



# Targeting fatty acid amide hydrolase as a therapeutic strategy for antitussive therapy

Michael A. Wortley<sup>1,4</sup>, John J. Adcock<sup>1,4</sup>, Eric D. Dubuis<sup>1</sup>, Sarah A. Maher<sup>1</sup>, Sara J. Bonvini<sup>1</sup>, Isabelle Delescluse<sup>2</sup>, Ross Kinloch<sup>2</sup>, Gordon McMurray<sup>2</sup>, Christelle Perros-Huguet<sup>2</sup>, Marianthi Papakosta<sup>2,3</sup>, Mark A. Birrell<sup>1</sup> and Maria G. Belvisi<sup>1</sup>

**Affiliations:** <sup>1</sup>Respiratory Pharmacology Group, Airway Disease Division, National Heart and Lung Institute, Faculty of Medicine, Imperial College London, London, UK. <sup>2</sup>Internal Medicine, Pfizer Global R&D, Sandwich, UK. <sup>3</sup>Inflammation and Immunology Research Unit, Pfizer Inc., Cambridge, MA, USA. <sup>4</sup>Both authors contributed equally to this work.

**Correspondence:** Maria G. Belvisi, Respiratory Pharmacology Group, Airway Disease Division, National Heart and Lung Institute, Faculty of Medicine, Imperial College London, Exhibition Road, London, SW7 2AZ, UK. E-mail: m.belvisi@imperial.ac.uk



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**Fatty acid amide hydrolase inhibition as a target for the development of novel, safe antitussive therapy**  
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**ABSTRACT** Cough is the most common reason to visit a primary care physician, yet it remains an unmet medical need. Fatty acid amide hydrolase (FAAH) is an enzyme that breaks down endocannabinoids, and inhibition of FAAH produces analgesic and anti-inflammatory effects. Cannabinoids inhibit vagal sensory nerve activation and the cough reflex, so it was hypothesised that FAAH inhibition would produce antitussive activity *via* elevation of endocannabinoids.

Primary vagal ganglia neurons, tissue bioassay, *in vivo* electrophysiology and a conscious guinea pig cough model were utilised to investigate a role for fatty acid amides in modulating sensory nerve activation in vagal afferents.

FAAH inhibition produced antitussive activity in guinea pigs with concomitant plasma elevation of the fatty acid amides *N*-arachidonylethanolamide (anandamide), palmitoylethanolamide, *N*-oleoylethanolamide and linoleoylethanolamide. Palmitoylethanolamide inhibited tussive stimulus-induced activation of guinea pig airway innervating vagal ganglia neurons, depolarisation of guinea pig and human vagus, and firing of C-fibre afferents. These effects were mediated *via* a cannabinoid CB<sub>2</sub>/G<sub>i/o</sub>-coupled pathway and activation of protein phosphatase 2A, resulting in increased calcium sensitivity of calcium-activated potassium channels.

These findings identify FAAH inhibition as a target for the development of novel, antitussive agents without the undesirable side-effects of direct cannabinoid receptor agonists.

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## Introduction

Chronic cough severe enough to interfere with the normal activities of daily life is thought to affect ~7% of the population [1]. It is often associated with inflammatory airway diseases including chronic obstructive pulmonary disease, asthma, idiopathic pulmonary fibrosis, lung cancer or conditions outside the lung such as gastro-oesophageal reflux and rhinosinusitis, and is also a side-effect of drug treatments such as angiotensin-converting enzyme inhibitors [2]. Treatment-resistant cough can also be idiopathic in origin and can account for 18–42% of patients at specialist cough clinics [3]. Despite its importance, the treatment options for cough are limited and recent evidence suggests over-the-counter cough medications are ineffective [4].

The cough reflex is regulated by vagal, sensory afferent nerves which innervate the airway [2, 5]. The cell bodies for airway sensory nerves are mostly housed in the nodose and jugular ganglia. There are several known sensory nerve subtypes present in the lung: some are more mechanically sensitive, *e.g.* the rapidly adapting receptors, slowly adapting receptors and the subtype known as the “cough” receptor, and some are more chemosensitive, *e.g.* C-fibres and A $\delta$  nociceptors. Ion channels present on these vagal nerve termini can be activated by a wide variety of stimuli to elicit cough and other reflexes. Transient receptor potential (TRP) channels are the main family of ion channels implicated in the initiation of sensory reflexes.

Previous studies have highlighted the role of cannabinoid CB<sub>2</sub> receptors in the modulation of airway sensory nerve activity and the cough reflex [6, 7]. However, directly administered cannabinoids are not devoid of undesirable side-effects [8]. The enzyme fatty acid amide hydrolase (FAAH) is an integral membrane protein found within the nervous system, and is responsible for the hydrolysis of the endocannabinoid *N*-arachidonylethanolamide (AEA; anandamide) and other related amidated signalling lipids, such as palmitoylethanolamide (PEA), *N*-oleoylethanolamide (OEA) and linoleoylethanolamide (LEA). These signalling lipids exert their effects through various different receptors such as cannabinoid CB<sub>1</sub>/CB<sub>2</sub> receptors and also *via* noncannabinoid receptors, including TRPV1, peroxisome proliferator-activated receptors (PPARs) and opioid receptors [9–11]. Elevating endogenous levels of AEA and other bioactive fatty acid amides (FAAs) through genetic deletion or pharmacological inhibition of FAAH elicits analgesic effects in pre-clinical models of pain and inflammation [12]. These lipid signalling molecules are synthesised and released on demand and act locally, as they are rapidly inactivated. This suggests that there may be therapeutic potential in modulating the levels of these ligands, thereby reducing the potential for side-effects that result from widespread systemic cannabinoid receptor activation.

Given CB<sub>2</sub> receptor agonists have been shown to produce antitussive activity in pre-clinical models of cough [6, 7], we hypothesised that inhibition of FAAH may represent a target for the development of novel antitussive agents. In these studies we utilised the tussive agents commonly used for clinical cough challenge studies, *i.e.* capsaicin and citric acid (which represents a low pH solution) [2].

## Material and methods

### Animals

*In vivo* and *ex vivo* experiments were conducted in male Dunkin–Hartley guinea pigs (300–500 g; 400–800 g for single-fibre *in vivo* studies) (Harlan, Bicester, UK and B&K Universal, Hull, UK) housed in a temperature-controlled (21°C) room with food and water freely available for at least 1 week before commencing experimentation. The experiments were performed in accordance with the UK Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) act 1986 and the ARRIVE guidelines [13].

### Measurement of PF-04862853 and FAA levels

In stand-alone pharmacokinetic characterisation studies, blood samples (saphenous vein bleeds) were taken at 0, 30, 60, 120, 180, 240, 300 and 360 min after oral administration. Plasma levels of the novel FAAH inhibitor PF-04862853 were measured in plasma by liquid chromatography-mass spectrometry (LC-MS) as described previously [14, 15]. Plasma was prepared as described previously [14, 15] in separate studies to assess AEA, PEA, OEA and LEA levels, also measured by LC-MS.

### Conscious guinea pig cough counting

PF-04862853 (1 mg·kg<sup>-1</sup>) or vehicle (0.5% methylcellulose/0.1% Tween 80 in saline) was administered *per os* 4 h prior to cough recording. The studies were blinded and oral dosing was performed by an independent operator. In all experiments the operator was blinded to the treatment groups. Conscious guinea pigs were placed in individual double-chamber plethysmography boxes (Buxco, Wilmington, NC, USA) and cough assessed to an aerosol of citric acid (aerosol of 0.3 M in saline for 10 min) as described previously [7, 16]. Animals were sacrificed at the end of the experiment.

### **Guinea pig and human isolated vagus nerve recording**

Recording of depolarisation of isolated guinea pig and human vagus was carried out as described previously [2, 6, 7, 16]. Briefly, nerves were stimulated twice with capsaicin (1  $\mu\text{M}$ ) or low pH (pH 5) perfused for 2 min (internal control) before vehicle/FAA was perfused for 10 min. Stimulation was then re-assessed in the presence of vehicle or FAA. After 5–10 min wash, nerves were stimulated at the end of each experiment to confirm viability. For relevant experiments, vehicle/antagonist/potassium channel blocker was perfused for 10 min prior to and in the presence of vehicle/FAA. For experiments to examine the role of  $G_{i/o}$  subunits, vehicle (saline) or pertussis toxin (PTX) was administered (25  $\mu\text{g}$ , intraperitoneally) 3 days prior to removal of the vagus nerves for the isolated vagal nerve recording experiments.

The vagus nerve was dissected from human donor *en bloc* lungs unsuitable for transplantation (obtained from the International Institute for the Advancement of Medicine, Edison, NJ, USA), except in one case where the vagus nerve was dissected from lung tissue of a lung transplant recipient (obtained from the Royal Brompton & Harefield NHS Foundation Trust, London, UK). Samples were from nine patients (seven males and two females; median (range) age 51 (27–67) years) with no history of respiratory disease, except the recipient sample that was from a cystic fibrosis patient. In all cases tissue was consented for use in scientific research. Ethics approval was obtained from the Royal Brompton & Harefield NHS Foundation Trust.

### **In vivo single-fibre recording**

Guinea pigs were anaesthetised with urethane (1.5  $\text{g}\cdot\text{kg}^{-1}$ , *i.p.*) supplemented with additional urethane as required. The trachea was cannulated to maintain blood gases and pH at physiological levels by means of artificial ventilation, with tidal volume 10  $\text{mL}\cdot\text{kg}^{-1}$  and 50–60 breaths $\cdot\text{min}^{-1}$  of laboratory air. A cannula was connected to an air pressure transducer (SenSym 647; Farnell, Leeds, UK) with a side-arm to a monitor to measure tracheal pressure. The right jugular vein and carotid artery were cannulated for injecting drugs and measuring systemic arterial blood pressure, respectively. Animals were paralysed by (intravenous) administration of vecuronium bromide; initially at 100  $\mu\text{g}\cdot\text{kg}^{-1}$ , *i.v.*, and maintained with 50  $\mu\text{g}\cdot\text{kg}^{-1}$  every 20 min. A cervical incision was made to locate both vagus nerves, which were cleared from the carotid artery and cut at the central end. The left vagus nerve was teased down until a single-fibre unit firing could be recorded using platinum electrodes. C-fibres used for these experiments were identified by the lack of spontaneous discharge and responsiveness to hyperinflation and deflation, responsiveness to capsaicin/citric acid administration, and possessing a conduction velocity  $<1\text{ m}\cdot\text{s}^{-1}$  [2, 17]. Upon identification of a C-fibre, the experimental protocol was as follows: stimuli (capsaicin 100  $\mu\text{M}$  for 15 s or citric acid 300 mM for 60 s) were administered as aerosols to provoke action potential firing, with time between stimulations to allow recovery to baseline activity levels. PEA (10  $\text{mg}\cdot\text{kg}^{-1}$ , *i.p.*) was then administered and then 30 min later the same stimuli were administered to evaluate the effect of PEA on action potential firing.

### **Imaging of intracellular calcium in isolated airway neurons**

Dissection of jugular ganglia, dissociation of neurons from tissue, identification of airway terminating neurons and assessment of intracellular free calcium  $[\text{Ca}^{2+}]_i$  levels using the fluorophore Fura-2 AM were carried out as described previously [2, 18]. 50 mM potassium chloride solution was applied at the start and end of each experiment for 10 s to assess cell viability and normalise responses. Neurons were stimulated twice with low pH (pH 6) or capsaicin (100 nM) for 1 min before vehicle or PEA (1 nM) was applied for 10 min. Stimulation was then re-assessed in the presence of vehicle or PEA. The criterion for a “responsive cell” was judged as an increase in  $[\text{Ca}^{2+}]_i$  of  $\geq 10\%$  of the response to the 50 mM potassium chloride solution.

### **Compounds, materials and solutions**

PF-04862853 (7-azaspiro[3.5]nonane urea) [14] was kindly supplied by Pfizer Global R&D (Cambridge, UK). SR141716A and SR144528 were purchased from Tocris Bioscience (Bristol, UK), and LEA was purchased from Cambridge Bioscience (Cambridge, UK). Fura-2 AM was purchased from Invitrogen (Paisley, UK). L-15 and Hanks’ balanced salt solution were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). All reagents not mentioned above were purchased from Sigma-Aldrich (St Louis, MO, USA).

For calcium imaging experiments, 50 mM potassium solution contained 50 mM KCl, 91.4 mM NaCl, 1 mM  $\text{MgCl}_2$ , 2.5 mM  $\text{CaCl}_2$ , 0.33 mM  $\text{NaH}_2\text{PO}_4$ , 10 mM glucose, 10 mM HEPES; pH adjusted to 7.4 at 37°C using KOH. For *in vitro* whole vagus and calcium imaging experiments, capsaicin and PEA were dissolved in 100% dimethylsulfoxide (DMSO) and solutions were diluted 1:1000 in the perfusing solution. This was Krebs–Henseleit solution [16] for vagus experiments and extracellular solution (5.4 mM KCl, 136 mM NaCl, 1 mM  $\text{MgCl}_2$ , 2.5 mM  $\text{CaCl}_2$ , 0.33 mM  $\text{NaH}_2\text{PO}_4$ , 10 mM glucose, 10 mM HEPES; pH

adjusted to 7.4 at 37°C using NaOH) for calcium imaging experiments. Final vehicle concentration for an individual drug was 0.1%, or up to 0.3% when combinations of drugs were perfused together.

#### Data analysis and statistics

A p-value of <0.05 was considered significant. Significance was assessed by the paired t-test (where comparisons are made in the presence of drug and compared with initial control responses on the same preparation), or by either the Mann-Whitney U-test or Kruskal-Wallis with Dunn's *post hoc* analysis (depending on the number of treatment columns), as indicated in the figure legends.

## Results

### PF-04862853 inhibits FAAH, elevates FAAs and inhibits cough in a conscious guinea pig model

PF-04862853 is known to achieve peak drug levels at 4 h post-dose, and demonstrates good oral bioavailability of 53% and 33% in rats and dogs, respectively [14, 15]. Our results in guinea pig paralleled these findings, with peak levels of PF-04862853 ( $1 \text{ mg}\cdot\text{kg}^{-1}$ , *p.o.*) occurring 2–4 h post-dose (figure 1a); we chose the 4 h time-point for further evaluations. PF-04862853 ( $1 \text{ mg}\cdot\text{kg}^{-1}$ , *p.o.*) significantly inhibited citric acid-evoked cough (figure 1b). This dose caused robust inhibition of FAAH activity in the same animals (~84%, similar to previous studies in rats and dogs [15]) with concomitant elevations in plasma AEA, PEA, OEA and LEA (~4–7-fold; figure 1c). These data indicate that the antitussive activity of the FAAH inhibitor is associated with peak PF-04862853 levels, FAAH enzyme inhibition and elevation of FAAs.

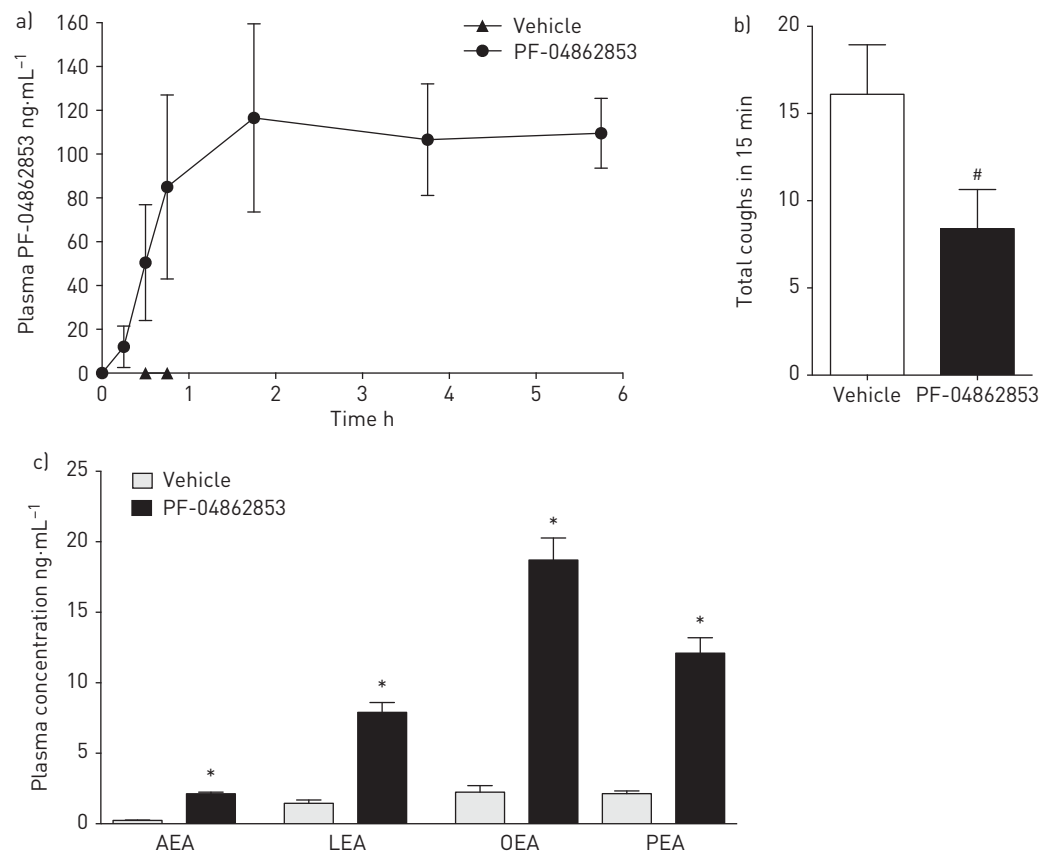
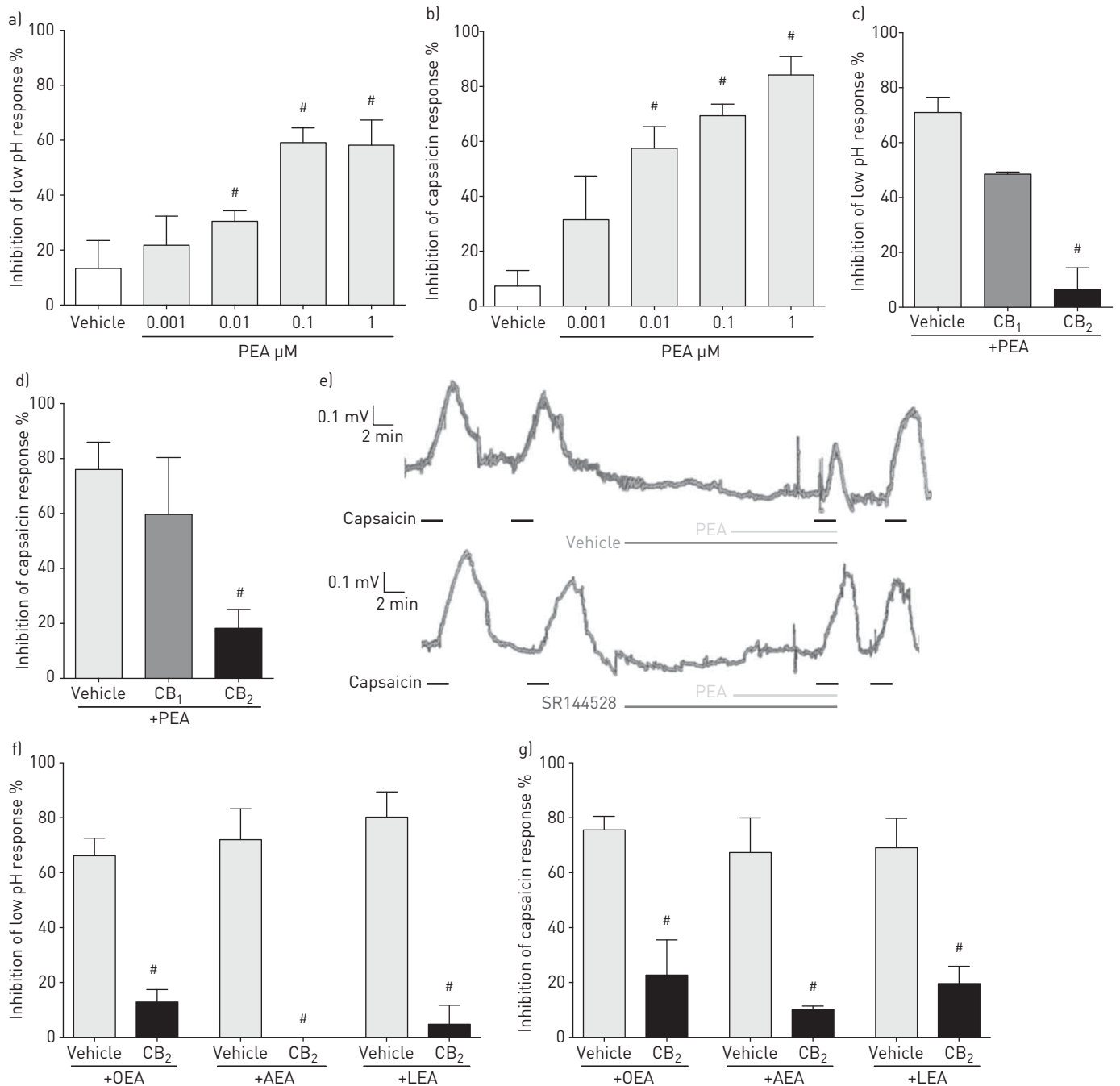


FIGURE 1 Oral dosing of PF-04862853 leads to elevation of fatty acid amides (FAAs) and inhibition of citric acid-evoked cough. AEA: *N*-arachidonylethanolamide; LEA: linoleylethanolamide; OEA: *N*-oleylethanolamide; PEA: palmitylethanolamide. a) Plasma levels of PF-04862853 at time-points after  $1 \text{ mg}\cdot\text{kg}^{-1}$ , *p.o.* b) Effect of PF-04862853 ( $1 \text{ mg}\cdot\text{kg}^{-1}$ , *p.o.*, 4 h post-dose) on the number of coughs evoked by 0.3 M citric acid. c) Plasma levels of the FAAs AEA, LEA, OEA and PEA following administration of PF-04862853 ( $1 \text{ mg}\cdot\text{kg}^{-1}$ , *p.o.*, 4 h post-dose). Vehicle: 0.5% methylcellulose/0.1% Tween 80 in saline. Data are presented as mean±SEM, n=8 animals per group. Compared with vehicle control group: #: p<0.05 (Mann-Whitney U-test); \*: p<0.05 (paired t-test).

### FAAs inhibit guinea pig isolated vagus nerve depolarisation via the CB<sub>2</sub> receptor

We examined the effects of PEA, AEA, OEA and LEA on tussive stimuli-induced depolarisation of guinea pig isolated vagus nerve. PEA (1 nM to 1  $\mu$ M) caused a concentration-related inhibition of both low pH- and capsaicin-induced depolarisation of isolated guinea pig vagus nerve (figure 2a and b). At a concentration of 100 nM (submaximal for PEA), all four FAAs caused substantial inhibition (60–80%) of



**FIGURE 2** Palmitoylethanolamide (PEA) and other fatty acid amides inhibit low pH- and capsaicin-induced vagus nerve depolarisation *via* activation of cannabinoid CB<sub>2</sub> receptors. OEA: *N*-oleoylethanolamide; AEA: *N*-arachidonylethanolamide; LEA: linoleoylethanolamide. a, b) Concentration-related inhibition by PEA of depolarisation of the isolated guinea pig vagus evoked by a) low pH (pH 5) or b) capsaicin (1  $\mu$ M). c–e) Effect of CB<sub>1</sub> (SR141716A; 10 nM) and CB<sub>2</sub> (SR144528; 10 nM) receptor antagonists on PEA (100 nM) inhibition of c) low pH (pH 5)- or d) capsaicin (1  $\mu$ M)-induced depolarisation of guinea pig vagus, with e) showing representative traces of experiments with vehicle or CB<sub>2</sub> antagonist effect on PEA inhibition of capsaicin responses. f, g) Effect of CB<sub>2</sub> (SR144528; 10 nM) receptor antagonist on OEA, AEA or LEA (100 nM) inhibition of f) low pH (pH 5)- or g) capsaicin (1  $\mu$ M)-induced depolarisation of guinea pig isolated vagus nerve. Vehicle: 0.1% v/v dimethylsulfoxide. Data are presented as mean  $\pm$  SEM, n=4 nerves from individual animals per group. Compared with vehicle control group: #: p<0.05 (Mann-Whitney U-test or Kruskal-Wallis with Dunn's *post hoc* analysis).

the depolarisation induced by low pH and capsaicin, which was significantly reversed by the CB<sub>2</sub> receptor antagonist SR144528 but not by the CB<sub>1</sub> receptor antagonist SR141716A (figure 2c–g).

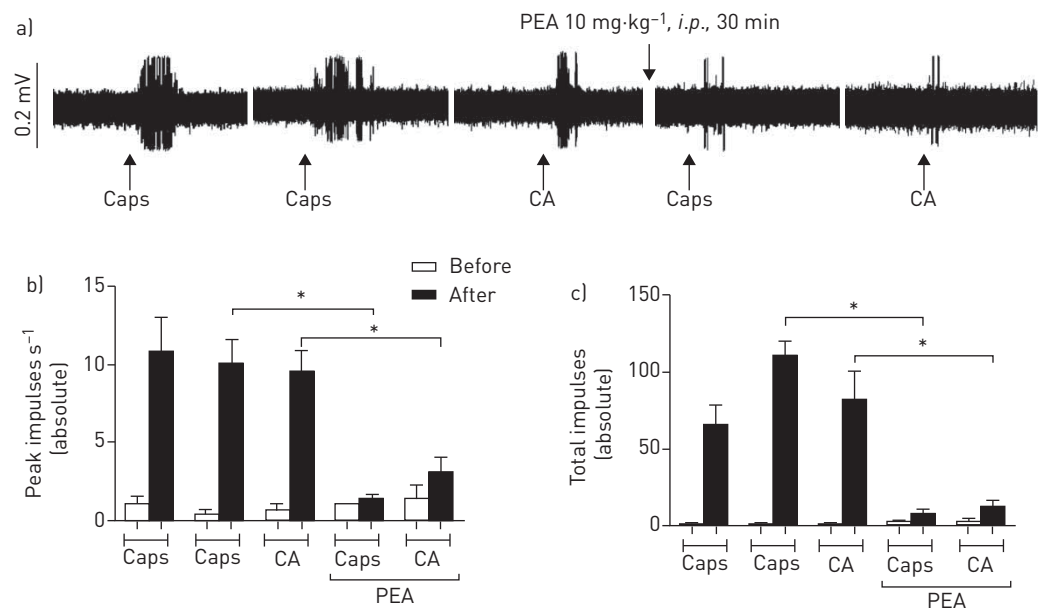
#### **PEA inhibits single-fibre firing in vivo and calcium influx in vitro in airway innervating nerves/neurons**

PEA (10 mg·kg<sup>-1</sup>, *i.p.*) significantly reduced both total action potential firing and firing frequency of single airway innervating C-fibres in response to activation with either an aerosol of citric acid (0.3 M) or capsaicin (100 μM) (figure 3a–c).

We also utilised an *in vitro* preparation, whereby we examined intracellular calcium flux in isolated jugular ganglion neurons stained with the retrograde tracer dye DiI, administered intranasally *in vivo*, to identify airway terminating neurons *in vitro*. PEA (1 nM) significantly abrogated the intracellular calcium signal induced by either low external pH (pH 6) or by capsaicin (1 μM) *in vitro* in jugular ganglia neurons stained with DiI (indicating airway innervating neurons) (figure 4a–d).

#### **PEA inhibits guinea pig isolated vagus nerve depolarisation via the G<sub>i/o</sub> signalling pathway and effects on calcium-activated potassium channels**

PTX was utilised to block signal transduction *via* G<sub>i/o</sub> subunits. In guinea pigs dosed with PTX, PEA inhibition of capsaicin-induced depolarisation was greatly abrogated compared with PEA inhibition in vagus nerves from vehicle-dosed control animals, indicating a role for G<sub>i/o</sub> signalling (figure 5a). Cannabinoid effects are often suggested to be at least partially mediated by effects on potassium channels; therefore, we examined the effect of various potassium channel blockers on PEA inhibition of vagus nerve depolarisation. Clotrimazole, paxilline and glibenclamide, blockers of intermediate (I<sub>KCa</sub>) and large (B<sub>KCa</sub>) conductance calcium-activated and ATP-sensitive (K<sub>ATP</sub>) potassium channels, respectively, had no significant effect on the inhibitory effects of PEA on guinea pig vagus nerve depolarisation. By contrast, apamin (1 μM), a blocker of small conductance (S<sub>KCa</sub>) calcium-activated potassium channels, almost abolished PEA inhibition (~85–90%) of capsaicin- and low pH-induced depolarisation (figure 5b and c). Consistent with an activity on S<sub>KCa</sub> channels, PEA inhibited depolarisation of guinea pig vagus nerve evoked by agonists of multiple excitatory ion channels examined (supplementary figure S1), *i.e.* the TRPA1



**FIGURE 3** Palmitoylethanolamide (PEA) inhibits citric acid (CA)- and capsaicin (Caps)-evoked C-fibre firing *in vivo*. a) Representative trace showing single C-fibre action potential firing to capsaicin (100 μM) or citric acid (0.3 M) aerosol for 15 and 60 s, respectively, prior to and then in the presence of PEA (10 mg·kg<sup>-1</sup>, *i.p.*, 30 min prior to capsaicin challenge). b) Peak firing frequency and c) total impulses were assessed in guinea pig airway innervating C-fibres in response to intratracheal aerosol administration of citric acid (0.3 M) or capsaicin (100 μM). Panels show spontaneous levels of firing immediately before and levels of firing after administration of citric acid or capsaicin. Control responses were established on each nerve before *i.p.* administration of PEA (10 mg·kg<sup>-1</sup> in vehicle 0.5% methylcellulose/0.1% Tween 80 in saline 10 mL·kg<sup>-1</sup>). Data are presented as mean±SEM, n=3 nerve fibres in individual animals. Compared with respective control groups: \*; p<0.05 (paired t-test).



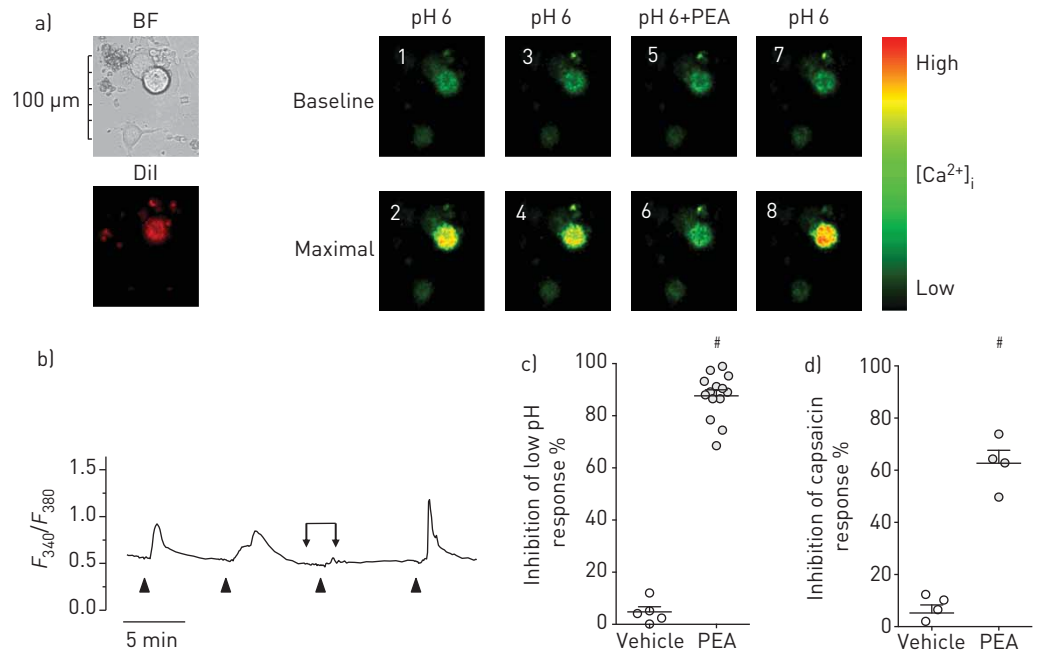


FIGURE 4 Palmitoylethanolamide (PEA; 1 nM) inhibits low pH- and capsaicin-induced increases in intracellular calcium in airway neurons. a) Representative images showing brightfield (BF) image of neurons with Dil emission indicating an airway terminating neuron. Images 1–8 indicate levels of intracellular calcium pre- and post-pH 6 stimulation. b) Trace of quantified intracellular calcium levels in the same neuron/experiment, with drug additions indicated by arrowheads (pH 6) and the brace/arrows (PEA; 1 nM).  $F_{340}/F_{380}$ : ratio of the fluorescence intensities at 340 and 380 nm excitation. c, d) PEA (1 nM) inhibition of c) low pH (pH 6)- or d) capsaicin (100 nM)-induced intracellular calcium increases in isolated airway jugular neurons from guinea pigs. Data are presented as mean  $\pm$  SEM,  $n=4-14$  cells per group,  $N=3-4$  animals. Vehicle: 0.1% v/v dimethylsulfoxide. Compared with vehicle control group: #:  $p < 0.05$  (Mann-Whitney U-test).

agonist acrolein (300  $\mu$ M), the synthetic TRPV4 agonist GSK1016790A (300 nM) or the endogenous TRPV4 agonist hypo-osmotic solution ( $-80$  mOsm).

As the SK<sub>Ca</sub> channel was implicated in the inhibitory actions of PEA, we examined the possible involvement of protein phosphatase 2A (PP2A), which forms a regulatory part of the SK<sub>Ca</sub> channel multiprotein complex along with casein kinase II (CKII) and calmodulin (CaM). CaM is bound to the SK<sub>Ca</sub> channel and translates calcium binding into channel opening; CKII phosphorylation of CaM decreases SK<sub>Ca</sub> calcium sensitivity (and therefore open probability), whereas PP2A dephosphorylation of CaM increases calcium sensitivity [19]. A PP2A inhibitor (okadaic acid; 1 nM) blocked the inhibitory effects of PEA on vagus nerve depolarisation, whereas a CKII inhibitor (4,5,6,7-tetrabromobenzotriazole; 10  $\mu$ M) inhibited depolarisation itself (figure 5d). These results suggest that PEA activates SK<sub>Ca</sub> channels by activation of PP2A, which dephosphorylates SK<sub>Ca</sub>-bound CaM to increase the channel's calcium sensitivity.

#### PEA inhibits human vagus nerve depolarisation via a CB<sub>2</sub>-PP2A-SK<sub>Ca</sub> channel mechanism

To increase the translational nature of this study we performed key experiments in a unique human vagal sensory nerve assay assessing depolarisation in a similar fashion to the guinea pig. PEA (100 nM) inhibited both low pH- and capsaicin-induced depolarisation of human vagus nerves by  $\sim 70-80\%$  (figure 6). Furthermore, PEA inhibition of depolarisation was completely reversed by the CB<sub>2</sub> receptor antagonist but not the CB<sub>1</sub> receptor antagonist (figure 6b) and substantially reversed ( $\sim 85\%$ ) by the SK<sub>Ca</sub> channel blocker (apamin; 1  $\mu$ M) (figure 6c). In preliminary experiments ( $n=2$ ), the PP2A inhibitor blocked the effects of PEA and an inhibitor of CKII mimicked the effects of PEA (figure 6d), suggesting PEA inhibition of human vagus nerve depolarisation is also mediated by the CB<sub>2</sub>-PP2A-SK<sub>Ca</sub> channel pathway.

### Discussion

Recent evidence suggests that CB<sub>2</sub> receptors mediate inhibition of airway sensory nerve activity and cough in animal models, highlighting the CB<sub>2</sub> receptor as a potential target for the treatment of cough [6, 7]. However, cannabinoids are associated with a variety of central effects such as sedation, nausea and addiction, which limit their use as therapies [20, 21]. The pharmacological blockade of FAAH has

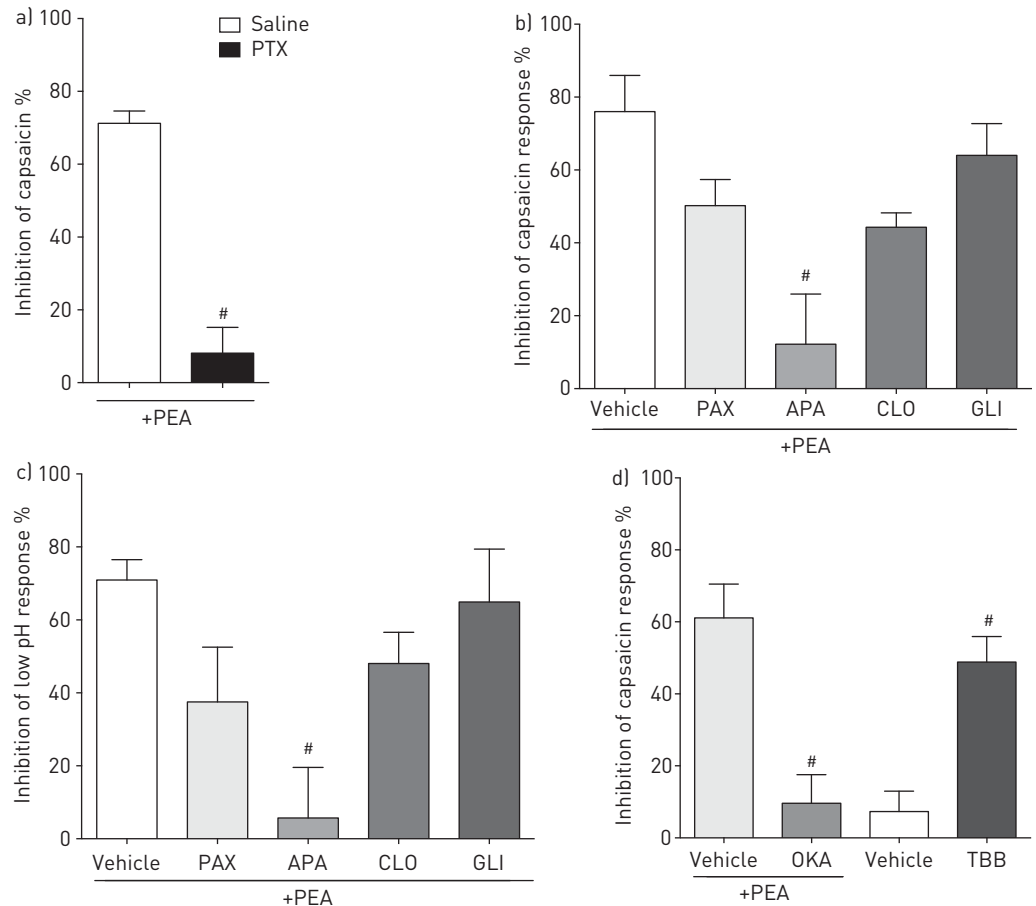


FIGURE 5 Palmitoylethanolamide (PEA) inhibits isolated vagus nerve depolarisation through a  $CB_2/G_{i/o}$ -coupled pathway and activation of small conductance calcium-activated potassium channels and phosphatase 2A. PTX: pertussis toxin; PAX: paxilline; APA: apamin; CLO: clotrimazole; GLI: glibenclamide; OKA: okadaic acid; TBB: 4,5,6,7-tetrabromobenzotriazole. a) Effect of PTX (dosed 25  $\mu$ g, *i.p.*, 3 days prior to the removal of nerve tissue) on PEA (100 nM) inhibition of capsaicin (1  $\mu$ M)-induced depolarisation of guinea pig vagus nerve. b, c) Effect of PAX (1  $\mu$ M), APA (1  $\mu$ M), CLO (10  $\mu$ M) or GLI (10  $\mu$ M) on PEA (100 nM) inhibition of b) capsaicin (1  $\mu$ M)- and c) low pH [pH 5]-induced depolarisation of guinea pig vagus nerve. d) Effect of OKA (1 nM) on PEA (100 nM) inhibition of capsaicin (1  $\mu$ M)-induced depolarisation and effect of TBB (10  $\mu$ M) on capsaicin (1  $\mu$ M)-induced depolarisation of isolated guinea pig vagus nerve. Vehicle: 0.1% v/v dimethylsulfoxide. Data are presented as mean  $\pm$  SEM,  $n=3-4$  nerves from individual animals per group. Compared with relevant vehicle control group: #:  $p < 0.05$  (Mann-Whitney U-test or Kruskal-Wallis with Dunn's *post hoc* analysis).

emerged as a potentially attractive strategy by which endocannabinoid levels are elevated and retain the beneficial effects of cannabinoid receptor activation to relieve pain [22], while avoiding the undesirable effects of global cannabinoid receptor activation.

Over the past decade, there have been advances in the development of FAAH inhibitors and initial interest focused on developing FAAH inhibitors to augment the actions of FAAs and reduce pain. More recently, emerging literature has shown that these FAAs, through interactions with a variety of receptors (extracellular and intracellular), can induce a diverse array of effects that include appetite suppression, modulation of lipid and glucose metabolism, vasodilatation, cardiac function, and inflammation [22]. However, to the best of our knowledge, the effects of FAAH inhibitors have not been studied in the airways. We hypothesised that inhibition of FAAH by a selective inhibitor may have antitussive effects. The FAAH inhibitor used in this study (*i.e.* PF-04862853) is a recently developed compound shown to have excellent potency, selectivity, pharmacokinetic properties and *in vivo* efficacy in a model of inflammatory pain [15].

PF-04862853, examined 4 h post-dose, inhibited cough in guinea pigs and was associated with increases in the plasma levels of FAAs (PEA, AEA, OEA and LEA). Although there are several subclasses of FAAs, the endogenous FAAs identified in the present study belong to the *N*-acylethanolamide subclass, and have been identified in mammalian tissues in the brain and in peripheral tissues [23–25]. These FAAs are



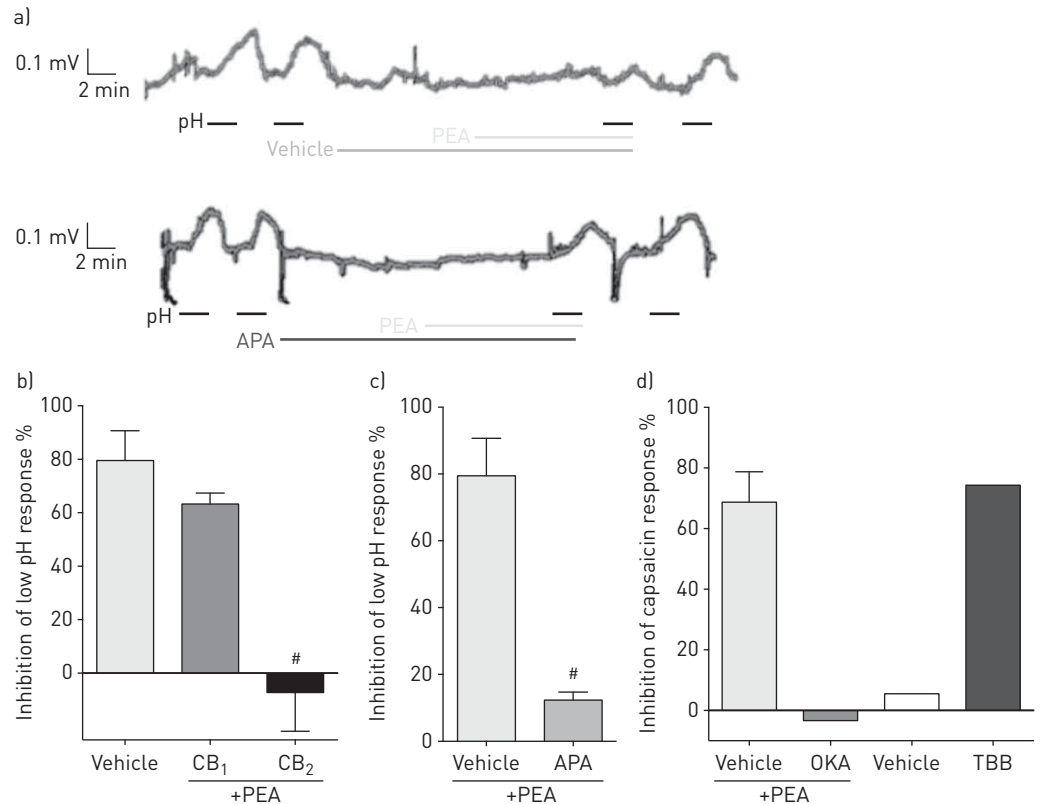


FIGURE 6 Palmitoylethanolamide (PEA) inhibits depolarisation in human vagus nerve *via* CB<sub>2</sub>-phosphatase 2A–small conductance calcium-activated potassium channel. APA: apamin; OKA: okadaic acid; TBB: 4,5,6,7-tetrabromobenzotriazole. a) Representative traces showing PEA inhibition of low pH responses in the presence of (top) vehicle or (bottom) APA (1 μM). b) Effect of CB<sub>1</sub> (SR141716A, 10 nM) and CB<sub>2</sub> (SR144528, 10 nM) receptor antagonists on PEA (100 nM) inhibition of low pH (pH 5)-induced depolarisation of human isolated vagus nerve. c) Effect of APA (1 μM) on PEA (100 nM) inhibition of low pH (pH 5)-induced depolarisation of human isolated vagus nerve. d) Effect of OKA (1 nM) on PEA (100 nM) inhibition of capsaicin (1 μM)-induced depolarisation of human isolated vagus nerve. Vehicle: 0.1% v/v dimethylsulfoxide. Data are presented as mean±SEM, n=4 nerves from individual animals per group, except where no SEM shown, n=2 nerves from individual animals. Compared with relevant vehicle control group: #: p<0.05 (Mann-Whitney U-test or Kruskal-Wallis with Dunn's *post hoc* analysis).

referred to as “endocannabinoids” because they can function as agonists of CB<sub>1</sub> and CB<sub>2</sub> receptors. Given that exogenous cannabinoids exert antitussive activity *via* action on CB<sub>2</sub> receptors on sensory nerves [6, 7], we hypothesised that these FAAs mediate the antitussive effects of FAAH inhibition by likewise inhibiting sensory nerve activation *via* the CB<sub>2</sub> receptor.

All the FAAs examined inhibited low pH- and capsaicin-evoked depolarisation of the guinea pig isolated vagus nerve, an effect reversed by the selective CB<sub>2</sub> receptor antagonist, but not the CB<sub>1</sub> receptor antagonist [26, 27]. Furthermore, PEA (used as the exemplar) also inhibited capsaicin- and citric acid-evoked action potential firing in guinea pig airway innervating C-fibres *in vivo*, and the intracellular calcium increases induced by capsaicin or low pH in isolated airway terminating guinea pig jugular neurons *in vitro*. The combination of these experiments strongly suggests that the FAAs elevated by FAAH inhibition act on CB<sub>2</sub> receptors on sensory nerves to mediate the antitussive effects of PF-04862853. Furthermore, in vagus nerve tissue from PTX-treated animals, PEA inhibition was lost, suggesting engagement of G<sub>i/o</sub> subunit signalling downstream of the CB<sub>2</sub> receptor. Although our data utilising the vagus nerve preparation would indicate a peripheral mechanism of action, central effects of FAAs cannot be disregarded.

Although the FAAs examined have been reported to activate CB<sub>2</sub> receptors [28–31], previous publications have also suggested effects on CB<sub>1</sub> receptors [29], PPAR (PPAR-α, -β and -γ) receptors [10, 31–33], and TRPV1 [9, 34, 35] and TRPM8 [36] ion channels. However, a role for CB<sub>2</sub> receptors has been clearly identified in these studies, which utilised highly selective pharmacological tools [26, 27], that is consistent with previous studies [6, 7]. However, it is still impossible to completely rule out a contribution from noncannabinoid receptors to the *in vivo* activity of FAAs.

Apamin, an inhibitor of SK<sub>Ca</sub> channels, blocked the inhibition by PEA of depolarisation of the guinea pig isolated vagus nerve, confirming previous reports that CB<sub>1</sub> and CB<sub>2</sub> receptor agonists can activate potassium channels [37]. SK<sub>Ca</sub> channels are one of a family of calcium-activated potassium channels and their activation causes potassium efflux from neurons, thereby decreasing their excitability. The SK<sub>Ca</sub> channel is part of a multiprotein complex along with CaM, PP2A and CKII. CaM is bound to the SK<sub>Ca</sub> channel and plays a key role in translating calcium binding into channel opening. The sensitivity of CaM, and therefore of the SK<sub>Ca</sub> channel, to calcium is regulated by phosphorylation/dephosphorylation, with phosphorylation by CKII resulting in decreased calcium sensitivity and dephosphorylation by PP2A resulting in increased calcium sensitivity [19]. Our finding that okadaic acid, a PP2A inhibitor, blocks the inhibitory activity of PEA on vagus nerves suggests that PEA acts to increase the sensitivity to calcium (and therefore the activity) of the SK<sub>Ca</sub> channel *via* activation of PP2A. Key datasets were repeated in human vagus nerve preparations with similar results obtained compared with guinea pig.

In conclusion, inhibition of FAAH produces antitussive activity in conscious guinea pigs. Our data indicate that this effect could be mediated by elevated FAAs, acting on cannabinoid (CB<sub>2</sub>) receptors on vagal sensory nerves, leading to activation of SK<sub>Ca</sub> channels, an effect that translates to human vagus nerves. To date, interest in this area has focused on developing FAAH inhibitors to reduce pain, and early clinical studies have indicated that a closely related analogue (*i.e.* PF-04457845) to the compound investigated here was well tolerated with no effect on cognitive functions at doses exceeding those required for maximal inhibition of FAAH activity and elevation of FAAs [38]. These data suggest that inhibition of FAAH with consequent elevation of FAAs could be an interesting and novel therapeutic approach to treat chronic cough. Although efficacy in pre-clinical and clinical capsaicin challenge studies (*e.g.* utilising TRPV1 antagonists) has not translated to efficacy in chronic cough patients, the fact that FAA ligands inhibit depolarisation of the vagus evoked by agonists of multiple excitatory ion channels gives a broader chance of success [39–41]. With clinical-ready compounds available, proof-of-concept clinical studies could be undertaken by adopting a re-positioning approach and utilising compounds from the arena of pain therapeutics. This strategy was successful recently with the P2X<sub>3</sub> inhibitor AF-219, which was found to demonstrate unprecedented efficacy in chronic cough in treatment-resistant patients [42].

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