

Online data supplement

Prostaglandin D₂ and the role of the DP₁, DP₂ and TP receptor in the control of airway reflex events.

Sarah A Maher¹, Mark A Birrell^{1,2}, John J Adcock¹, Michael A Wortley¹, Eric D Dubuis¹, Sara J Bonvini¹, Megan S Grace³, Maria G Belvisi^{1,2}

METHODS

Assessing calcium movement in airway jugular ganglia cells

1,1'-Diocetadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI) is weakly fluorescent in water but highly fluorescent and photostable when incorporated into membranes. DiI has an extremely high extinction coefficient at its longest-wavelength absorption maximum (550 nm) though modest quantum yields, and short excited-state lifetimes (~1 nanosecond) in lipid environments. This dye uniformly labels neurons *via* lateral diffusion in plasma membranes at a rate of 0.2-0.6 mm a day in fixed specimens and 6mm a day *in vivo* due to active dye transport process. DiI was chosen to label neurons in this study for its maximum absorption (520-550 nm) and emission fluorescence wavelengths (570 nm). This avoids light contamination with the other dyes used which will be used for the calcium imaging (Fura 2, absorption 340/380 nm, emission 510 nm).

Fourteen days after administering the dye, the ganglia were extracted from surrounding tissue under a microscope and incubated at 37 °C in Papain solution (20U/ml) and collagenase (2 mg/ml)/dispase II (2.4 mg/ml) to break down the extracellular matrix and collagen. Ganglia were mechanically freed from tissues, remaining axons and satellite cells. The mixture of tissue and cells obtained were centrifuged through layers of medium with increasing densities. Tissues and small cells were trapped in the interface between the two medium and the neuron collected from the cell pellet at the bottom of the high density medium. Cells were plated onto D-polylysine / Laminin-coated culture dishes and primary cultured for 24 hours with complete F12 medium to allow full adherence of the cells.

For Calcium imaging the cells were loaded with 6μM Fura-2 acetoxymethyl ester (Fura-2 AM) for 40 minutes. To perform experiments, neuronal cells were rinsed and maintained in extracellular solution (ECS) to de-esterify for at least 30 min. Viability of the cells were assessed by applying 50mM potassium solution (K_{50}) at the start and finish of experiments. PGD_2 (1μM) or

capsaicin (0.1 μ M) was applied for 70 seconds and washed until returned to baseline. For inhibition experiments, the inhibitor was applied for 10 minutes and the agonist re-applied in the presence of inhibitor. After a wash period, the agonist was re-applied. All recordings were performed at 37 °C. Intracellular calcium change was recorded and analysed using NIH software Image J.

Recording from single afferent nerve fibres *in vivo*

Guinea pigs were anaesthetised with urethane (1.5 g/kg) intraperitoneally. If required, anaesthesia was supplemented with additional urethane. The trachea was cannulated and bronchospasm was measured with an air pressure transducer (SenSym 647) connected to a side arm of the tracheal cannula. Blood gases and pH were maintained at physiological levels by artificial ventilation (Ugo Basile small animal ventilator), with a tidal volume of 10 ml/kg and 50-60 breaths per min of laboratory air. The right jugular vein and carotid artery were cannulated for injecting drugs and measuring systemic arterial blood pressure (Gould P23XL transducer), respectively. Body temperature was continuously monitored with a rectal thermometer and maintained at 37°C with a heated blanket and control unit (Harvard Apparatus). Animals were paralysed with vecuronium bromide, initially administered at a dose of 0.10 mg/kg i.v., followed every 20 min with 0.05 mg/kg i.v. to maintain paralysis. The depth of anaesthesia was frequently assessed by monitoring the response of heart rate and blood pressure to noxious stimuli. Both cervical vagus nerves were located, via a cervical incision, and dissected free from the carotid artery, sympathetic and aortic nerves; both vagus nerves were cut at the central end, posterior to the vagal ganglia. The left vagus nerve was used for sensory nerve fibre recording and was cleared of its surrounding fascia. The skin and muscle in the neck at either side of the incision were lifted and tied to a metal ring to form a well, which was filled with light mineral oil. Bipolar Teflon-coated platinum electrodes (exposed at the tips) were used for recording purposes, using fascia positioned on one electrode for a reference. The vagus nerve was placed on a small black perspex plate to facilitate subsequent dissection. Thin filaments of nerve were teased from the vagus nerve, under a binocular microscope, and placed on

the second electrode until a single active unit, or one of not more than two or three units, was obtained. Action potentials were recorded in a conventional manner using electrodes connected to a pre-amp headstage (Digitimer NL100K). The signal was amplified (x5000, Digitimer NL104), filtered (LF30Hz – HF8.5kHz, Digitimer NL125) and passed through a Humbug noise reducer (AutoMate Scientific) before input sampling and recording. All signals were sampled (50 kHz) and recorded using the Spike 2 software data acquisition system via a CED Micro1401 interface. The software allowed pulse train counting over selected time periods. In addition, monitoring of the input signal to the Spike software was also carried out on a digital storage oscilloscope (Tektronix DPO 2012). The input signal was also fed through an audio amplifier to a loud speaker. All animals were killed at the end of the experiments with an overdose of pentobarbitone.

Conduction velocities were measured to distinguish slow conducting non-myelinated C-fibres from fast conducting myelinated A-fibres by stimulating the vagus nerve close to the thorax with bipolar silver electrodes, using a supra threshold voltage at 0.5 ms, 1 Hz (Grass stimulator). The corresponding action potential was recorded in the nerve fibre under observation. The stimulus and the recorded action potential were captured on the Spike software in a single sweep and the time interval between them was measured to calculate the velocity using the distance from the cathode stimulating electrode and the recording electrode. Aerosols were generated by an Aerogen nebulizer (Buxco Nebulizer Control) connected to the ventilator, and arranged so that the inspired air passed through the medication chamber before entering the lungs of anaesthetised animals via the tracheal cannula. Single vagal nerve fibres were identified as originating from the three groups of airway sensory nerve endings, i.e., slowly adapting stretch receptors (SARs), irritant receptors (rapidly adapting stretch receptors, RARs, A δ -fibres) and pulmonary/bronchial C-fibre receptors using several criteria [1]. These included pattern of spontaneous discharge, response to hyperinflation and deflation, adaptation indices (AIs), and response to capsaicin aerosol administration and conduction velocities. As a rule, a fibre that had no obvious pattern to the spontaneous activity (often very sparse), didn't respond to hyperinflation or hyperdeflation and

responded to capsaicin aerosol was considered for further investigation. Finally, verification of a C-fibre was confirmed at the end of the experiment by determining conduction velocity. After surgery the animals were allowed to stabilize for at least 30 min. Following identification of a lung afferent fibre and its sensitivity to capsaicin aerosol, the ensuing protocol was pursued: After a control baseline recording of at least 2 min, capsaicin (100 μ M) was administered by aerosol for 15 s and the changes in fibre activity, intra-tracheal pressure and blood pressure were continuously recorded until baseline or a steady state was re-established. Agonists were each aerosolised for 60 seconds at 10 minute intervals while recording variables.

In vitro vagus nerve preparation

Male guinea pigs or mice were culled and the vagus nerves, caudal to the nodose ganglion, were carefully dissected and placed in Krebs-Henseleit solution (KHS). Human trachea, with branches of the cervical vagus nerve still attached was obtained from donor patients for a heart or heart/lung transplant or post mortem from the International Institute for the Advancement of Medicine (USA). Relevant approvals were obtained from the Royal Brompton and Harefield Trust Ethics Committee.

Segments of human and guinea-pig vagus nerves were cleared of connective tissue and carefully desheathed. Nerve trunks were mounted in a 'grease-gap' recording chamber as previously described [2, 3]. Briefly, the nerve was drawn longitudinally through a narrow channel (2mm diameter, 10mm length) in a Perspex block. The centre of the channel was filled with petroleum jelly, injected through a side arm electrically isolating the extracellular space between the two ends of the nerve. One end of the nerve was constantly superfused with KHS at a flow rate of approximately 2ml/min. The other nerve ending remained in a second, smaller chamber containing oxygenated KHS throughout the experiments. Ag/AgCl electrodes (Mere 2 Flexible reference electrodes, World Precision Instruments (WPI)), filled with KHS, made contact at either end of the nerve trunk and recorded DC potential via a DAM 50 differential amplifier (WPI). DC voltages were amplified $\times 10$, filtered at 1000 Hz, and sampled at 5 Hz. Our experiment set up enables two

nerves to be recorded simultaneously. The temperature of the perfusate was maintained at 37°C by means of a water bath. Drugs were applied at known concentrations into the perfusing solution of the first channel only, and depolarising responses recorded onto a chart recorder (Lectromed Multi-Trace 2).

Agonists were applied for 2 minutes after which the nerve was washed with KHS and the maximum depolarisation recorded. For antagonist experiments, two reproducible responses to agonists were acquired. The antagonist or vehicle was perfused for 10 minutes and immediately after the agonists was reapplied for 2 minutes in the presence of the antagonist. The degree of inhibition was then recorded and a final agonist's response achieved to illustrate that the nerve was still viable. Concentrations of antagonists (shown in results section) were selected that were approximately 100-fold the pA₂ value (defined as the negative logarithm of the molar concentration of an antagonist that would produce a twofold shift in the concentration–response curve for an agonist) as is common practice.

In vitro tracheal organ bath preparation

The guinea-pig trachea was opened longitudinally opposite to the smooth muscle strip. The epithelium was left intact and transverse segments were prepared and sutured and suspended from a force transducer in a 10ml organ bath containing KHS warmed to 37°C and bubbled with 95% O₂ / 5% CO₂. Indomethacin was present (10µM) as a non-selective cyclooxygenase inhibitor to prevent the formation of endogenous prostanoids. Changes in force were measured isometrically using a force displacement transducer connected to a data acquisition system as previously described [4]. Tissues were washed every 20 minutes for one hour until the baseline was stable. Supramaximal responses to 1mM acetylcholine (Ach) were established in all tissues. Antagonist or vehicle (0.1% DMSO) was added to the bath for 30 minutes after which a cumulative concentration-response curve was constructed. After the experiment, the tissue was washed and viability confirmed by a response to 1mM Ach.

Compounds and Materials

Indomethacin and acetylcholine were purchased from Sigma Aldrich (Poole UK).

In vivo cough

PGD₂ was dissolved in PBS on the day of experiment at 1mg/ml and diluted.

In vivo single fibre recording

Capsaicin was dissolved in 1% ethanol and 1% tween 80 in 0.9% saline to working solution. PGD₂, 15(R)-15-methyl PGD₂, BW245C were dissolved in PBS to 100µg/ml on the day of experiment. Vecuronium Bromide (Sigma-Aldrich, Poole, Dorset, U.K.) was dissolved in distilled at 2.5/mg ml stock and diluted in distilled H₂O as required.

In vivo staining of airways neurons

The lipophilic retrograde tracer dye DiI (DiI_{C₁₈(3)})-(1,1'-dioctacetyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) (Invitrogen) was prepared as a stock of 25mg/ml diluted in 100% ethanol and kept in the dark at room temperature up to 1 year. On the day of staining, the dye was diluted in 0.9% sterile saline at a concentration of 625ug/ml. A dose of 1ml/kg was used for staining.

In vitro experiments

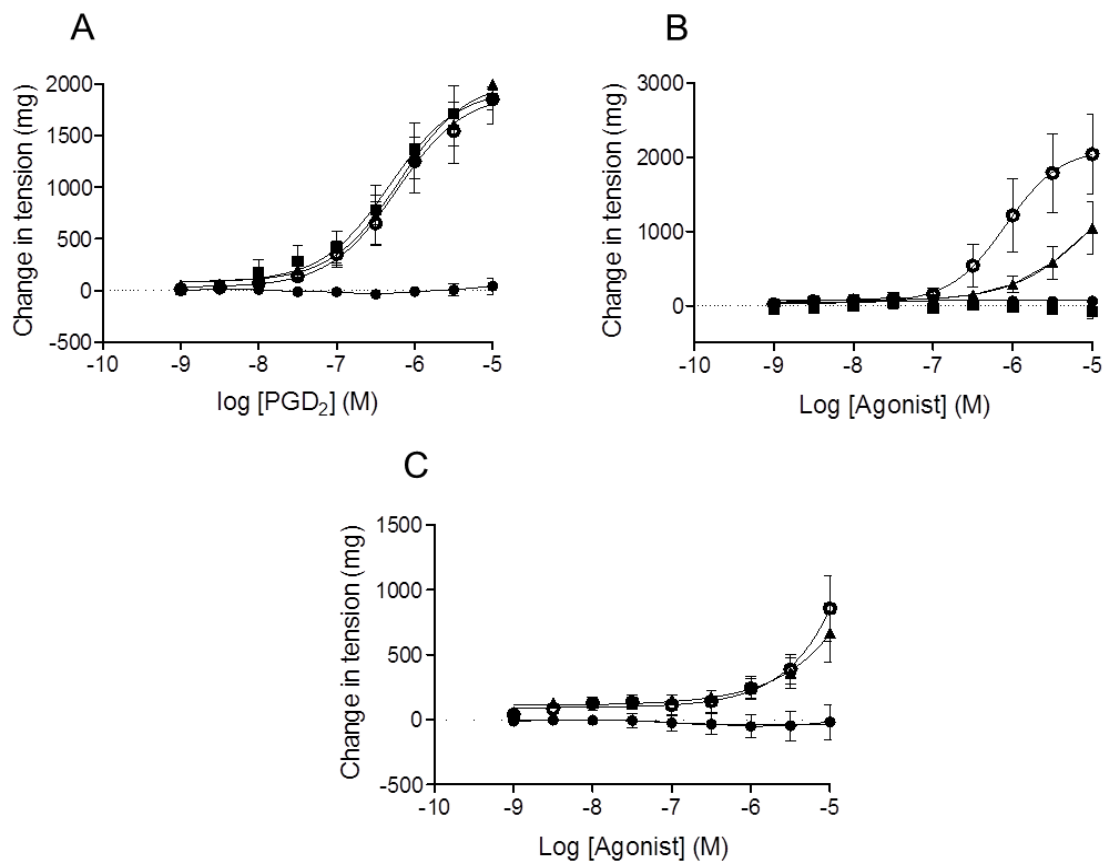
KHS (mM: NaCl 118; KCl 5.9; MgSO₄ 1.2; CaCl₂ 2.5; NaH₂PO₄ 1.2; NaHCO₃ 25.5; glucose 5.6), Extracellular solution (ECS, in mM: 5.4 KCl, 136 NaCl, 1 MgCl₂, 2.5 CaCl₂, 0.33 NaH₂PO₄, 10 D-Glucose, 10 HEPES; 297mOsm, pH adjusted to 7.4 with NaOH at 37°C) and 50mM hyperpotassium solution (K50, in mM: 50 KCl, 101.4 NaCl, 1 MgCl₂, 2.5 CaCl₂, 0.33 NaH₂PO₄, 10

D-Glucose, 10 HEPES; 297mOsm, pH adjusted to 7.4 with NaOH at 37°C) solutions were made fresh daily.

Fura2-AM (Molecular Probes) stocks were prepared as 10µl aliquots in DMSO and frozen at -20°C. To load the cells, Fura2-AM was diluted in 2.5ml ECS. PGD₂, PGE₂, 15(R)-15-methyl PGD₂, BW245C were dissolved in Ethanol and diluted 1:1000 in krebs or ECS on the day of experiment. BWA868C, CAY10471, SQ29548, HC-030031, JNJ17203212 and capsaicin were dissolved in DMSO and diluted 1:1000 in KHS or ECS on the day of experiment.

Reference List:

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Supplementary figure 1

Identification of the receptors involved in PGD₂ mediated effects in the airway smooth muscle. (A) Concentration response curve of PGD₂ in guinea-pig tracheal smooth muscle strips in the presence of vehicle (0.1% DMSO, open circles) TP antagonist (1 μ M SQ29548, closed circles), DP₁ antagonist (10 μ M BWA868C, closed square) or DP₂ antagonist (1 μ M CAY10471, closed triangle). (B) Concentration response curve of Vehicle (0.1% Ethanol, closed circle) PGD₂ (Open circle) BW245C (DP₁ agonist, closed square) and 15(R)-15-methyl PGD₂ (DP₂ agonist, closed triangle) in guinea pig tracheal smooth muscle strips. (C) Concentration response curve of DP₂ agonist 15(R)-15-methyl PGD₂ in the presence of vehicle (0.1% DMSO, open circles), TP antagonist (1 μ M SQ29548, closed circles) or DP₂ antagonist (1 μ M CAY10471 closed triangles). Data represents mean \pm SEM, n=4-6