On line Supplement

TRP channels mediate the tussive response to prostaglandin E_2 and bradykinin

Megan Grace, Mark A. Birrell, Eric Dubuis, Sarah A. Maher, Maria G. Belvisi

METHODS

Animals

Male Dunkin-Hartley guinea-pigs (300-500g) and C57BL/6 mice (18-20g) were purchased from Harlan (Bicester, Oxon, U.K.), and housed in temperature-controlled (21°C) rooms with food and water freely available for at least 1 week before commencing experimentation. Breeding pairs of mice devoid of the TRPA1 (*Trpa1*$^{-/-}$) or TRPV1 (*Trpv1*$^{-/-}$) gene that had been backcrossed on to the C57BL/6 background were obtained from Jackson Laboratories (Bar Harbour, ME). Breeding colonies were maintained at Imperial College, London. Experiments were performed in accordance with the U.K. Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act of 1986.

Isolated vagal ganglia

Cell dissociation

Guinea-pigs were sacrificed by injection of pentobarbitone (20 mg/kg, i.p.). Nodose and jugular ganglia were dissected free of adhering connective tissue and isolated by enzymatic digestion. Ganglia were incubated with activated papain (Sigma, Papain type 200U/ml in Ca^{2+}-free, Mg^{2+}-free Hanks' balanced salt solution) for 30 minutes at 37°C followed by incubation for 40 minutes with type 4 collagenase (Worthington, 2mg/ml) and Dispase II (Roche, 2.4mg/ml), with gentle agitation every 5 minutes. The neurons were dissociated by tituration with fire-polished glass Pasteur pipettes of decreasing tip pore size and washed by centrifugation for 1 minute (1400 rpm, 380g) at 37°C. The supernatant was decanted and the pellet of tissues and cells carefully homogenised in HBSS at room temperature. Cells were separated from the remaining tissue by two centrifugations at 22°C: 8 min (1400 rpm, 380g) in L15 medium containing 20% Percoll (v/v) and 2 min (2300 rpm, 1030g) in L15 medium. The cells were resuspended in complete F-12 medium and plated in poly-d-lysine/Laminin

(22.5 ug/ml) coated fluorodishes. Neurons were allowed to adhere for 2h in a 37°C, 5% CO2 environment, then gently flooded with 2ml of complete F12 medium (10% FBS, 1% penicillin 10,000U/ml – streptomycin 10mg/ml). Plates were used for experimentation within 24h.

Calcium imaging

Intracellular free calcium ($[Ca^{2+}]_i$) measurements were performed in dissociated jugular and nodose neurons. Fluorodishes were loaded with Fluo-4 AM (6 μM, Invitrogen) for agonist experiments, or Fluo-2 AM (3 μM, Invitrogen) for antagonist experiments for 40 min in the dark at 25°C and allowed to rest for 30 min in the dark at 25°C. After washing, a fluoro dish was placed in a full incubation chamber mounted on the stage of a widefield inverted microscope Zeiss Axiovert 200 (Carl Zeiss Inc., NY, USA) and held at 37°C. Signals were recorded using an Hamamatsu EM-CCD C9100-02 camera run by Simple PCI software. CA Xenon gas Arc lamp Cairn ARC Optosource Illuminator, a Quad filter set Ex 485-20 Bs 475-495 Em 510-53120x and a LD Plan-Neofluar AIR Korr objective were used to generate excitatory signal and record emission.

Neurons were constantly superfused with 37°C ECS buffer using an in house designed pressurized solution-changing perfusion system allowing complete bath (600μl volume) replacement in 3s. Prior to experiments, the cells were superfused for 10 min with ECS-only. 50 mM potassium chloride solution (K50) was applied at the start and end of each experiment for 10s to assess cell viability and normalise responses. Stock solutions of agonists and antagonists were diluted 1/1000 in ECS to make a working solution. Capsaicin or acrolein was applied for 10-20s, PGE_2 for 20-30s and BK for 40-60s *(*N=4-6 animals, n=15-26 cells per drug tested*)*. After each application of drug, cells were washed with ECS until complete recovery of baseline $[Ca^{2+}]_i$. Images were acquired with a frequency of 1Hz from 30s prior to

drug application and for 2min afterward, and at 0.2Hz otherwise. To take into account the multiphasic responses obtained in some cells the area under curve of calcium signal (total elevation of calcium above resting level over time or calcium flux) was used to measure responses, which were normalised to calcium flux generated by application of K50. Only neurons producing a fast response to K50 which was washable within 5min, and that had diameter of over 20 μm were analysed.

The ability of TRPA1-selective (HC-030031) and TRPV1-selective (JNJ17203212) antagonists to inhibit submaximal acrolein (10 μM) and capsaicin (1 μM)-induced changes in $[Ca^{2+}]_i$ was investigated in jugular cells. CR curves were established for HC-030031 (0.001, 0.01, 0.1, 1 μM) or vehicle (0.1% DMSO) against acrolein; and JNJ17203212 (1, 10, 100 μM) or vehicle (0.1% DMSO) against capsaicin. $[Ca^{2+}]_i$ responses were recorded using Fura-2, with only one concentration of antagonist assessed per plate. Once appropriate concentrations of the antagonists had been determined, the effect of 0.1 μM HC-030031 on capsaicin and 10 μM JNJ17203212 on acrolein stimulated $[Ca^{2+}]_i$ elevation was investigated to establish that there was no off-target effect at the concentration chosen. These antagonist concentrations were subsequently used to inhibit PGE_2 (1 μM) and BK (10 μM) responses in jugular neurons (N=3-4, n=10-19).

Isolated vagus nerve preparation

Initially, concentration-response curves were established for the agonists. Concentrations of vehicle (distilled water or ethanol, 0.1% vol/vol), PGE_2 or BK were applied to guinea-pig (PGE_2 3-100 μM; BK 1-30 μM) or mouse (PGE_2 and BK 0.1-100 μM) nerves in a random order for 2 minutes each, including a wash period to retain baseline membrane potential between stimulations. No more than five stimulations were generated per section of nerve. From this, a submaximal dose of each agonist was chosen for future experiments: 10 μM

PGE_2; and 1 μM BK in the mouse and 3 μM BK in the guinea-pig. The ability of PGE_2 (10μM) and BK (3μM) to stimulate human vagus nerves was also determined. Human vagal tissue (n = 6 patients, 2 male, 27-72 year old donors with no respiratory disease) was obtained from two sources – transplant tissue surplus to requirements (Harefield Hospital, UK); and purchased from IIAM (International Institute for the Advancement of Medicine, Edison, NJ). In all cases, the tissue was consented for use in scientific research and ethics approval obtained.

The GPCR through which PGE_2 induces cough has been identified as the EP_3 receptor [S1]. However, the GPCR through which BK signals has not yet been comprehensively investigated using selective ligands. Therefore, the ability of B_1 (1μM R715) or B_2-selective (10μM WIN 64338) antagonists to inhibit BK-induced human, guinea-pig and mouse sensory nerve depolarisation was examined. These were based on using concentrations 10-fold higher than the reported antagonist affinity for the mouse (B_1; [S2]) or guinea-pig (B_2; [S3]) receptor. There has been a suggestion that BK may activate sensory nerves indirectly by inducing production of prostaglandins. To investigate this, a general cyclooxygenase inhibitor (10μM indomethacin) was tested according to our standard antagonist protocol to see if BK responses were altered. In addition, depolarisation to BK was assessed in both wild type and genetically modified mice with the EP_3 gene deleted ($EP_3^{-/-}$). Tetrodotoxin (TTX, 3μM) was also used to assess whether depolarisation to these tussive agents was sensitive to sodium channel blockade indicating a role for sensory nerve activation. The concentration of TTX was based on previous functional experiments in isolated airway tissue shown to inhibit neuronal responses [S4].

Concentration-response curves were established for TRPA1-selective (HC-030031) and TRPV1-selective (capsazepine and JNJ17203212) antagonists, or vehicle (dimethyl sulfoxide [DMSO], 0.1% vol/vol) using previously established submaximal doses of the selective

agonists acrolein (300 μM) and capsaicin (1 μM). From this, the dose of antagonist exhibiting maximal inhibition of its receptor was chosen for further experiments. To demonstrate that these antagonists were not exhibiting off-target effects, 10 μM HC-030031 was also tested against the TRPV1 agonist capsaicin; and 10 μM capsazepine and 100 μM JNJ17203212 were tested against the TRPA1 agonist acrolein. The ability of TRP-selective antagonists to inhibit PGE_2 and BK-induced sensory nerve depolarisation was comprehensively profiled in wild type mouse and guinea-pig tissue (n=6), and was also assessed in human tissue when available (n=2-3). Concentrations of both agonists and antagonists used for human vagus nerve were the same as for guinea-pig experiments. To further confirm that the inhibition observed with selective antagonists was due to inhibition of the TRPA1 or TRPV1 ion channel, we performed parallel experiments using vagal tissue from *Trpa1*$^{-/-}$ and *Trpv1*$^{-/-}$ mice. Knockdown of the TRPA1 or TRPV1 gene was confirmed in the genetically modified mice using standard genotyping techniques.

Conscious guinea-pig cough model

Conscious unrestrained guinea-pigs were placed in individual plastic transparent whole-body plethysmograph chambers (Buxco, Wilmington, NC, USA), and cough detected as previously described [S1, S5]. Concentration-responses were established for capsaicin (15-90 μM, n=10), acrolein (10-300 mM, n=12), PGE_2 (30-300 μg/ml; n=8), BK (0.3-10 mg/ml; n=4), or appropriate vehicle (1% ethanol, 1% Tween 80 in 0.9% sterile saline; 0.9% sterile saline; 0.1M phosphate buffer; or 0.9% sterile saline, respectively). Stimuli were aerosolised for 5 min (capsaicin and acrolein) or 10 min (PGE_2 and BK). Coughs were counted for 10 min, both with the Buxco cough analyser and by a trained observer. From these experiments, a submaximal dose of agonist was identified for further experiments.

To determine an appropriate dose of TRPA1 and TRPV1-selective antagonist, guinea-pigs were injected i.p. with HC-030031 (30-1000 mg/kg), JNJ17203212 (10-1000 mg/kg) or appropriate vehicle (0.5% methylcellulose in 0.9% saline or 15% solutol in 5% dextrose solution, respectively). One hour later the guinea-pigs were exposed to 5 minutes of a submaximal dose of TRP-selective agonist (100 mM acrolein or 60 μM capsaicin). Coughs were counted during this period, and for a further 5 minutes post-stimulation (10 minutes total). Once a concentration had been established that maximally inhibited its own receptor, this concentration was tested against the alternate agonist to confirm receptor selectivity at the chosen dose. Guinea-pigs received two i.p. injections of either: (a) TRPA1 vehicle (0.5% methylcellulose in 0.9% saline) plus TRPV1 vehicle (15% solutol in 5% dextrose solution); (b) TRPA1 antagonist (300 mg/kg HC-030031) plus TRPV1 vehicle; (c) TRPA1 vehicle plus TRPV1 antagonist (100 mg/kg JNJ17203212); or (d) TRPA1 antagonist plus TRPV1 antagonist. One hour later the guinea-pigs were exposed to 10 minutes of aerosolised PGE_2 (300 μg/ml, n=12) or BK (3 mg/ml, n=10-11), and the number of coughs counted during this period.

Compounds and Materials

PGE_2 was purchased from Cayman Chemical (Ann Arbor, Michigan, USA). The TRPA1 inhibitor HC-030031 was purchased from ChemBridge (San Diego, USA). The TRPV1 inhibitor JNJ17203212 was a kind gift from Glaxo SmithKline, who also provided the excipient Solutol (BASF, Ludwigshafen, Germany). All other agents were purchased from Sigma-Aldrich (Poole, Dorset, U.K.)

Isolated vagal ganglia experiments: Fluo-4 AM and Fura-2 AM were purchased from Molecular Probes/Invitrogen. L-15 and Hanks' balanced salt solution (HBSS) were purchased

from Gibco/Invitrogen (Carlsbad, CA, USA). The 50mM potassium solution (K50) contained (in mM): 50 KCl, 91.4 NaCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 0.33 NaH_2PO_4, 10 glucose, 10 HEPES; pH adjusted to 7.4 at 37°C using KOH). Capsaicin was dissolved in 100% DMSO, PGE_2 in 100% ethanol, and BK in dH_2O. Stock solutions were diluted 1/1000 in extracellular solution (ECS) buffer (in mM: 5.4 KCl, 136 NaCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 0.33 NaH_2PO_4, 10 glucose, 10 HEPES; pH adjusted to 7.4 at 37°C using NaOH) to the desired final concentrations. Final vehicle concentration was 0.1% for the calcium measurements.

In vitro vagus experiments: All Krebs salts were obtained from BDH (Dorset, U.K.), and Krebs Hanseleit solution was made fresh on a daily basis (mM: NaCl 118; KCl 5.9; $MgSO_4$ 1.2; $CaCl_2$ 2.5; NaH_2PO_4 1.2; $NaHCO_3$ 25.5; glucose 5.6). TTX stock was dissolved in 20mM citric acid. All other agonists and antagonsits were dissolved in 100% distilled water, DMSO or ethanol. Aliquots were diluted down 1/1000 in Krebs solution for testing.

In vivo cough experiments: Acrolein was supplied in liquid form at 15M. A 1M stock was made in saline (0.9%) and diluted in saline to 100mM. A 10mM stock of capsaicin was made in ethanol and diluted in vehicle to obtain 1% ethanol and 1% Tween 80 in 0.9% saline. Bradykinin was dissolved in 0.9% saline to working solution, accounting for the acetate conversion factor (1.06). PGE_2 was dissolved in 0.1M phosphate buffer to a working solution. HC-030031 (300 mg/ml) was suspended in vehicle (0.5% methyl cellulose in sterile saline); JNJ17203212 (100 mg/ml) was suspended in vehicle (15% solutol in 5% dextrose solution or 0.5% methyl cellulose in sterile saline), total dosing volume 10 ml/kg i.p.

RESULTS

Characterising agonist responses in isolated vagal ganglia

Capsaicin increased intracellular free calcium ($[Ca^{2+}]_i$) in jugular neurons with an EC_{50} of 1.43 ± 0.13 μM and a maximum response (R_M) of 75 ± 14% at 10 μM (N=5, n=24), but on average triggered a small response in nodose neurons (R_M 5 ± 2% at 10 μM, EC_{50} 1.62 ± 0.14 μM; N=5, n=18). It should be noted that robust responses to capsaicin are observed in a small population of nodose neurons [S6, S7]; and in the capsaicin-responsive nodose cells in this study we did see some significant increases in $[Ca^{2+}]_i$. However, our data represent an overview of all responding and non-responding cells, of which 62.5% of jugular neurons and 16.7% of nodose neurons were responsive to capsaicin stimulation (Supplementary Figure 1A & 1C). Different types of response were observed for acrolein in the jugular compared to nodose ganglia. Primary jugular cells responded in a multi-phasic pattern, showing periods of repetitive sharp $[Ca^{2+}]_i$ elevations, with an EC_{50} of 3.4 ± 0.39 μM and 126 ± 18% R_M at 30 μM (N=6, n=24). Whereas, acrolein-induced $[Ca^{2+}]_i$ elevations in the nodose ganglia were mono-phasic, with an EC_{50} of 8.14 ± 1.1 μM and 163 ± 31% R_M at 30 μM (N=6, n=26) (Supplementary Figure 1B & 1D). Interestingly, 54.2% of jugular neurons and 53.8% of nodose neurons responded to acrolein stimulation.

Figure Legends

Figure S1. Characterisation of TRPA1- and TRPV1-selective agonists in the *in vitro* primary ganglia and isolated vagus nerve preparations.

Panels A-D: Cocentration responses showing increases in intracellular calcium ($[Ca^{2+}]_i$) for TRPV1 selective (capsaicin) and TRPA1 selective (acrolein) agonists in primary neurons isolated from (A & B) guinea-pig jugular and (C & D) nodose ganglia. In each panel, histograms show an increase in $[Ca^{2+}]_i$ for increasing concentrations of tussive agent. To take into account multiphasic shapes of some responses and their lengths, the calcium flux (area under curve) generated by applications of tussive agents is normalised, and expressed as

percentage of response to K50. The response obtained is expressed as percentage of response to K50. The tussive agent used is indicated above each set of histograms and the concentration below each bar in μM (N=5-6, n=18-26). The trace in the lower left shows a typical recording of the light intensity over time following exposure to the agonist. Time and duration of drug application are indicated by a black bar above the trace. Time scale is given by the 1 minute length-equivalent black bar shown below the trace. On the bottom right are display images taken during the recording. Time of the snapshot is indicated below each picture with zero being the start of tussive agent application. The pseudo colour code used for light intensity in the pictures is represented on the right of each set of images.

Panels E & F: Perfusion for 2 minutes of capsaicin (black bars) or acrolein (white bars) activated (E) guinea-pig and (F) mouse isolated vagus nerves in a concentration-dependent manner, measured as depolarisation of the nerve in mV (n=6).

Panel G: Perfusion for 2 minutes of capsaicin (1 μM) or acrolein (300 μM) but not vehicle (0.1% DMSO v/v) activated human isolated vagus nerves, measured as depolarisation of the nerve in mV (n=4-8). Data is expressed as mean ± s.e.m of *n* observations. Veh = vehicle.

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