airway-terminating neurons, and the resultant increase in intracellular calcium was monitored via the fluorescence of the Fura2 calcium fluorophore (see Methods 2.4.3). The capsaicin response was recorded either until baseline was recovered, or for 10min (whichever was sooner), and the resultant area under the curve for the increased fluorescence (see Methods 2.4.6) was normalised to an initial response to K_{50} (50mM potassium solution; 15s) recorded in the same cell. Data displayed as mean±SEM, bold number indicates number of animals, italics number indicates number of cells, percentage indicates proportion of cells responding to capsaicin (with arbitrary cut-off of 10% of K50 response), * p<0.05 as determined by Mann-Whitney U test comparing CS-exposed to air-exposed groups.*