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PKCε stimulation of TRPV1 orchestrates carotid body responses to asthmakines

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Key points

- We have previously shown that carotid body stimulation by lysophosphatidic acid elicits a reflex stimulation of vagal efferent activity sufficient to cause bronchoconstriction in asthmatic rats.
- Here, we show that pathophysiological concentrations of asthma-associated prototypical Th2 cytokines also stimulate the carotid bodies.
- Stimulation of the carotid bodies by these asthmakines involves a PKCε–transient receptor potential vanilloid 1 (TRPV1) signalling mechanism likely dependent on TRPV1 S502 and T704 phosphorylation sites.
- As the carotid bodies' oxygen sensitivity is independent of PKCε–TRPV1 signalling, systemic blockade of PKCε may provide a novel therapeutic target to reduce allergen-induced asthmatic bronchoconstriction.
- Consistent with the therapeutic potential of blocking the PKCε–TRPV1 pathway, systemic delivery of a PKCε-blocking peptide suppresses asthmatic respiratory distress in response to allergen and reduces airway hyperresponsiveness to bradykinin.

Abstract The autonomic nervous system orchestrates organ-specific, systemic and behavioural responses to inflammation. Recently, we demonstrated a vital role for lysophosphatidic acid in stimulating the primary autonomic oxygen chemoreceptors, the carotid bodies, in parasympathetic-mediated asthmatic airway hyperresponsiveness. However, the cacophony of

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stimulatory factors and cellular mechanisms of carotid body activation are unknown. Therefore, we set out to determine the intracellular signalling involved in carotid body-mediated sensing of asthmatic blood-borne inflammatory mediators. We employed a range of *in vitro* and rat *in situ* preparations, site-directed mutagenesis, patch-clamp, nerve recordings and pharmacological inhibition to assess cellular signalling. We show that the carotid bodies are also sensitive to asthma-associated prototypical Th2 cytokines which elicit sensory nerve excitation. This provides additional asthmatic ligands contributing to the previously established reflex arc resulting in efferent vagal activity and asthmatic bronchoconstriction. This novel sensing role for the carotid body is mediated by a PKCε-dependent stimulation of transient receptor potential vanilloid 1 (TRPV1), likely via TRPV1 phosphorylation at sites T704 and S502. Importantly, carotid body oxygen sensing was unaffected by blocking either PKCε or TRPV1. Further, we demonstrate that systemic PKCε blockade reduces asthmatic respiratory distress in response to allergen and airway hyperresponsiveness. These discoveries support an inflammation-dependent, oxygen-independent function for the carotid body and suggest that targeting PKCε provides a novel therapeutic option to abate allergic airway disease without altering life-saving autonomic hypoxic reflexes.

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Introduction

The autonomic nervous system is a potent modulator of inflammation (Pavlov & Tracey, 2017). Recent interest has focused on the role of the parasympathetic nervous system, often referred to as the 'cholinergic anti-inflammatory reflex' (Borovikova *et al.* 2000; Pavlov & Tracey, 2017). Activation of this reflex alleviates systemic inflammation caused by bacterial-induced sepsis (Borovikova *et al.* 2000). However, in response to lung-targeted allergens (McQueen *et al.* 2007; Talbot *et al.* 2015), or prolonged inflammation (Pongratz & Straub, 2013) parasympathetic activation appears to be pro-inflammatory. Under such conditions, Major Basic Protein (released from invading eosinophils) and IgE are strongly upregulated (Froidure et al. 2016). The Major Basic Protein appears to antagonise the muscarinic M2 receptor brake on cholinergic nerve terminals causing increased acetylcholine (ACh) release (Fryer & Jacoby, 1998; Jacoby etal. 2001), whereas IgE upregulation appears to cause a change in the balance of nicotinic and muscarinic receptor expression in mast cells such that non-ACh reponders become responders (Kleij & Bienenstock, 2005; Masini *et al.* 1985; Cyphert *et al.* 2009). Consequently, ACh likely provides critical drive to the pro-inflammatory cytokine cascade (Kleij & Bienenstock, 2005; Cyphert *et al.* 2009; Mazzone & Undem, 2016). However, what causes parasympathetic activation in response to localized allergens or inflammation is not well understood. This may have significant importance for prevalent allergic and/or inflammatory diseases such as asthma.

The defining feature of allergen-induced asthma is the heightened sensitivity to an allergenic trigger (i.e. airway hyperresponsiveness; AHR), resulting in early- and late-phase responses (O'Byrne & Inman, 2003; Cockcroft & Davis, 2006). During the early phase (occurring within 15 min and lasting 1–2 h), inflammatory mediators produced by the lungs increase (Weersink *et al.* 1994). These asthmakines – predominantly lysophosphatidic acid (LPA) and asthma-associated prototypical Th2 cytokines including IL4, IL5, IL13 and CCL11 (eotaxin) (Kim *et al.* 2020) – likely contribute to the late-phase asthmatic response in which airways are invaded by granulocytes and lymphocytes (Toward & Broadley, 2004; Nabe *et al.* 2005; Gauvreau *et al.* 2015). The mechanisms causing the early- and late-asthmatic responses are likely multifaceted involving interaction between pulmonary immune cells and parasympathetic (vagal efferent) innervation as described above (Talbot *et al.* 2015; Drake *et al.* 2018).

Consistent with an essential role for parasympathetic innervation in asthma, we and others have shown that cervical vagotomy drastically suppresses acute asthmatic bronchoconstriction (Kesler & Canning, 1999; Wagner & Jacoby, 1999; Liu *et al.* 2014; McAlexander *et al.* 2015; Jendzjowsky *et al.* 2018). Indeed, parasympathetic nerve endings in the lung release ACh (Kleij & Bienenstock,

2005; Cyphert *et al.* 2009; Mazzone & Undem, 2016), substance P (Hunter & Undem, 1999), calcitonin gene-related peptide (Assas *et al.* 2014) and vasoactive intestinal peptide (Talbot *et al.* 2015); and receptors for these neurotransmitters are found in multiple cell types in the lung, including airway epithelium (Mazzone & Undem, 2016), fibroblasts (Kahler *et al.* 2001; Matthiesen *et al.* 2006), macrophages (Ichikawa *et al.* 1995; Koarai *et al.* 2012), neutrophils (Renshaw *et al.* 2009), eosinophils (Costello *et al.* 1997), T-lymphocytes (Blum *et al.* 1992; Tayebati *et al.* 1999), innate lymphoid cells (Talbot *et al.* 2015) and mast cells (Cyphert *et al.* 2009). Thus, the parasympathetic nervous system has both direct and indirect means of activating (and inactivating) airway smooth muscle and has potentially powerful influences over the cytokine cascades involved in other aspects of AHR (Mazzone & Undem, 2016). Although evidence suggests that the lung surveys its environment (Belvisi, 2002; Undem & Potenzieri, 2012), it appears then that immune responses to allergens are governed at least in part from outside the lung (Nadel & Widdicombe, 1962; Jordan, 2001; Mazzone & Canning, 2002; Habre *et al.* 2010; Fernández *et al.* 2011; Nardocci *et al.* 2015; Jendzjowsky *et al.* 2018).

The carotid bodies, the primary peripheral autonomic and respiratory oxygen sensors, are involved in a multitude of regulatory responses (Zera *et al.* 2019) and are vital ganglia which synapse onto vagal brainstem centres (Nadel & Widdicombe, 1962; Iscoe & Fisher, 1995; Jordan, 2001; Mazzone & Canning, 2002; Habre *et al.* 2010; Jendzjowsky *et al.* 2018), thus triggering AHR. Recently, we discovered that the carotid bodies play an important role in initiating allergen-induced parasympathetic activity, causing bronchoconstriction (Jendzjowsky *et al.* 2018). Importantly, we demonstrated that LPA activation of carotid body afferents stimulate parasympathetic nuclei in the brainstem, sufficient to cause bronchoconstriction. Further, carotid body denervation, vagotomy, and pharmacologic inhibition of LPA receptors (LPAr) reduced the increase in lung resistance associated with AHR in anaesthetized asthmatic (ovalbumin-sensitized; OVA) Brown Norway (BN) rats. The presence of a similar carotid body bronchoconstriction reflex in human asthma is likely because, in a mixed cohort of asthmatic and emphysema patients on ventilatory assistance, carotid body denervation caused an immediate reduction of airway resistance (Winter & Whipp, 2004). Nevertheless, significant knowledge gaps remain that impede translation, including the promiscuity of the carotid body to other asthmakines and the cellular signalling involved.

In this study, we set out to test the hypothesis that multiple asthmakines stimulate the carotid body via a PKCε–transient receptor potential vanilloid 1 (TRPV1) pathway. We report that multiple blood-borne asthma-associated prototypical Th2-cytokines along with LPA activate the carotid body–petrosal ganglia complex by a common PKCε–TRPV1-dependent pathway. Activation of TRPV1 by PKCε involves TRPV1 T704 and S502 sites, not S800. To test the utility of targeting this pathway for treatment, we further demonstrate that the systemic delivery of PKCε-blocking peptide in OVA BN rats reduces allergen-induced bronchoconstriction and AHR. Importantly, disruption of PKCε signalling within the carotid body does not alter the carotid bodies' sensitivity to oxygen, thereby preserving the potentially life-saving acute hypoxic ventilatory response. Thus, targeting PKCε may provide a new therapeutic option to treat asthmatic AHR.

Methods

Ethical approval

Experimental procedures were approved by the University of Calgary Animal Care and Use Committee (Protocol numbers AC15-0061 and AC16-0204) in accordance with the Canadian Council of Animal Care, were carried out according to the guidelines laid down by the University of Calgary Animal Care and Use Committee, conform to the principles and regulations as described by Grundy (2015) and comply with the ethics policies of *The Journal of Physiology*.

Animals

Male Brown Norway (BN/Crl, p28–35, 80–120 g) and Sprague Dawley rats (p28–38, 80–150 g) were purchased from Charles River (QC). Food and water were provided *ad libitum* and all rats were housed in a temperature (23°C) and humidity (30-50%) controlled room with a 12/12 light–dark cycle. Where appropriate, the use of rat strain is described in the experimental procedures below.

Chemicals and reagents

Oleoyl-lysophosphatidic acid (18:1 LPA), and εV1-2 (PKCε-inhibitor peptide) were purchased from Cayman Chemical (Cayman, Ann Arbor, MI). AMG9810 was purchased from Tocris Biosciences (Oakville, ON). Cytokines were purchased from the following vendors: IL4, IL13 and CCL11 (eotaxin; Cedarlane Burlington, ON), IL5 (R&D biosystems, Oakville, ON), IL1β (Prospecbio, East Brunswick, NJ), IL6 and TNFα (Millipore-Sigma, Oakville, ON). Ovalbumin, pertussis toxin, aluminium hydroxide, $MgSO_4$, NaH_2PO_4, KCl, $NaHCO_3$, NaCl, glucose, sucrose, $CaCl_2$, Tween 20, dimethyl sulfoxide,

pancuronium bromide, AM095 and, Ki16425 were purchased from Millipore-Sigma (Oakville ON).

Statistics and analysis

All statistical analysis was performed in SigmaPlot *vs*. 14.0 (Systat Software San Jose, CA). Normally distributed data were analysed using parametric two-sided statistical tests and presented as means ± SD; the Student–Newman–Keuls *post hoc* test was used which corrects for multiple comparisons. A P value <0.05 was considered statistically significant. Specific tests are indicated with appropriate experiments below.

Human embryonic kidney cells, site-directed mutagenesis and electrophysiology

Mutation of the rat TRPV1 residues (S502; T704; S800, cDNA obtained from Ardem Patapoutian, Scripps Research Institute) was done using site-directed mutagenesis with the QuikChange II site-directed mutagenesis kit (Agilent Santa Clara, CA) and QIAfilter Plasmid Mini kit (Qiagen) for DNA transfection. The mutations were verified by sequencing and sub-cloned into the original pcDNA3 plasmid to eliminate undesirable mutations elsewhere in the vector (Basso *et al*. 2019).

Human embryonic kidney (HEK) 2933 tsA-201 cells (ECACC Cat# 96121229, RRID:CVCL_2737), known to express a predominance of LPAr1 (Meyer zu Heringdorf *et al*. 2001), were grown to 80% confluence at 37°C (5% CO_2) in Dulbecco's modified Eagle's medium (+10% fetal bovine serum, 200 units ml^{-1} penicillin and 0.2 mg ml^{-1} streptomycin (Invitrogen, Carlsbad, CA, USA)). Cells were dissociated with trypsin (0.25%)-EDTA before plating on glass coverslips treated with 25% poly-ornithine and 2 μg ml^{-1} laminin (Millipore-Sigma, Oakville, ON). Cells were transfected with 1.5 μg of rat TRPV1 wild-type or mutant and 0.3 μg of GFP, as a transfection marker, using the calcium phosphate method (Iftinca *et al*. 2020). Cells were washed 8 h after transfection; electrophysiological recordings were conducted 24 h after transfection.

Whole-cell patch-clamp experiments on HEK cells transiently expressing either rat TRPV1 wild-type or mutants (S502A, T704A or S800G) were performed 16–24 h after transfection with calcium phosphate. The cells were placed in a 2 ml bath perfused with a solution of (in mM): 140.0 NaCl, 1.5 $CaCl_2$, 2.0 $MgCl_2$, 5.0 KCl, 10.0 Hepes, 10.0 D-glucose (pH 7.4 adjusted with NaOH) on the stage of an inverted epi-fluorescence microscope (Olympus IX51, Olympus America Inc., USA). HEK cells expressing the channel were identified via GFP fluorescence and were maintained at -60 mV throughout the recordings. Borosilicate glass (Harvard Apparatus Ltd., UK) pipettes were pulled and polished to 2–5 MΩ resistance with a DMZ-Universal Puller (Zeitz-Instruments GmbH., Martinsried, Germany). Pipettes were filled with an internal solution containing (in mM): 120.0 $CsCl_2$, 2.0 EGTA, 10.0 Hepes, 3.0 $MgCl_2$, 2.0 ATP Na_2, 0.5 GTP, pH 7.2 adjusted with CsOH. Patch-clamp experiments were performed using an Axopatch 200B amplifier (Molecular Devices Corp, Sunnyvale, CA) and pClamp 10.4 software was used for data acquisition and analysis (Axon Instruments). Data were filtered at 1 kHz (8-pole Bessel) and digitized at 10 kHz with a Digidata 1440 A converter (Axon Instruments). Average cell capacitance of recorded HEK cells was 27.25 ± 1.3 pF. Only the cells that exhibited a stable voltage control throughout the recording were used for analysis. All experiments were conducted at room temperature (22 ± 2°C). In the experiments using the εV1-2 (5 μM) PKCε inhibitor, cells were pre-incubated for 30 min at 37°C. To test the role of PKCε in the phosphorylation-dependent inhibition of TRPV1 tachyphylaxis (decreased response to capsaicin), we used a protocol of 5 s consecutive applications of saturating concentration of capsaicin (1 μM), 1 min apart (Lukacs *et al*. 2013). To assess the effect of LPA-mediated activation of PKCε on TRPV1 tachyphylaxis, we applied LPA (10 μM) for 60 s between the second and third applications of capsaicin. Mutants of TRPV1 in the PKC phosphorylation consensus sites were used to determine which phospho site was required for LPA-mediated inhibition of TRPV1 tachyphylaxis. Data analysis and offline leak subtraction were completed in Clampfit 10.4 (Axon Instruments). Wild-type analysis was compared with one-way ANOVA, mutant analyses were completed with unpaired t tests.

Asthmatic model

Brown Norway rats were sensitized to OVA (0.1 mg) with pertussis toxin (0.5 ng) and aluminium hydroxide as adjuvant (0.15 g) dissolved in saline (1 ml, OVA) for three consecutive days (Days 1, 2, and 3, I.P.) and challenged with 5% OVA (dissolved in 0.9% saline, 3 ml) and aerosolized on days 15, 18 and 21 for 10 min. The commercially acquired OVA was not tested for endotoxin but was purified by agarose gel electrophoresis (Sigma Millipore A5503). Therefore, it is possible that trace amounts of LPS may have been introduced with injection and aerosolization. Pertussis toxin was delivered as adjuvant alongside aluminium hydroxide as pertussis toxin augments the late-phase asthmatic response (Heuer *et al*. 1996), may suppress T_{reg} populations (Chen *et al*. 2006) as well as assist in increasing both Th2 and Th1 immune responses (Ryan *et al*. 1998; Hofstetter *et al*. 2002) giving an asthmatic phenotype resembling human asthma.

En bloc perfused carotid body preparation

Sprague Dawley rats were heavily anaesthetized with isoflurane-saturated air (animals were placed in a bell jar with gauze soaked in isoflurane). Once deeply anaesthetized the animal was killed by decapitation and exsanguination, then the carotid bifurcation, including the carotid body, carotid sinus nerve, and superior cervical ganglion, was quickly removed and transferred to a beaker (100 ml) containing carbogen (95% O_2, 5% CO_2) equilibrated physiological saline (1 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 4 mM KCl, 24 mM $NaHCO_3$, 115 mM NaCl, 10 mM glucose, 12 mM sucrose, and 2 mM $CaCl_2$). After ∼20 min, the carotid bifurcation was transferred to a recording chamber (A.R., custom made) and the common carotid artery was immediately cannulated for luminal perfusion with physiological saline (as above) with a peristaltic pump set at 15 ml min^{-1} to maintain a constant pressure of 100 mmHg in a custom recording chamber with a built-in water-fed heating circuit. The perfusate was equilibrated with computer-controlled gas mixtures of 100Torr PO_2 and 35Torr PCO_2 balanced with N_2 and recirculated throughout the experiments (yielding pH ∼7.4) and heated to 37 ± 0.5°C. The carotid sinus region was bisected, and the carotid sinus nerve was de-sheathed. Chemosensory discharge was recorded extracellularly from the whole de-sheathed carotid sinus nerve, hooked to a platinum electrode and lifted into a thin film of paraffin oil. A reference electrode was placed close to the carotid artery bifurcation. Nerve activity was monitored using a differential AC amplifier (model 1700, AM Systems), secondary amplifier (model AM502, Tektronix, Beaverton, OR), filtered (300 Hz low cut-off, 5 kHz high cut-off), displayed on an oscilloscope, rectified, integrated (200 ms time constant), and stored on a computer using an analogue-to-digital data acquisition system (Digidata 1322A, Axon Instruments; Axoscope 9.0). All preparations were exposed to a brief hypoxic (Hx, 60 Torr PO_2) challenge to determine viability. Preparations that failed to show at least a 50% increase in integrated nerve activity, as compared with the preceding baseline, during this challenge were discarded. After this challenge, preparations were left undisturbed for ∼30 min to stabilize before the experimental protocols described below.

Sprague Dawley Rats ($n = 66$):

1a) Infusion of vehicle (0.1% DMSO, 300 μl) then LPA (5 μM, dissolved in PBS);
1b) Infusion of Ki16425 (5 μM dissolved in 0.1% DMSO (Jendzjowsky *et al.* 2018)), then LPA (5 μM);
1c) Infusion of εV1-2 (5 μM, dissolved in 0.1% DMSO (Liron *et al.* 2007)), then LPA (5 μM), then Ki16425 (5 μM dissolved in DMSO);
1d) Infusion of AM095 (10 μM, dissolved in 0.1% DMSO (Ruisanchez *et al.* 2014)), then LPA (5 μM).

The concentration of DMSO used in this study (0.1%), does not affect baseline carotid sinus nerve activity or the hypoxic response (Roy *et al.* 2012).

2a) IL4 (0.5 nM, dissolved in PBS), IL13 (0.5 nM, dissolved in PBS), CCL11 (eotaxin, 0.18 nM, dissolved in PBS) were infused followed by AMG9810 (10 μM);
2b) εV1-2 (5 μM) was infused, followed by IL4 (0.5 nM), IL13 (0.5 nM), CCL11 (eotaxin, 0.18 nM, all dissolved in PBS) then AMG9810 (10 μM);
2c) IL4 (0.5 nM, dissolved in PBS) was infused followed by AMG9810 (10 μM);
2d) IL5 (0.15 nM, dissolved in PBS) was infused followed by AMG9810 (10 μM);
2e) IL13 (0.5 nM, dissolved in PBS) was infused followed by AMG9810 (10 μM);
2f) Eotaxin (0.18 nM, dissolved in PBS) was infused followed by AMG9810 (10 μM);
2g) IL1 (0.2 nM, dissolved in PBS), IL6 (0.8 nM, dissolved in PBS), TNFα (0.8 nM, dissolved in PBS) was infused followed by AMG9810 (10 μM).

Neural recordings were analysed offline using custom software (written in VEE by R.J.A.W.). The final 1 min of carotid sinus nerve activity during each condition was rectified, summed and expressed as integrated neural discharge. The neural responses for different conditions were normalized to the baseline (normoxic) condition. Differences between drug/cytokine conditions were analysed by one-way ANOVA, unpaired t test or paired t test where appropriate.

Lung mechanics and respiratory distress

Two cohorts of OVA-sensitized BN rats were used for these experiments (see above).

Cohort 1 ($n = 24$): To investigate the utility of systemic PKCε blockade in abrogating bronchoconstriction, OVA-sensitized rats were anaesthetized with isoflurane (5%, balance O_2) and instrumented for surgery. The femoral artery and vein were cannulated for the measurement of arterial pressure, and the infusion of intravenous anaesthetic, alfaxan (15 mg $kg\,min^{-1}$ I.V. delivered by syringe pump; Kent Scientific, Torrington CT) and the jugular vein was cannulated for the delivery of saline and drugs described below. Upon induction of I.V. anaesthetic, isoflurane administration was ceased, and the plane of anaesthesia was constantly monitored by pedal withdrawal reflex, and the stability of blood pressure and heart rate. The trachea was cannulated, and the rat was subsequently subject to neuromuscular blockade

Table 1. Cytokine concentration in asthma and healthy controls

Substance	Normal (pg ml^{-1})	Asthma (pg ml^{-1})	Stimulated asthma (pg ml^{-1})
IL1β (serum)[2, 4]	169.2 ± 17.2	1927.3 ± 3958.4	1465.7 ± 1741.6
IL4 (serum)[2,3,4]	8.5 ± 1.5	63.1 ± 26.9	67.9 ± 24.8
IL5 (serum)[2]	4.4 ± 1.1	69.1 ± 99	–
IL6 (serum)[2,3,4]	12.5 ± 1.5	62.5 ± 142.5	62.7 ± 70.9
IL13 (serum)[2,3,4]	11.5 ± 0.6	36.2 ± 75.5	43.9 ± 87.9
TNFα (serum)[2,3,4]	36 ± 6	262.8 ± 348.5	237.4 ± 217.1
Eotaxin (plasma)[1,2,3]	30.8 ± 6.2	350 ± 190	520 ± 250
Lysophosphatidic acid (plasma)[5]	1746.1 ± 170.2	2051.6 ± 279.4	3649.3 ± 733.4

Values are presented as means ± SD from serum or plasma (as indicated). Cytokine concentrations used in *en bloc* carotid body experiments are calculated from [1]Lilly *et al*. 1999; [2]Kokkonen *et al*. 2010; [3]Kleiner *et al*. 2013; [4]Meyer *et al*. 2014 and are representative of asthmatic human values (normal values are given for comparisons). Lysophosphatidic acid concentrations are taken from [5]Jendzjowsky *et al*. 2018 and represent concentrations from ovalbumin-sensitized asthmatic rats.

with pancuronium bromide (1 mg kg^{-1}, *ia*, dissolved in 0.9% saline) and attached to the Flexivent respirator system (SCIREQ, Montreal QC) for ventilation and measurement of airway mechanics. Upon administration of neuromuscular blockade, blood pressure and heart rate were assessed throughout the experiment where neuromuscular blockade was used to provide assurance on adequate depth of anaesthesia. Once surgical procedure and intervention (antagonist injection, below) were complete, rats were allowed to stabilize for 30 min while being ventilated. Bradykinin (0.4 mg) was nebulized for 45 breaths at 1, 10 and 20 min following an initial saline baseline challenge. Single frequency manoeuvres (Snapshot 90 measurement of whole lung resistance) were repeated five times during baseline and each bradykinin inhalation and the average of the five manoeuvres was taken to calculate total lung resistance (R_L) for each time point. OVA-sensitized rats were randomly separated into four different groups: vehicle (0.1% DMSO balance 0.9% saline, 300 μl), LPAr blockade (Ki16425; 5 mg kg^{-1} I.V. (Jendzjowsky *et al.* 2018), dissolved in 0.1% DMSO balance 0.9% saline, 300 μl), PKCε blockade (εV1-2; 7.5 mg·kg^{-1} I.V. (Inagaki Koichi *et al.* 2008), dissolved in 0.1% DMSO balance 0.9% saline, 300 μl), LPAr+ PKCε blockade (εV1-2; 7.5 mg·kg^{-1} + Ki16425; 5 mg·kg^{-1} I.V., 300 μl). Upon completion of experiments, animals were killed with an overdose of anaesthetic (alfaxan 50 mg·kg^{-1} I.V.) and exsanguination (terminal procedure). Absolute and normalized responses (normalized to the initial saline baseline challenge) to bradykinin were analysed using two-way repeated measures ANOVA (group × time).

OVA Cohort 2 ($n = 6$): To demonstrate the therapeutic effectiveness of the antagonist intervention following allergen provocation, OVA rats underwent separate plethysmograph experiments. Rats were OVA-sensitized as described above, but on day 15, following OVA exposure, rats were treated with saline; on days 18 and 21, aerosolized OVA was delivered for 10 min, followed 10 min later by PKCε antagonist (εV1-2, 7.5 mg·kg^{-1} dissolved in 0.1% DMSO balance 0.9% saline, 300 μl (Inagaki Koichi *et al.* 2008)) or TRPV1 antagonist (AMG9810 10 μmol·kg^{-1} dissolved in 0.1% DMSO, and then diluted with saline (Peles *et al.* 2009)) delivered I.P. (total volume ∼0.3 ml) randomized between days. Rats were then placed in a plethysmograph (Buxco, DSI systems Minneapolis, MN; total time after the beginning of aerosolization = 20 min) and inspiratory–expiratory time ratio (Ti:Te) and expiratory time (Te) were measured for 3 h in order to attain the early- and initial late-onset phase of asthmatic responses (Jendzjowsky *et al.* 2018). Upon completion of experiments, animals were killed with CO_2 inhalation and exsanguination. Five-minute averages of each variable were calculated. Data were analysed using a two-way repeated measures ANOVA (time × group).

Results

The response of the carotid body to asthmakines

Previously, we demonstrated that the carotid body responds vigorously to LPA at a concentration congruent with that in asthmatic arterial plasma (Jendzjowsky *et al.* 2018). However, asthma causes an increase in the plasma concentrations of other asthma-associated prototypical Th2 cytokines (IL4, IL5, IL13 and eotaxin) and several non-Th2 inflammatory cytokines (IL1, IL6, TNFα; Table 1). As receptors for these cytokines are likely to be expressed in the carotid body (as reported in a supplementary RNAseq dataset of carotid bodies (Chang *et al.* 2015)), we investigated whether these additional asthmakines also stimulate naïve carotid bodies. Using the perfused *en bloc* carotid body preparation (Cummings & Wilson, 2005; Roy *et al.* 2012) (Fig. 1*A*) and concentrations reported in

asthmatic blood (Table 1), we found that a cocktail of prototypical Th2 cytokines was almost as efficacious as LPA (Fig. 1*B*) at stimulating the carotid sinus nerve. Moreover, individual cytokines also induced substantive responses (eotaxin > IL5 > IL4 > IL13; Fig. 1*B*). A cocktail of asthma-associated non-Th2 cytokines also stimulated the carotid body but were less efficacious (Fig. 1*B*).

Interaction between LPA receptors and TRPV1 requires PKCε

To get a handle on the cellular mechanisms by which inflammatory mediators stimulate the carotid body, we first focused on LPA because it produces the most robust response (Figs 1*B* and 2*A*, *F*) and previously we demonstrated that these responses were dependent on TRPV1 (Jendzjowsky *et al.* 2018). First, we established which of the LPAr present in the carotid body (Chang *et al.* 2015; Jendzjowsky *et al.* 2018) are responsible for carotid body excitation. We deduced that LPAr1 is likely the most important because it has been most associated with TRPV1 signalling (Pan *et al.* 2010; Wang *et al.* 2015; Koda *et al.* 2016) and is highly expressed in the carotid body (Chang *et al.* 2015; Jendzjowsky *et al.* 2018). Consistent with this, the LPAr1&3-specific antagonist (Ki16425, 5 μM (Jendzjowsky *et al.* 2018); Fig. 2*B*, *E* and *F*) and LPAr1-specific antagonist (AM095, 10 μM (Ruisanchez *et al.* 2014), Fig. 2*C* and *F*) blunt the response to LPA and AMG9810 does not further significantly reduce the response to LPA following LPAr1&3 blockade (Fig. 2*E* and *F*). As LPAr1 activates PKCε (Pan *et al.* 2010; Wang *et al.* 2015; Koda *et al.* 2016) and TRPV1 is phosphorylated by PKCε (Pan *et al.* 2010; Wang *et al.* 2015; Koda *et al.* 2016), we tested whether the effects of LPA were blocked by the potent PKCε-inhibitor peptide (εV1-2, 5 μM (Inagaki Koichi *et al.*, 2008), Fig. 2*D* and *F*).

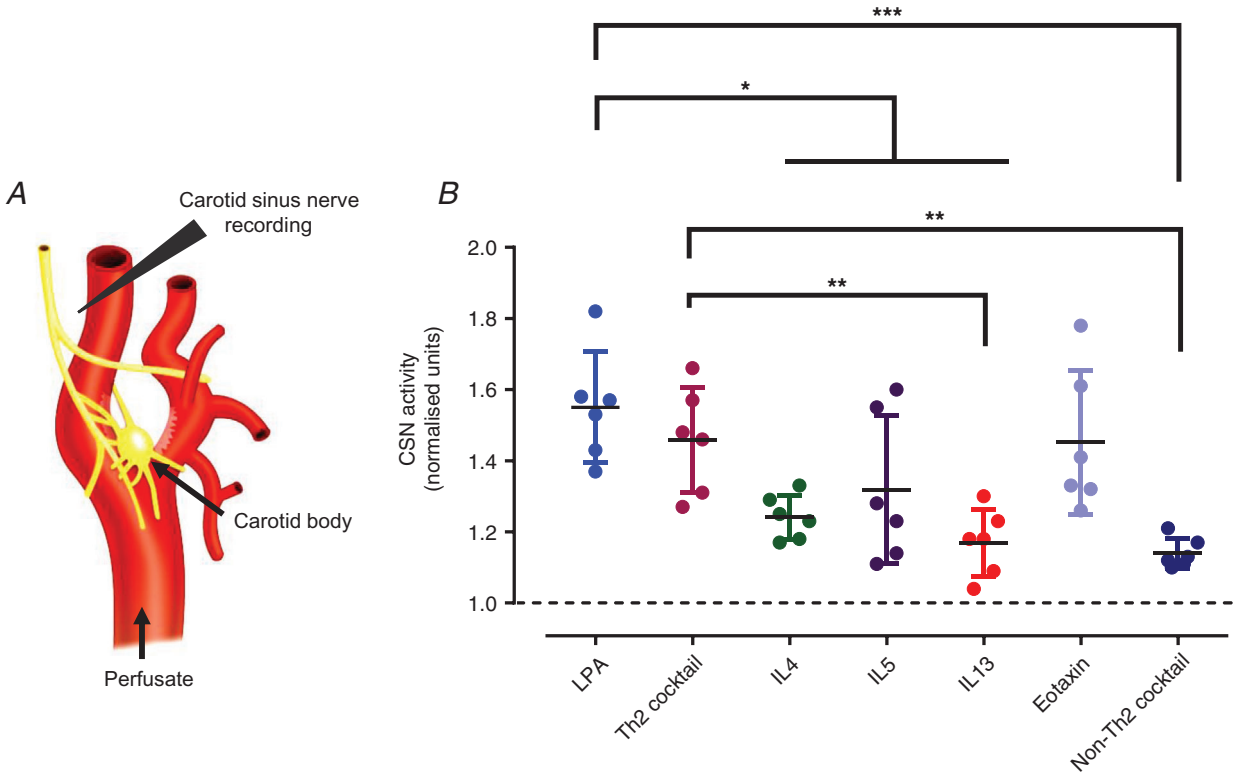

Figure 1. Asthmakines stimulate the carotid body
A, the *en bloc* perfused carotid body preparation was used to record chemosensory afferents in the carotid sinus nerve (CSN) from naïve Sprague Dawley rats (Cummings & Wilson, 2005; Roy *et al.* 2012). *B*, lysophosphatidic acid (LPA 18:1, 5 μM), asthma-associated prototypical Th2 cytokine cocktail (IL4, 0.5 nM; IL13, 0.5 nM; eotaxin, 0.18 nM) individual Th2 cytokines of IL4 (0.5 nM), IL5 (0.15 nM), IL13 (0.5 nM), eotaxin (0.18 nM), or the asthma-associated non-Th2 cytokine cocktail (IL1, 0.2 nM; IL6, 0.8 nM; TNFα, 0.8 nM) stimulate the carotid body with differing effect. Summary data, $n = 6$/group: one-way ANOVA $F_{6,41} = 7.200$, $P < 0.0001$. Student–Newman–Keuls *post hoc*: LPA *vs*: Th2 cocktail $P = 0.278$, IL4 $P = 0.006$*, IL5 $P = 0.041$*, IL13 $P < 0.001$*, eotaxin $P = 0.471$, non-Th2 cocktail $P < 0.001$***; Th2 cocktail *vs*: IL4 $P = 0.061$, IL5 $P = 0.226$, IL13 $P = 0.012$**, eotaxin $P = 0.937$, non-Th2 cocktail $P = 0.006$**. CSN activity is normalized to baseline activity. Data are presented as means ± S.D. [Colour figure can be viewed at wileyonlinelibrary.com]

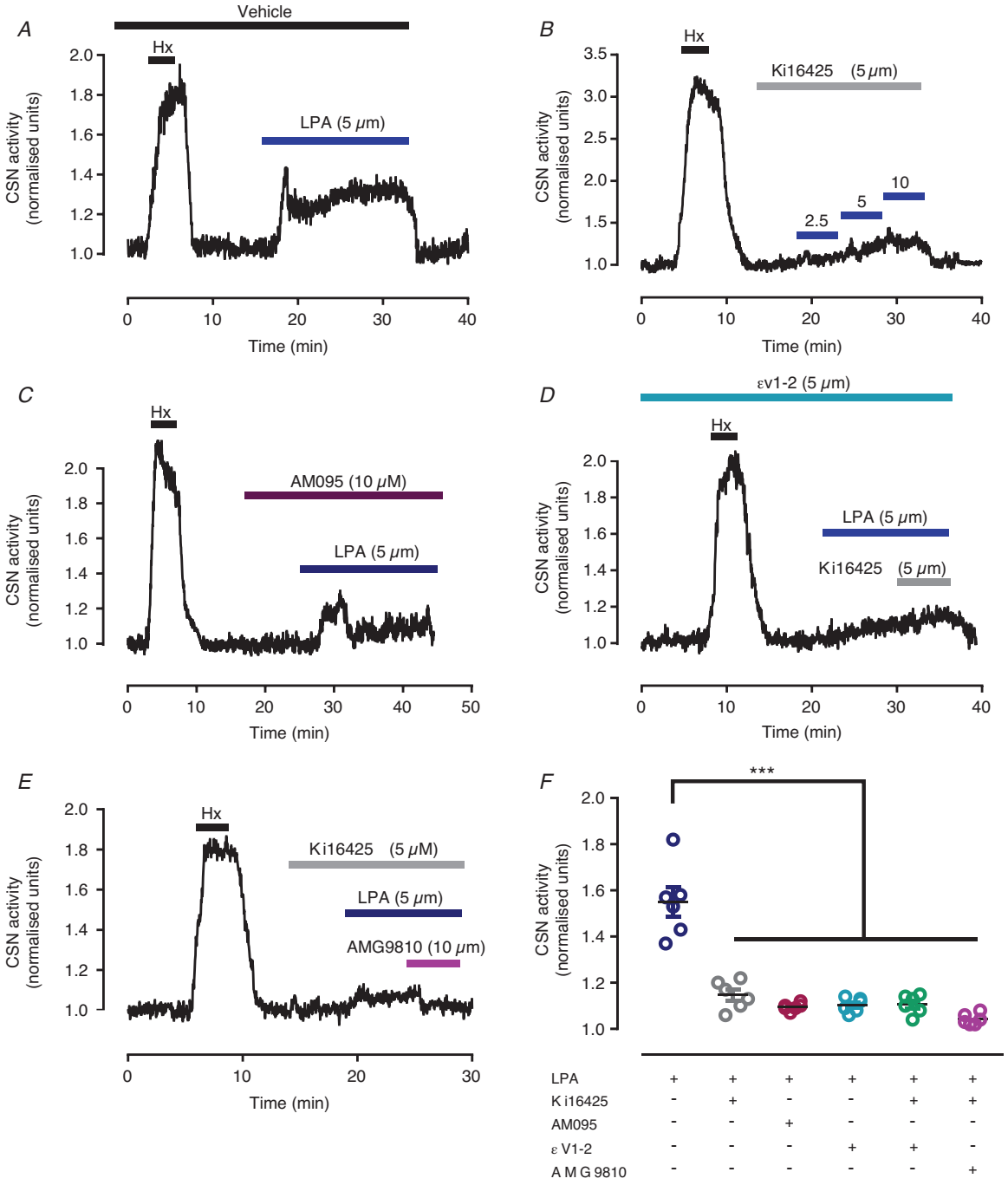

Figure 2. LPA-mediated carotid body excitation occurs via PKCε excitation of TRPV1 in carotid bodies from naïve Sprague Dawley rats
A, lysophosphatidic acid (LPA) increases carotid sinus nerve (CSN) activity when delivered in conjunction with dimethylsulfoxide (DMSO, vehicle). *B*, CSN activity in response to LPA dose response is reduced by LPAr1&3 blockade (Ki16425, 5 μM) pretreatment. *C*, the response to LPA is equally reduced by pretreatment with LPAr1-specific blockade (AM095, 10 μM). *D*, pretreatment with PKCε inhibitor peptide (εV1-2, 5 μM) reduces carotid body stimulation in response to LPA, which is not further suppressed by LPAr1&3 blockade (Ki16425). Hx = hypoxia test of viability. *E*, pre-treatment with LPAr1&3 blockade (Ki16425, 5 μM) reduces carotid body stimulation in response to LPA, which is marginally suppressed by TRPV1 blockade (AMG9810 10 μM). Hx = hypoxia test of viability. F, Summary data of responses to LPA (comparing 5 μM responses from each preparation; n = 6/group) one-way ANOVA $F_{5,35} = 39.957$ $P < 0.0001$. Student–Newman–Keuls *post hoc*: LPA *vs*: Ki16425 $P < 0.001$***, AM095 $P < 0.001$***, εV1-2 $P < 0.001$***, εV1-2+Ki16425 $P < 0.001$***, Ki16425+AMG9810 $P < 0.001$***. CSN activity is normalized to baseline activity. Data are presented as means ± SD. [Colour figure can be viewed at wileyonlinelibrary.com]

The reduction in the carotid body's response to LPA with the PKCε-inhibitor peptide was comparable to the LPAr antagonists (Fig. 2*F*), suggesting that a PKCε-dependent pathway dominates the interaction between LPAr1 and TRPV1.

Regulation of TRPV1 by PKCε

Given that PKCε likely mediates LPA's effects on TRPV1, we probed the site of regulation. Using HEK cells expressing TRPV1 mutants lacking one of the three intracellular phosphorylation sites, S800G, T704A, S502A, we quantified the effects of LPA on TRPV1 desensitized by repeated bouts of capsaicin (Fig. 3); channel phenotype did not affect the capsaicin desensitization (Fig. 3*D*–*G*). As expected, cells expressing wild-type TRPV1 channels exhibited pronounced inward currents in response to LPA (10 μM) overcoming capsaicin-induced desensitization (Fig. 3*B* and *G*). In the S800 mutant, LPA had a similar effect as in the wild-type (Fig. 3*D* and *G*). However, in the T704 (Fig. 3*E* and *G*) and S502 (Fig. 3*F* and *G*) mutants, or wild-type treated with the PKCε inhibitor peptide (εV1-2, 5 μM, Fig. 3*C* and *E*), LPA's restorative effect was abolished. These data suggest that LPA's effects are mediated by PKCε phosphorylation of TRPV1 at T704 and S502 and demonstrate the efficacy of the PKCε-inhibitor peptide (Fig. 3).

Th2 cytokines also stimulate the carotid body via a PKCε- and TRPV1-dependent pathway

Having demonstrated that asthma-associated Th2 cytokines delivered at physiological concentrations (in combination (Figs 1*B* and 4) or separately (Fig. 5)) strongly augment carotid body excitation, we reasoned that they might also employ the PKCε–TRPV1 pathway. The pore-blocking TRPV1 antagonist (AMG9810, 10 μM (Gavva *et al.* 2005)) diminished the excitation elicited by the Th2 cocktail (Fig. 4*A* and *B*) and individual cytokines (Fig. 5). Importantly, PKCε inhibitor peptide pretreatment (εV1-2, 5 μM; Fig. 4*C* and *D*) also diminished excitation to the Th2 cocktail. Interestingly, subsequent TRPV1 antagonist delivery caused a further reduction in Th2 cytokine-induced activity, suggesting some TRPV1 activation also occurs via a non-PKCε-dependent pathway (Fig. 4*C* and *E*). In contrast, asthma-associated concentrations of non-Th2 cytokines produce only mild carotid body stimulation (Figs 1*B* and 4*F*, G), consistent with previous findings (Shu *et al.* 2007; Fan *et al.* 2009; Fernández *et al.* 2011). Nonetheless, non-Th2 cytokine-induced excitation was also reduced by the TRPV1 antagonist (AMG9810, 10 μM; Fig. 4*F* and *G*).

In summary, multiple asthma-associated cytokines activate the carotid body, consistent with a role for the carotid body in sensing inflammation and mediating systemic autonomic effects. This suggests that the carotid body's immune-sensing capability is a potential target for pharmaceutical intervention in inflammatory and allergic disease.

The role of the carotid body PKCε–TRPV1 pathway in asthma

Given that we used concentrations of asthma-associated Th2 cytokines reported in asthmatic plasma (Lilly *et al.* 1999; Kokkonen *et al.* 2010; Kleiner *et al.* 2013; Meyer *et al.* 2014; Akiki *et al.* 2017) (Table 1), and the carotid bodies produce bronchoconstriction (Nadel & Widdicombe, 1962; Iscoe & Fisher, 1995; Mazzone & Canning, 2002; Habre *et al.* 2010; Jendzjowsky *et al.* 2018), we suggest that multiple asthmakines are capable of activating the PKCε–TRPV1 pathway, and – in different circumstances or disease states – different combinations may trigger the carotid body bronchoconstriction reflex.

Bolstered by our *ex vivo* data suggesting PKCε involvement in the carotid body sensing inflammatory mediators, we next tested whether systemic PKCε blockade abrogates AHR. We used anaesthetized artificially ventilated *in vivo* preparations to test a role for the PKCε-signalling in asthmatic OVA BN rats by measuring lung function with the Flexivent system. Previously, using this model, we showed that bradykinin increased lung resistance in asthmatic BN rats; and this response was significantly reduced by severing the afferent (carotid body) arm of the reflex, efferent arm of the reflex (vagus), LPAr blockade, TRPV1 blockade or combined LPAr+TRPV1 blockade (Jendzjowsky *et al.* 2018). Here we report additional experiments (see protocol in Fig. 6*A*) demonstrating that the systemic delivery of LPAr1&3 blocker (Ki16425; 5 mg kg^{-1} I.V. (Jendzjowsky *et al.* 2018)), PKCε-inhibitor peptide (εv1-2. 7.5 mg·kg^{-1} I.V. (Inagaki Koichi *et al.*, 2008)), or combined pretreatment diminished the bradykinin-induced increase in lung resistance to the same degree (Fig. 6*B* and *C*). These data suggest that PKCε along with LPAr1&3 play a significant role in mediating bronchoconstriction.

Allergen-induced changes in breathing pattern are PKCε- and TRPV1-dependent

To determine whether PKCε acts through TRPV1 to mediate allergen-induced respiratory difficulty in conscious animals we measured expiratory duration (Te) and the inspiratory–expiratory duration ratio (Ti:Te) following allergen challenge in conscious OVA BN rats using whole-body plethysmography (Fig. 6*D*). Ten minutes following allergen exposure, Te was reduced equally in rats injected with PKCε-inhibitor peptide

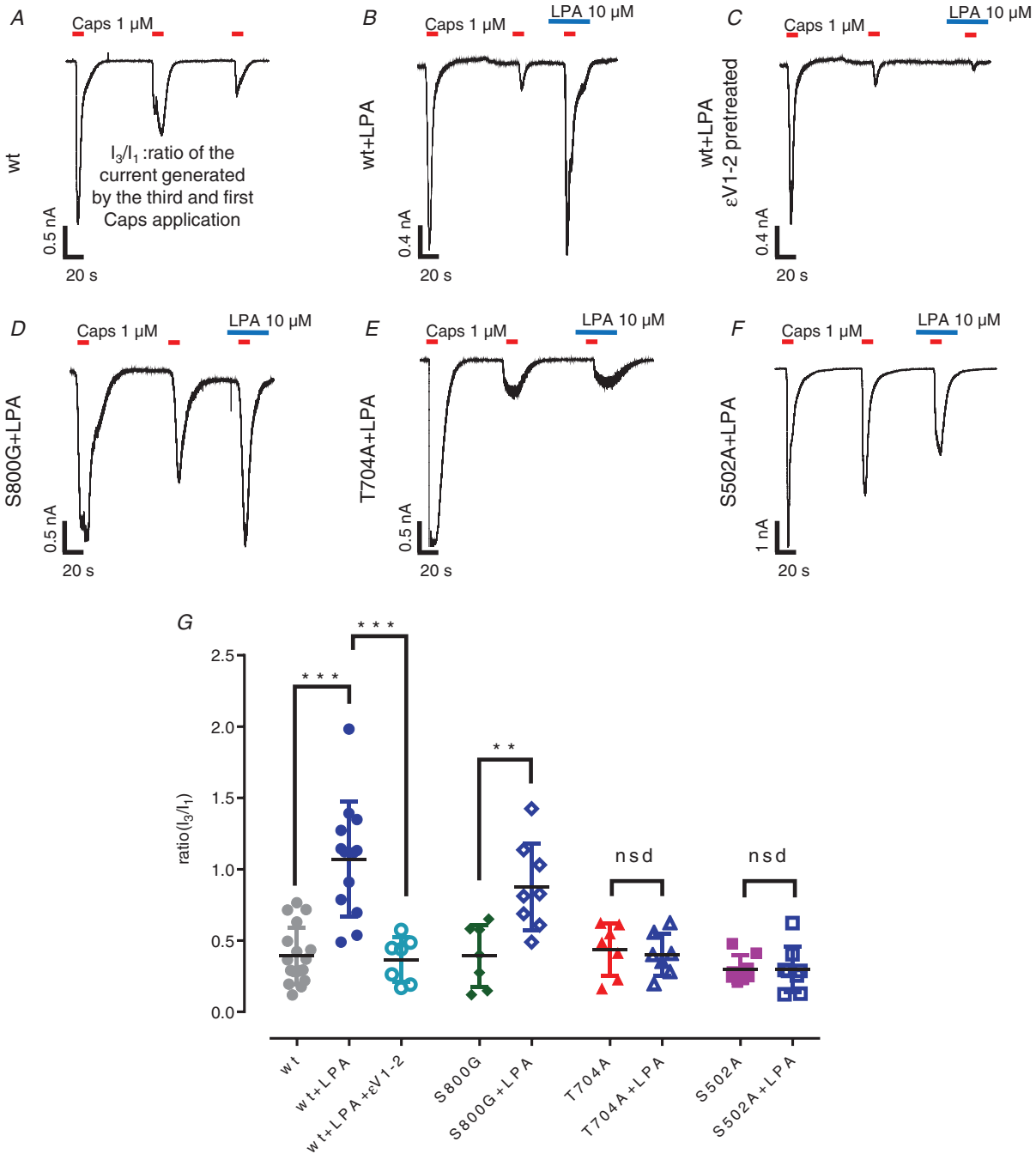

Figure 3. LPA-mediated potentiation of TRPV1 occurs via PKCε-dependent phosphorylation of S502 and T704 in human embryonic kidney cells
A, repeated capsaicin application (Caps, 1 μM) desensitizes TRPV1 function. *B*, lysophosphatidic acid (LPA, 10 μM) restores TRPV1 function after Caps desensitization. *C*, PKCε-inhibitor peptide (εV1-2, 5 μM) abolishes the LPA-mediated block of TRPV1 desensitization. *D*, mutation of the S800 residue to G does not compromise the potentiation of TRPV1 by LPA. *E*, mutation of the T704 residue to A inhibits the potentiation of TRPV1 by LPA. *F*, mutation of the S502 residue to A inhibits the potentiation of TRPV1 by LPA. *G*, summary data of LPA-mediated potentiation of TRPV1 (ratio of the current generated by the third and first Caps application, I_3/I_1): One-way ANOVA $F_{2,35} = 25.625$, $P < 0.0001$ wt ($n = 18$) *vs.* wt+LPA ($n = 13$) *vs.* wt+LPA+εV1-2 ($n = 7$), Student–Newman–Keuls *post hoc*: wt *vs.* wt+LPA $P < 0.001$***, wt *vs.* wt+LPA+εV1-2 $P = 0.841$, wt+LPA *vs.* wt+LPA+εV1-2 $P < 0.001$***. Summary data unpaired *t* test: $t_{13} = 3.479$ (S800G ($n = 7$) *vs.* S800G+LPA ($n = 8$)) $P = 0.004$**. Summary data unpaired *t* test: $t_{12} = 0.410$ (T704A ($n = 7$) *vs.* T704A+LPA ($n = 7$)) $P = 0.689$. Summary data unpaired *t* test: $t_{14} = 0.00167$ (S502A ($n = 8$) *vs.* S502A+LPA ($n = 8$)) $P = 0.999$. Abbreviation: nsd, no significant difference. Data are presented as means ± SD. [Colour figure can be viewed at wileyonlinelibrary.com]

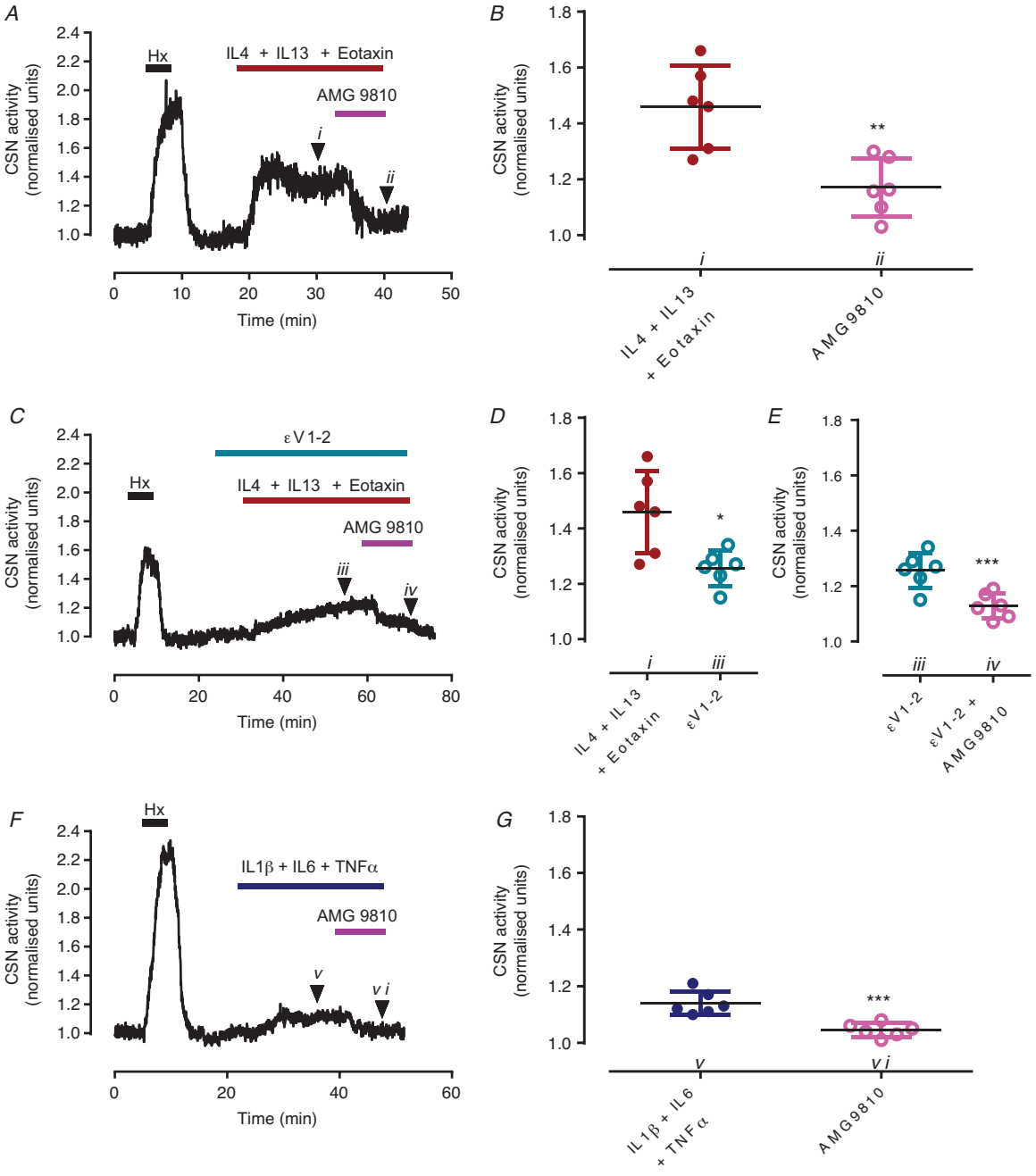

Figure 4. Th2-related cytokines augment carotid body excitation through PKCε phosphorylation of TRPV1 in carotid bodies from naïve Sprague Dawley rats
A, asthma-associated Th2 cytokines (IL4, 0.5 nM; IL13, 0.5 nM; eotaxin, 0.18 nM) significantly augment carotid sinus nerve (CSN) activity (time point of measurement – *i*, as indicated by arrowhead) which was suppressed by AMG9810 (TRPV1 antagonist, 10 μM, time point of measurement – *ii*, as indicated by arrowhead). *B*, summary data: Th2 (*i*) *vs.* AMG9810 (*ii*) paired *t* test $T_5 = 4.730$ $P = 0.0052$**. *C*, Th2 cytokine-mediated carotid body excitation is suppressed by PKCε inhibitor peptide (εV1-2, 5 μM, time point of measurement – *iii*, as indicated by arrowhead), and further suppressed by AMG9810 (TRPV1 antagonist, 10 μM, time point of measurement – iv, as indicated by arrowhead). *D*, summary data $n = 6$/group: Th2 (*i*) *vs.* PKCε inhibitor peptide + Th2 (*iii*) unpaired *t* test $T_{10} = 3.047$, $P = 0.0123$*. *E*, summary data $n = 6$/group: PKCε inhibitor peptide + Th2 (*iii*) *vs.* PKCε-inhibitor peptide + Th2 + AMG9810 (I.V.) paired *t* test $T_5 = 9.164$, $P = 0.0003$***. *F*, asthma-associated non-Th2 cytokines (IL1, 0.2 nM; IL6, 0.8 nM; TNFα, 0.8 nM) demonstrate dearth CSN excitation (time point of measurement – *v*, as indicated by arrowhead) which was suppressed by AMG9810 (10 μM, time point of measurement – *vi*, as indicated by arrowhead). *G*, summary data $n = 6$/group: Non-Th2 (*v*) *vs.* AMG9810 (*vi*) paired *t* test $T_5 = 8.497$ $P = 0.0004$***. Carotid sinus nerve activity is normalized to baseline activity. Data are presented as means ± S.D. [Colour figure can be viewed at wileyonlinelibrary.com]

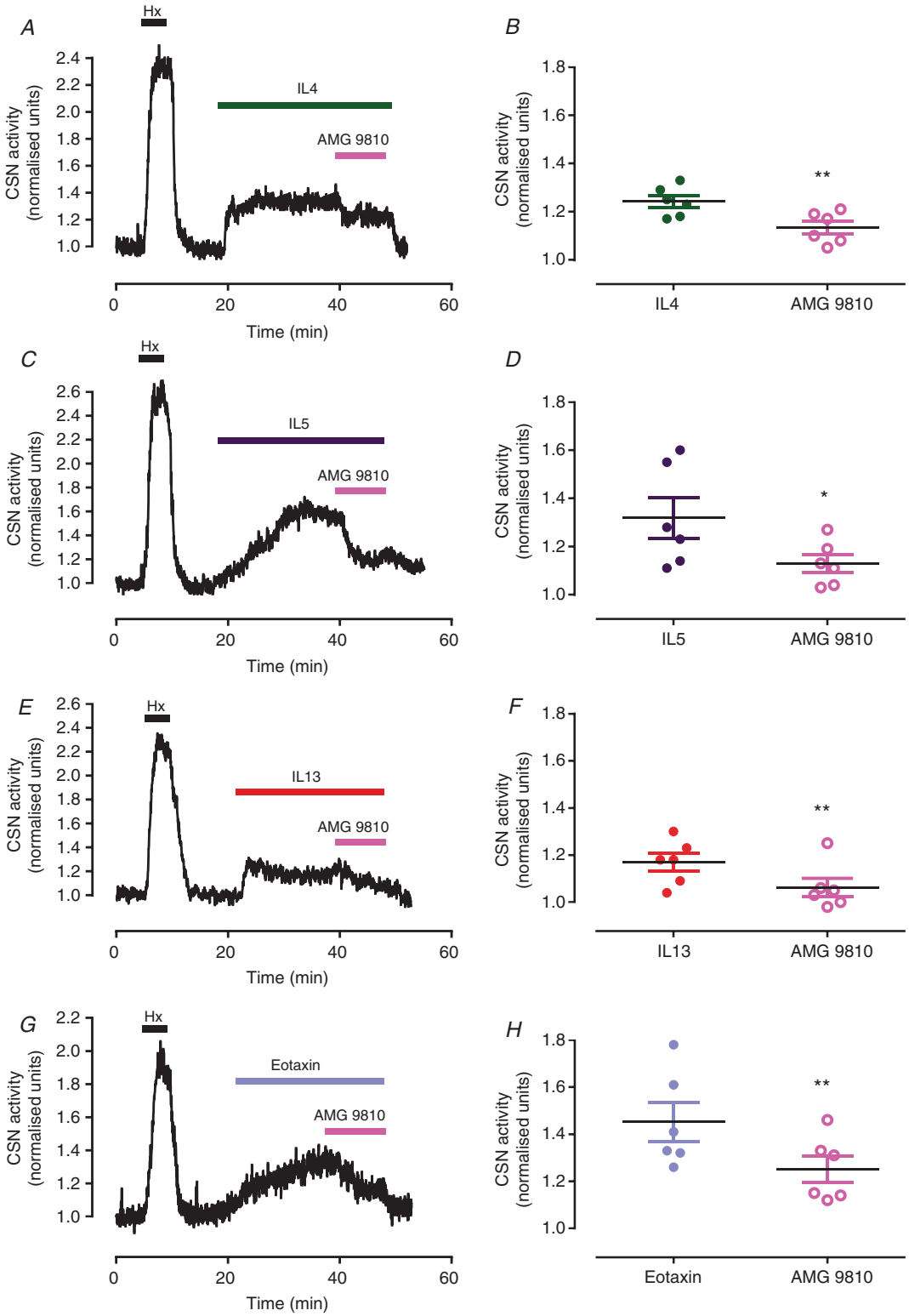

Figure 5. Carotid sinus nerve excitation in response to individual Th2 cytokines
All panels $n = 6$. *A*, IL4-induced carotid sinus nerve (CSN) excitation is reduced by AMG9810. *B*, paired *t* test $T_5 = 9.777$ $P = 0.00019$**. *C*, IL5-induced CSN excitation is reduced by AMG9810. *D*, paired *t* test $T_5 = 3.780$ $P = 0.0129$*. *E*, IL13-induced CSN excitation is reduced by AMG9810. *F*, paired *t* test $T_5 = 4.364$ $P = 0.00726$**. *G*, eotaxin-induced CSN excitation is reduced by AMG9810. *H*, paired *t* test $T_5 = 5.270$ $P = 0.00327$**. (Hx = hypoxia test of viability). Data are presented as means ± SD. [Colour figure can be viewed at wileyonlinelibrary.com]

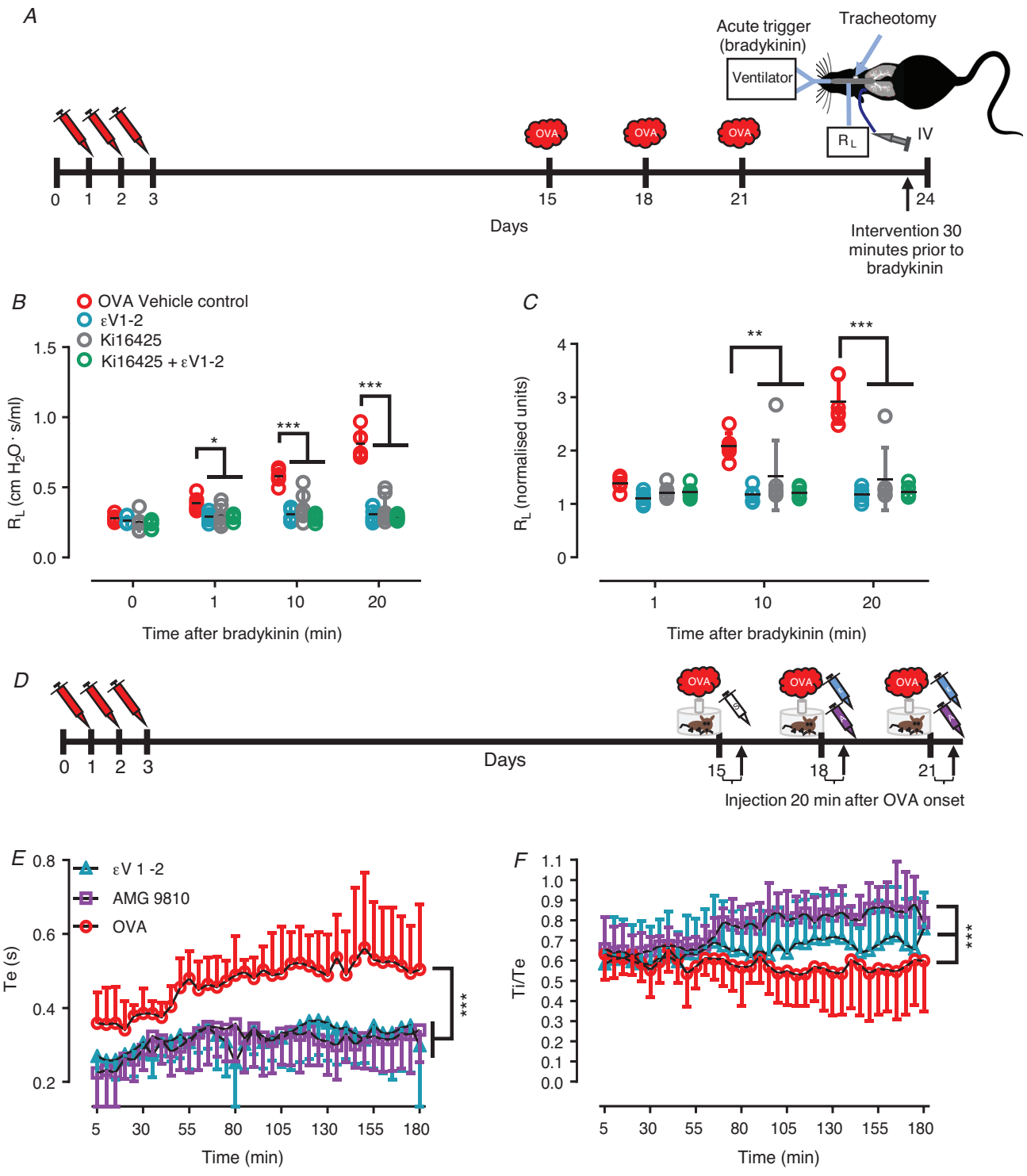

Figure 6. Carotid body-mediated lung resistance is mediated by PKCε excitation of TRPV1
A, ovalbumin sensitization and challenge (OVA) protocol (see Methods, *OVA Cohort 1*) to test carotid body–bronchoconstricting pathway in Brown Norway (BN) rats. OVA and naïve BN rats were exposed to nebulized saline (baseline) and three consecutive 45 breath nebulizations of 0.4 mg bradykinin at 1, 10 and 20 min while measuring R_L. *B*, bradykinin had group-specific effects: Absolute R_L, two-way repeated measures ANOVA $F_{3,20}$ (condition) = 41.585 $P < 0.001$, $F_{3,60}$ (time) = 67.342 $P < 0.001$, $F_{9,95}$(condition × time) 29.447 $P < 0.001$. Student–Newman–Keuls *post hoc*: bradykinin caused a marked increase in R_L compared with baseline (0 time point) in OVA (vehicle-treated, red, $n = 6$) which was reduced by PKCε-inhibitor peptide (εV1-2, 7.5 mg·kg^{-1} I.V., teal, $n = 6$; 0: $P = 0.603$; 1: $P = 0.020$*; 10: $P < 0.001$***; 20: $P < 0.001$***), LPAr1&3 blockade (Ki16425,

5 mg·kg^{-1}I.V., grey, $n = 6$; 0: $P = 0.638$; 1: $P = 0.018$*; 10: $P < 0.001$***; 20: $P < 0.001$***) and combined PKCε-inhibitor peptide + LPAr1&3 blockade (εV1-2+Ki16425, mint $n = 6$; 0: $P = 0.553$; 1: $P = 0.024$*; 10: $P < 0.001$***; 20: $P < 0.001$***). *C*, R_L normalized to saline nebulization two-way repeated measures ANOVA $F_{3,20}$ (condition) = 22.194 $P < 0.001$, $F_{2,40}$ (time) = 22.986 $P < 0.001$, $F_{6,71}$ (condition × time) = 14.258 $P < 0.001$. Student–Newman–Keuls *post hoc*: bradykinin caused a marked increase in R_L compared with baseline (0 time point) in OVA (vehicle-treated, red, $n = 6$) which was reduced by PKCε-inhibitor peptide (εV1-2, 7.5 mg·kg^{-1}I.V., teal, $n = 6$; 1: $P = 0.423$; 10: $P < 0.001$**; 20: $P < 0.001$**), LPAr1&3 blockade (Ki16425, 5 mg·kg^{-1}I.V., grey, $n = 6$; 1: $P = 0.617$; 10: $P = 0.003$**; 20: $P < 0.001$**) and combined PKCε-inhibitor peptide + LPAr1&3 blockade (εV1-2+Ki16425, mint, $n = 6$; 1: $P = 0.375$; 10: $P < 0.001$**; 20: $P < 0.001$**). *D*, OVA-sensitization protocol (see Methods, *OVA Cohort 2*). *E*, expiratory time (Te); two-way repeated measures ANOVA $F_{2,540}$ (group) = 196.4, $P < 0.0001$, $F_{35,540}$ (time) = 2.911, $P < 0.0001$, $F_{70,540}$ (group × time) = 0.6206, $P = 0.9928$; Student–Newman–Keuls *post hoc*: $P < 0.0001$***, difference between OVA (red, $n = 6$) and TRPV1 blockade (AMG9810, 10 μM·kg^{-1}I.P., purple, $n = 6$) and OVA (red) and PKCε-inhibitor peptide (εV1-2, 7.5 mg·kg^{-1}I.P., teal, $n = 6$) treatment conditions. *F*, inspiratory:expiratory time (Ti:Te); two-way repeated measures ANOVA $F_{2,540}$ (group) = 64.69, $P < 0.0001$, $F_{35,540}$ (time) = 0.7876, $P = 0.805$, $F_{70,540}$ (group × time) = 0.6787, $P = 0.978$; Student–Newman–Keuls *post hoc*: $P < 0.0001$***, different between all treatment conditions. Data are presented as means ± S.D. [Colour figure can be viewed at wileyonlinelibrary.com]

(εV1-2, 7.5 mg·kg^{-1} I.P. (Inagaki Koichi *et al.*, 2008)) or the TRPV1-blocker (AMG9810, 10 μM kg^{-1} I.P. (Peles *et al.* 2009)) compared with vehicle-injected rats (DMSO, 300 μl; see OVA in Fig. 6*E*); and both PKCε-inhibitor peptide and TRPV1-blockade groups had improved Ti:Te (Fig. 6*F*). Of note, the TRPV1 blockade-treated rats had slightly improved Ti:Te compared with PKCε-inhibitor peptide-treated rats, suggesting that some TRPV1 activation involved in the regulation of Ti occurs independently of PKCε in conscious animals. Nonetheless, these data support a major role for the PKCε–TRPV1 signalling pathway in bronchoconstriction and respiratory difficulty following asthmatic provocation.

The PKCε–TRPV1 pathway is not involved in oxygen sensing

The carotid body is the primary arterial oxygen sensor, triggering cardiorespiratory and arousal responses during severe hypoxia and/or asphyxia. Any surgical procedure or pharmaceutical that compromises this vital function of the carotid body is likely to be short-lived. Therefore, we tested whether the PKCε–TRPV1 pathway affects carotid body oxygen sensing. Importantly, the carotid body's hypoxic response is unchanged by the PKCε-inhibitor peptide (εV1-2 5 μM; Fig. 7). These critical data add to our previous finding that the TRPV1 antagonist AMG9810 does not affect carotid body responses to hypoxia-hypercapnia (Roy *et al.* 2012, 2018), and demonstrate that targeting the PKCε–TRPV1 pathway will not degrade the essential role of the carotid bodies as blood gas chemoreceptors.

Discussion

Carotid body activation of the autonomic nervous system can play a decisive role in the regulation of lung resistance (Nadel & Widdicombe, 1962; Iscoe & Fisher, 1995; Jordan, 2001; Mazzone & Canning, 2002; Habre *et al.* 2010; Jendzjowsky *et al.* 2018). Previously, we showed that the carotid body-stimulated parasympathetic bronchoconstriction reflex is particularly important in asthma and demonstrated that this activation is caused in part by an allergen-induced increase in circulating LPA (Jendzjowsky *et al.* 2018). Here we show that other asthmakines, namely prototypical Th2 cytokines, also strongly stimulate the carotid body and that both LPA and Th2 cytokines stimulate the carotid body through a common PKCε–TRPV1 pathway. This discovery, coupled with the fact that the autonomic nervous system modulates the function of almost every organ in the body (Zera *et al.* 2019), demonstrates that the carotid bodies must play a significant role in autonomic orchestration of local and/or systemic responses to inflammation in general.

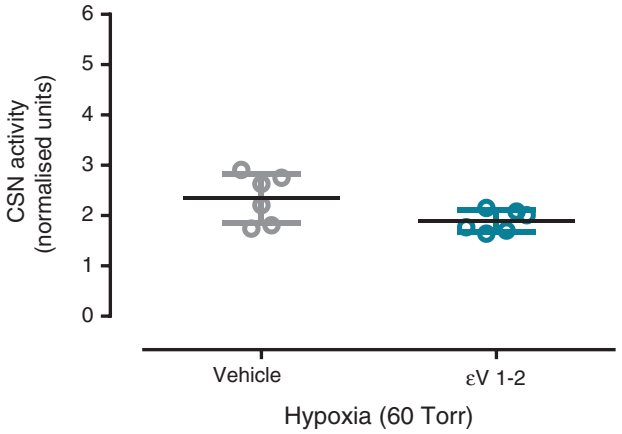

Figure 7. Carotid sinus nerve excitation in response to hypoxia
PKCε-inhibitor peptide (εV1-2, 5 μM, $n = 6$) does not suppress the hypoxic response to 60 Torr O_2 in Sprague Dawley rat carotid bodies treated with vehicle ($n = 6$), unpaired *t* test $T_{10} = 2.036$ $P = 0.07$. Data are presented as means ± SD. [Colour figure can be viewed at wileyonlinelibrary.com]

Spurred by the general applicability of this work, we investigated the intracellular mechanism by which asthmakines stimulate the carotid body. As with LPA, we discovered that the carotid body excitation by asthma-associated Th2 cytokines likely works through a TRPV1 pathway, similar to allergic pruritus (Oetjen *et al.* 2017), and demonstrate that these effects involve PKCε, probably via TRPV1 sensitization by phosphorylation at T704 and S502. Congruent with these data, we show for the first time that pharmacological inhibition of PKCε decreases allergen-induced bronchoconstriction in the OVA BN rat. Importantly, this inflammation-dependent PKCε–TRPV1 pathway is independent of carotid body oxygen sensitivity. Therefore, targeting this pathway may be a viable option to treat asthmatic bronchoconstriction and other inflammatory and/or allergen-directed diseases.

The present study aimed to investigate the intracellular mechanism of asthmakine-mediated signalling in the carotid body, critical for triggering asthmatic attacks. In pain (Pan *et al.* 2010; Koda *et al.* 2016) and pruritus (Wang *et al.* 2015) pathways, phospholipid-mediated stimulation of dorsal root ganglia C-fibres involves PKCε co-localizing with TRPV1. This is reported to involve TRPV1 phosphorylation at S800 (Koda *et al.* 2016) increasing channel open state probability (Mandadi *et al.* 2006; Pan *et al.* 2010; Wang *et al.* 2015). Given that a noteworthy population of carotid body afferents are of C-fibre lineage (Kumar & Prabhakar, 2012), we reasoned that an analogous cellular mechanism occurs in the carotid body–petrosal ganglia complex (Pan *et al.* 2010). We demonstrate that inhibiting PKCε can prevent LPA effects on wild-type HEK cells and site-directed mutagenesis of TRPV1 phosphorylation residues T704 and S502, but not S800, greatly diminishes the LPA effects on TRPV1. Thus our data are in apparent contrast to Nieto-Posadas *et al.* that suggest a direct effect of LPA on the c-terminus of TRPV1 (Nieto-Posadas *et al.* 2012). We conclude that either LPA's effects on TRPV1 are indirect or LPA has a direct effect on TRPV1 which is critically dependent on channel phosphorylation state. Similarly, Th2 cytokine effects were also highly dependent on PKCε. Though in contrast to LPA, the PKCε blocking peptide was less efficacious than the TRPV1 antagonist AMG9810, indicating that some TRPV1 activation is independent of PKCε. This PKCε-independent effect may indicate direct stimulation of TRPV1 by asthmakines or it may result from concomitant effects of other PKC isoforms. Importantly, these studies also demonstrated the efficacy of the PKCε inhibitor peptide in blocking the LPA to TRPV1 pathway.

Our data, whether obtained from isolated *en bloc* carotid bodies, *in vivo* anaesthetized preparations or conscious animals, are entirely consistent with a PKCε–TRPV1-dependent pathway likely to involve carotid body activation as being a critical step in the aetiology of AHR. Specifically, results from experiments using the selective PKCε-inhibitor peptide (Liron *et al.* 2007) demonstrate that asthmakines stimulate the carotid body via PKCε. Moreover, systemic PKCε blockade effectively blocked allergen-induced AHR, suggesting a vital role for TRPV1-dependent allergen-induced bronchoconstriction and respiratory difficulties in asthmatic animals. We therefore propose that a PKCε–TRPV1-dependent mechanism drives the carotid body bronchoconstriction reflex in asthma, as summarized in Fig. 8.

Several important questions remain regarding neuronal reflex control of lung resistance. These include if, when and how vagal and other extra-pulmonary pathways act in concert with the carotid body to regulate airway calibre and inflammation, and more generally, whether this pathway can be exploited to treat asthma and other inflammatory/allergen diseases in humans?

These data reveal a novel role for PKCε in mediating asthmakine stimulation of the carotid body and point to PKCε as a primary driver of allergen-induced asthmatic bronchoconstriction. However, a number of limitations in our study should be noted. The LPAr antagonists, PKCε inhibitor peptide, and TRPV1 antagonists (Jendzjowsky *et al.* 2018) are each effective in reducing allergen-induced asthmatic bronchoconstriction in the OVA BN rat. Therefore, we have highlighted the PKCε–TRPV1 pathway in mediating LPA and Th2 cytokine effects. However, Th2 cytokines may act through additional pathways and the precise localization of receptors and the interplay of cell types within the carotid body asthmakine signalling pathway requires further investigation. For example, we did not specifically test whether PKCε or LPAr1/3 blockade directly affected smooth muscle contractility and may have acted in alternate pathways of bronchoconstriction. However, we note that these antagonists given in isolation do not alter smooth muscle contractility (Inagaki Koichi *et al.*, 2008; Staiculescu *et al.* 2014).

Our focus has been on the novel effects of asthmakines on the carotid body and our data suggest that these effects play a primary role in allergen-induced asthmatic bronchoconstriction. However, we recognize that asthmakines likely have broad systemic effects on the inflammatory signalling cascades and the nervous system. It remains possible that the role of different systems (e.g. carotid body, vagal afferents, inflammatory cells) is highly non-linear such that each is required to reveal the full effects of allergens. This will necessitate separate studies to investigate the effects of the carotid body PKCε–TRPV1 pathway on bronchoalveolar lavage fluid, inflammatory cells, or airway remodelling. Moreover, the precise location of the cytokine and LPA receptors in the carotid body (type I *vs.* type II cells) have not been delineated. However, LPA receptors have been found in dorsal root

ganglia glia (Robering *et al.* 2019) and neurons (Pan *et al.* 2010; Nieto-Posadas *et al.* 2012) suggesting pre- and post-synaptic importance.

It should also be noted that the model and experimental approach used in these studies has limitations. With regards to the experimental approach, we did not include a non-sensitized control group, or measure blood gases during our invasive assessment of respiratory mechanics, and relied on the specificity of TRPV1 antagonist AMG9810. Instead, we banked on data from our previous study (Jendzjowsky *et al.* 2018), the fact that similar conclusions were drawn from studies of respiration in conscious animals using plethysmography, and/or previous literature on the specificity and efficacy of AMG9810 (Gavva *et al.* 2005).

With regards to the model, while the asthmatic OVA BN rat exhibits most, if not all, the symptoms of human asthma (Bice *et al.* 2000), it relies on a single allergen (i.e. ovalbumin) and has a limited time window (i.e. months) (Haczku *et al.* 1994; Swirski *et al.* 2006). In contrast, asthma in human individuals differs by allergen (e.g. house dust mite, pollen or chemicals), durations (e.g. decades) and disease severity (e.g. occasional wheezing and asthmatic cough to emergency hospitalization) (Holgate *et al.* 2015). Therefore, we might expect that the asthmakine cocktail released in response to allergen to differ between human individuals and over time. Importantly, we demonstrate for the first time that physiological concentrations of multiple asthma-associated Th2 cytokines IL4, IL5, IL13 and eotaxin were surprisingly efficacious at stimulating the carotid body, increasing activity by >50%; remarkably, our data suggest they also use a PKCε and TRPV1-dependent mechanism. In contrast, asthma-associated concentrations of prominent

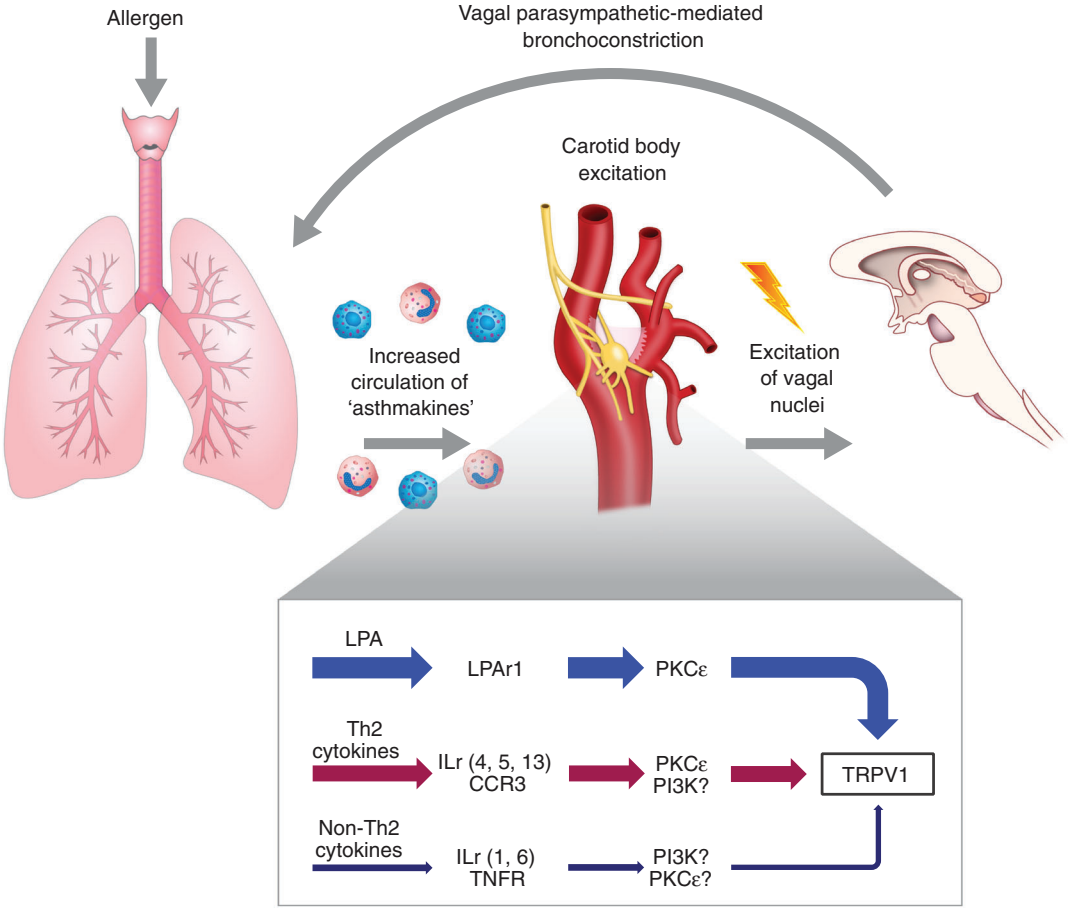

Figure 8. Asthmakine stimulation of carotid body-mediated bronchoconstriction works through TRPV1
A predominant role of lysophosphatidic acid (LPA) stimulation of LPA receptor 1 (LPAr1), PKCε phosphorylation and ultimately TRPV1 stimulation leads to carotid body-mediated stimulation of brainstem vagal centres (nucleus ambiguous and dorsal motor nucleus of the vagus) and subsequent efferent vagal excitation and ensuing bronchoconstriction. Asthma-associated prototypical Th2 cytokines work in parallel with LPA by activating respective interleukin receptor (ILr 4, 5, 13) and eotaxin receptor (CCR3) mediated PKCε and/or PI3K stimulation of TRPV1. To a lesser degree, asthma-associated non-Th2 cytokines may also activate carotid body-mediated bronchoconstriction via ILr (1, 6) and TNFα receptor (TNFR) stimulation of PKCε and/or PI3K stimulation of TRPV1. [Colour figure can be viewed at wileyonlinelibrary.com]

non-Th2 cytokines (IL1, IL6 and TNFα) only increased carotid body activity by ∼14% (Fan *et al*. 2009; Fernández *et al*. 2011; Shu *et al*. 2007). Nonetheless, this increase in activity was also TRPV1-dependent.

In summary, our data suggest that multiple asthmakines are capable of eliciting carotid body-mediated bronchoconstriction, providing additional support for an important role for the carotid body in asthma, and suggest that PKCε and/or TRPV1 may be good targets for new pharmaceutical treatments.

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Additional information

Data availability statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Competing interests

N.G.J., A.R. and R.J.A.W. declare the following competing interests. U.S. Patent Application No. 62/534638, Status: provisional patent; 'Method to Abate Acute Airway Hypersensitivity and Asthma Attacks'. Purpose: for the use of TRPV1 and LPAr blockade as a treatment for respiratory distress associated with acute asthmatic attack. R.J.A.W recently founded a University of Calgary spinoff company (AazeinTX.Inc) with the aim of developing pharmaceuticals to disrupt this pathway; R.J.A.W., N.G.J. and A.R. hold financial interests in AazeinTx. The remaining authors declare no competing interests.

Author contributions

Site-directed mutagenesis and patch-clamp recordings were conducted in the laboratory of C.A., all other experiments were conducted in the laboratory of R.J.A.W.; N.G.J., R.J.A.W., C.A. and M.M.K. conceived and designed the research; N.G.J., A.R., N.O.B., M.I., B.A.H. and F.V. performed the experiments; N.G.J., A.R., N.O.B., M.M.K., M.I., B.A.H. and C.A. analysed the data; N.G.J., A.R., M.I., C.A., M.M.K. and R.J.A.W. interpreted the results of the experiments; N.G.J., A.R., M.I., N.O.B. and R.J.A.W. prepared the figures; N.G.J. and R.J.A.W. drafted the manuscript. All authors edited and revised the manuscript. All authors approved the final version of the manuscript, agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Keywords

airway hyperresponsiveness, asthma, carotid body, cytokines, lysophosphatidic acid, neuro-immune interaction, PKCε, Th2, TRPV1

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Statistical Summary Document

Translational perspective

Our data demonstrate that multiple ligands stimulate secondary messengers (protein kinase c-epsilon) to activate the transient receptor potential vanilloid one channel in the carotid sinus nerve post-synaptic to carotid body neuroendocrine cells. By inhibiting these secondary messengers we show the ability to abate asthmatic bronchoconstriction. These findings provide evidence that targeting of intracellular signalling mechanisms may be a specific way to abate neuronal reflexes of airway hyperresponsiveness in asthma without disrupting other reflexes mediated by the carotid body, such as hypoxic sensing.