Role of chloride channels in bradykinin-induced guinea pig airway vagal C-fibre activation

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We tested the hypothesis that an ionic current carried by chloride ions contributes to bradykinin (BK)-induced membrane depolarization and activation of vagal afferent C-fibres. In an *ex vivo* innervated trachea/bronchus preparation, BK (1 μM) consistently produced action potential discharge in vagal afferent C-fibres with receptive fields in the trachea or main stem bronchus. The Ca^{2+}-activated Cl^- channel (CLCA) inhibitor, niflumic acid (NFA, 100 μM), significantly reduced BK-induced action potential discharge to 21 $\pm$ 7% of the control BK response. NFA did not inhibit capsaicin-induced or citric-acid-induced action potential discharge in tracheal C-fibres. The inhibitory effect of NFA was mimicked by another CLCA inhibitor, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB, 100 μM). NFA also inhibited the BK-induced inward current in gramicidin-perforated whole-cell patch-clamp recordings of capsaicin-sensitive jugular ganglion neurones retrogradely labelled from the airways. NFA did not inhibit the BK-induced increase in intracellular free Ca^{2+}. The TRPV1 inhibitor, iodo-resiniferatoxin (1 μM), also partially inhibited BK-induced action potential discharge, and the combination of iodo-resiniferatoxin and NFA virtually abolished the BK-induced action potential discharge. We concluded that in vagal afferent C-fibres, BK evokes membrane depolarization and action potential discharge through the additive effects of TRPV1 and Cl^- channel activation.

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Many chemicals activate primary afferent nerves by interacting with ionotropic receptors (or ligand-gated ion channels). Ligands that can open ionotropic receptors include capsaicin and endovallinoids (TRPV1 receptor), 5-HT ($5HT_3$ receptor), nicotine (nicotinic receptor), and ATP (P2X receptors). These substances interact with their receptors causing cation channel opening and membrane depolarization. Afferent nerve terminals can also be stimulated by G-protein-coupled receptor agonists. The transduction of G-protein-coupled receptor stimulation and terminal membrane depolarization in visceral afferent nerves are poorly understood.

Bradykinin (BK) is a prototypical nociceptive nerve stimulant that directly activates afferent C-fibres in both the somatosensory and viscerosensory system (Couture *et al.* 2001). BK is known to activate airway vagal afferent C-fibres by stimulating the BK B_2 receptor (Fox *et al.* 1993; Kajekar *et al.* 1999). The BK B_2 receptor is a G-protein-coupled receptor in which the G-protein is usually Gq (Wilk-Blaszczak *et al.* 1994). Studies at the level of the cell body have raised several potential mechanisms by which BK B_2 receptor stimulation may activate C-fibre terminals within the tissue compartment. BK B_2 receptor activation can lead to an inhibition of certain Ca^{2+}-activated K^+ channels (Weinreich & Wonderlin, 1987) in addition to the activation of certain Na^+ channels (Burgess *et al.* 1989). Stimulation of BK B_2 receptors can also lead to the opening of TRPV1 channels via the production of lipoxygenase products of arachidonic acid (Hwang *et al.* 2000), or from phosphatidylinositol 4,5-phosphate (PtdIns(4,5)P_2), a downregulator of TRPV1 activity (Chuang *et al.* 2001).

Less is known about the mechanism by which G-protein-coupled receptors evoke action potential discharge at the level of the terminals within peripheral tissue. BK B_2 receptor activation effectively and consistently evokes action potential discharge in vagal C-fibres in guinea pig and mouse airways studied *ex vivo* (Fox *et al.* 1993; Kajekar *et al.* 1999; Kollarik & Undem, 2004). Consistent with the hypotheses supported by studies at the cell body, nonselective TRPV1 antagonists, capsazepine and ruthenium red, inhibit the BK responses in guinea pig airway C-fibres (Carr *et al.* 2003). Likewise, in airways obtained from TRPV1–/– mice, the BK-induced

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action potential discharge was significantly, but only modestly, reduced compared with that observed in the airways of wild-type controls (Kollarik & Undem, 2004). These data indicate that both TRPV1 and non-TRPV1 ion channels are likely to be involved in the transduction between BK B_2 receptor activation and action potential discharge.

Recently, it has been proposed that a primary mechanism by which BK evokes an inward current in vagal sensory neurones is by opening a Ca^{2+}-activated Cl^- channel (CLCA; Oh & Weinreich, 2004). In the present study we addressed the hypothesis that Cl^- channels play a role in BK-induced action potential discharge in C-fibre terminals within the guinea pig isolated trachea. We provide data in support of the hypothesis that BK B_2 receptor activation of airway C-fibre terminals is the additive result of TRPV1 and Cl^- channel activation. When both of these channels are blocked, BK-induced action potential discharge is essentially abolished.

Methods

All experiments were approved by the Johns Hopkins Animal Care and Use Committee. Male Hartley guinea pigs (100–300 g, Hilltop Laboratory Animals, Inc., Scottsdale, PA, USA) were used.

Extracellular recording of tracheal/bronchial C-fibres

The animals were killed by 100% CO_2 asphyxiation in a custom-built induction cage (over 90% of the chamber volume per min) followed by exsanguination. The innervated isolated trachea/bronchus preparation was prepared as previously described (Riccio *et al.* 1996). Briefly, the airways and lungs with their intact right-side extrinsic innervation (vagus nerve including nodose and jugular ganglia) were taken and placed in a dissecting dish containing Krebs bicarbonate buffer solution composed of (mM) 118 NaCl, 5.4 KCl, 1.0 NaH_2PO_4, 1.2 $MgSO_4$, 1.9 $CaCl_2$, 25.0 $NaHCO_3$ and 11.1 dextrose, and equilibrated with 95% O_2 and 5% CO_2 (pH 7.2–7.4). Indomethacin (3 μM) was added to the buffer to suppress prostanoid release from the tissue. Connective tissue and both lungs were trimmed away leaving the larynx, trachea and right main bronchus with their intact nerves. The airways were opened by cutting longitudinally through the ventral surface of the larynx, trachea and right main bronchus, which were then pinned mucosal side up to the larger compartment of a custom-built two-compartment recording chamber which was lined with silicone elastomer (Sylgard). The right nodose and jugular ganglia were gently pulled into the adjacent compartment of the chamber through a small hole and pinned. Both compartments were separately superfused with the buffer with a flow rate of 6 ml min^{-1}. The buffer was warmed by a warming jacket (39–42°C) to keep airway tissues and ganglia at 37°C.

A sharp glass electrode was pulled by a Flaming Brown micropipette puller (P-87; Sutter Instruments, Novato, CA, USA) and filled with 3 M NaCl solution. The electrode was gently inserted into the jugular ganglion so as to be placed near the cell bodies. The recorded action potentials were amplified (Microelectrode AC amplifier 1800; A-M Systems, Everett, WA, USA), filtered (0.3 kHz of low cut-off and 1 kHz of high cut-off), and monitored on an oscilloscope (TDS340; Tektronix, Beaverton, OR, USA) and a chart record (TA240; Gould, Valley View, OH, USA). The scaled output from the amplifier was captured and analysed by a Macintosh computer using NerveOfIt software (Phocis, Baltimore, MD, USA). For measuring conduction velocity, an electrical stimulation (S44; Grass Instruments, Quincy, MA, USA) was applied on the core of the receptive field. The conduction velocity was calculated by dividing the distance along the nerve pathway by the time delay between the shock artifact and the action potential evoked by electrical stimulation. If a C-fibre (<1 m s^{-1}) was found, the recording was started.

Citric acid (10 mM, 200 μl) or BK (1 μM, 1 ml) was applied directly to the receptive field for 10 s by using a pipette-man or a syringe with an 18 gauge blunt needle. An interval of 30 min was allowed between each repeated application. Niflumic acid (NFA), 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) and iodo-resiniferatoxin (I-RTX) were applied through the perfusion line for 15 min prior to BK or citric acid application.

Labelling and cell dissociation

Tracheal afferent neurones were retrogradely labelled using DiI (DiC18(3); Molecular Probes, Eugene, OR, USA) solution (0.2%, 0.3 ml; dissolved in 10% DMSO and 90% normal saline). Under anaesthesia (50 mg kg^{-1} of ketamine and 2.5 mg kg^{-1} of xylazine; I.P.), the trachea was surgically exposed, and DiI was injected into the tracheal lumen using a 27 gauge needle 7–9 days before an experiment.

After the animals were killed by 100% CO_2 asphyxiation, the jugular ganglia were rapidly dissected and cleared of adhering connective tissue. Isolated ganglia were incubated in the enzyme buffer (1 mg ml^{-1} collagenase type 1A and 1 mg ml^{-1} dispase II in 5 ml Ca^{2+}-, Mg^{2+}-free Hanks' balanced salt solution) for 2 h at 37°C. Neurones were dissociated by trituration with three glass Pasteur pipettes of decreasing tip pore size, then washed by centrifugation (three times at 700 *g* for 45 s) and suspended in L-15 medium containing 10% fetal bovine serum (FBS). The cell suspension was transferred onto circular 25 mm glass coverslips (Bellco

Glass, Inc., Vineland, NJ, USA) coated with poly D-lysine (0.1 mg ml^{-1}). After the suspended neurones had adhered to the coverslips for 2 h, the neurone-attached coverslips were flooded with the L-15 medium (10% FBS) and used within 24 h. For chelating intracellular Ca^{2+}, in some experiments BAPTA-AM (30 μM × 40 min) was added to the medium before recording.

Whole-cell patch clamp

The jugular neurones labelled from the airways were identified under a fluorescent microscopy using 560 nm of excitation filter and 480 nm of emission filter. To maintain intracellular signal pathways and a native intracellular Cl^- concentration, a gramicidin-perforated whole-cell patch-clamp technique was employed using Multiclamp 700A amplifier and Axograph 4.9 software (Axon Instruments). A pipette (1.5–3 MΩ) was filled with a pipette solution composed of (mM) 140 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, 11 EGTA and 10 dextrose; titrated to pH 7.3 with KOH; 304 mosmol l^{-1}. Gramicidin was dissolved in DMSO and mixed with the pipette solution for a final concentration of 1 μg ml^{-1} just prior to each recording. Cell membrane potential was held at –60 mV. During the experiments, the cells were continuously superfused (6 ml min^{-1}) by gravity with Locke solution; composition (mM): 136 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2.2 $CaCl_2$, 1.2 NaH_2PO_4, 14.3 $NaHCO_3$ and 10 dextrose (pH 7.3–7.4). All recordings were done at 35°C.

Intracellular Ca^{2+} measurement

The intracellular Ca^{2+} measurements were done with dissociated jugular neurones irrespective of DiI labelling in six animals. The coverslip was loaded with Fura-2 AM (8 μM) in L-15 media containing 20% FBS and incubated for 40 min at 37°C. The coverslip was placed in a custom-built chamber (600 μl bath volume) that was superfused with Locke solution (at 35°C) for 20 min before the experiment by an infusion pump (8 ml min^{-1}).

Changes in intracellular free Ca^{2+} were measured by digital microscopy (Universal: Carl Zeiss, Inc., Thornwood, NY, USA) equipped with in-house equipment for ratiometric recording of single cells. A field of cells was monitored by sequential dual excitation, 352 and 380 nm, and the analysis of the image ratios used methods previously described (MacGlashan, 1989). The ratio images were acquired every 6 s. Superfused buffer was stopped 20 s prior to drug applications. In each experiment the cells on the coverslip were exposed to BK for 1 min (in the presence or absence of antagonists). This was followed 5 min later with a brief exposure to capsaicin (30 s, 1 μM). Beginning at least 5 min after the capsaicin exposure, the cells were also exposed to KCl (20 s, 75 mM) and then ionomycin (1 min, 1 μM). The KCl was used as an indicator of voltage-sensitive cells, and the ionomycin was used to obtain a maximum obtainable response. Between each stimulus, the cells were washed with fresh buffer for at least 4 min for the cells to recover prior to the addition of the second stimulus. Each set of images for the Ca^{2+} measurements also included a brightfield image of the field of cells under study. Cells that had an average diameter (long and short axis) of over 15 μm were analysed. Those cells that failed to respond to capsaicin were considered healthy neurones only if they responded to KCl with a rapid rise in Ca^{2+}.

Drug preparations and applications

BK (1 μM) and HOE140 (1 μM) were diluted from the aliquotted stock solution (1 mM dissolved in distilled water). Stock solutions of NFA (100 mM), NPPB (100 mM), I-RTX (1 mM) and BAPTA-AM (30 mM) were dissolved in DMSO and diluted with buffer. Capsaicin (0.3 or 1 μM) was diluted from a stock solution (10 mM, dissolved in ethanol) with the buffer. I-RTX was purchased from Tocris Cookson (Ellisvill, MO, USA). BK, HOE140, NFA, NPPB, BAPTA-AM, capsaicin and ionomycin were purchased from Sigma-Aldrich (St Louis, MO, USA). Fura-2 AM was purchased from Molecular Probes (Eugene, OR, USA). L-15 and Hanks' balanced solution were purchased from Gibco Invitrogen Corp. (Carlsbad, CA, USA).

Data analysis

There was little (<1 Hz) or no background activity in the extracellular recordings. In >90% of the experiments, a single unit was recorded. In those instances where more than one unit was being recorded, standard spike analysis was employed to distinguish the tracheal fibre from the background. The action potential discharge evoked by BK stimulation was quantified off-line and segregated into consecutive 1 s bins. The response was considered to be terminated when the number of spikes in the bins declined to <2 × baseline. The total number of action potentials recorded following BK, citric acid or capsaicin application was counted. For Ca^{2+} measurements, the increase in intracellular free Ca^{2+} induced by BK was normalized and expressed as a percentage of the increase induced by ionomycin (1 μM). The neurones were considered to have responded to BK (1 μM) or capsaicin (1 μM) if the drug-induced peak increase was greater than 3 × standard deviation of the baseline intracellular Ca^{2+} concentration. All data are expressed as means ± S.E.M. Student's *t* test, paired *t* test, Mann–Whitney Rank Sum test, one-way ANOVA test with Student–Newman–Keul's test for multiple comparisons, and χ^2 test were used for

statistical analysis when appropriate, and a P value less than 0.05 was taken as a significant difference.

Results

Thirty-one jugular capsaicin-sensitive C-fibres were recorded (one fibre per animal). BK evoked action potential discharge in 29/31 capsaicin-sensitive C-fibres innervating the guinea pig trachea/bronchus. Consistent with our previous findings (Carr *et al.* 2003), there was evidence of desensitization when the tissue was exposed to two consecutive BK (1 μM) treatments administered at 30 min intervals. The total number of action potentials evoked by the first BK response averaged 100 ± 11 ($n = 26$). The second BK response, obtained 30 min later, averaged 63 ± 3% of the first response (Fig. 1).

NFA (100 μM), a CLCA inhibitor, applied for 15 min prior to the second BK (1 μM) application, significantly inhibited the BK response as compared with the control. In the presence of NFA, the BK averaged 21 ± 7% of the first response ($P < 0.01$ compared with vehicle control, $n = 5$, Fig. 1). NFA had no effect on the citric acid response. The number of action potentials evoked by citric acid (10 mM) averaged 75.8 ± 14.3 and 74.0 ± 22.6 in the absence and presence of NFA, respectively ($n = 4$).

The inhibitory effect of NFA on BK responses was mimicked by a different CLCA blocker, NPPB. In the presence of NPPB (100 μM), the BK response averaged 28 ± 7% of the first response ($P < 0.05$, $n = 5$, Fig. 1).

We utilized a paired experimental design for the data in Fig. 1, as it allowed for the evaluation of a single nerve response before and after treatment with the inhibitors. The data obtained with this paired design, however, could be interpreted as NFA and NPPB enhancing the rate of BK desensitization (or inhibiting recovery from desensitization), rather than inhibiting the BK response *per se*. To address this issue, another series of experiments was carried out using a nonpaired design, such that the tissue was treated only once with BK, 30 min after either NFA or vehicle was applied to the tissue. The BK response was normalized to the response to capsaicin (0.3 μM) added at the end of the experiment. NFA did not affect the capsaicin response, which averaged 140 ± 46 and 161 ± 30 action potentials in control and NFA-pretreated tissues, respectively ($P > 0.1$). In control tissues ($n = 9$), the BK response averaged 109 ± 26% of the capsaicin response. In NFA-treated tissue, the BK response averaged 21 ± 6% of the capsaicin response ($P < 0.05$, $n = 5$, Fig. 2).

Using the gramicidin-perforated-patch technique, we found that BK induced membrane depolarization in tracheal labelled jugular ganglion neurones. Consistent with the observations at the nerve terminals within the airway, the BK (1 μM)-induced membrane depolarization reached the threshold for action potential discharge at the cell body. The selective $GABA_A$ receptor agonist muscimol (1 μM) also produced membrane depolarization and action potential discharge in BK-sensitive neurones ($n = 2$, Fig. 3*A*). This indirectly supports the hypothesis that the reversal potential of Cl^- in BK-sensitive neurones was above the action potential threshold.

BK caused a large inward current in airway labelled voltage-clamped jugular neurones. To study intracellular Ca^{2+} involvement in the mechanism of BK-induced inward current, the neurones were loaded with 30 μM BAPTA-AM. After BAPTA pretreatment, the amplitude of BK-induced inward current averaged 4.7 ± 2.2 pA pF^{-1}

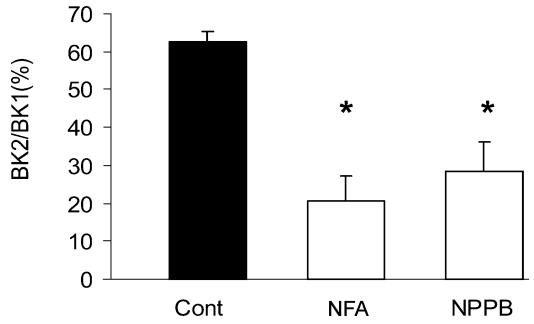

Figure 1. The effect of the Ca^{2+}-activated Cl^- channel (CLCA) blockers niflumic acid and NPPB on bradykinin-induced activation of jugular C-fibres in guinea pig airways
Niflumic acid (NFA; $n = 5$, 100 μM) and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB; $n = 5$, 100 μM) were applied 15 min prior to the second bradykinin (BK; 1 μM) application. The interval between the two BK applications was 30 min. In the control group, the second response to BK was desensitized to 63 ± 3% of the first response to BK ($n = 5$). Both NFA and NPPB reduced the BK response to 21 ± 7 and 29 ± 8%, respectively. They were significantly different from control group (*$P < 0.05$).

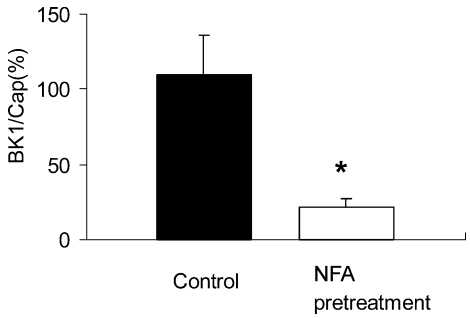

Figure 2. The effect of NFA on the response to the first BK application to guinea pig airway jugular C-fibres
To determine whether NFA facilitates desensitization or blocks the response to BK, the preparation was pretreated with NFA (100 μM) for 30 min, and then the response to BK (1 μM) was normalized to the capsaicin (0.3 μM) response (BK1/Cap%). The response to capsaicin was not different between the control ($n = 9$) and NFA-pretreated groups ($n = 5$) in the number of evoked action potentials (140 ± 46 and 161 ± 30, respectively). BK1/Cap (%) in the NFA-pretreated group was significantly smaller than vehicle treated group (21 ± 6% *versus* 109 ± 26%, $P < 0.05$, *t* test).

($n = 7$) compared with 15.3 ± 4.7 pA pF^{-1} in naïve neurones ($n = 12$). When BK (1 μM) was applied at 10 min intervals, the amplitude of the inward current induced by the second BK exposure averaged less than 20% of the first response. Because of this desensitization, BK was applied only once to a neurone, and the ability of NFA to reverse the response was quantified. A 30 s application of BK induced an inward current with a peak occurring at ~20 s, followed by a slow decay back to baseline (Fig. 3*B*). The half-life ($t_{1/2}$) from the peak current back to baseline averaged 18 ± 4 s ($n = 12$). When NFA was added 15 s after the onset of the BK exposure, the BK-induced inward current was quite abruptly reversed in 5/5 neurones with a $t_{1/2}$ of 1 ± 0.1 s ($n = 5$, $P < 0.05$ compared with control, Fig. 3*C*). In 4/5 neurones, a small component of the BK current remained after NFA treatment (Fig. 3*C*). This NFA-resistant component of the current averaged (407 ± 154 pA) or ~20% of the peak current.

We next tested whether NFA nonselectively inhibited the signalling pathway between B_2 receptor activation and its induced changes in intracellular Ca^{2+}. To this end, the BK-induced increases in intracellular free Ca^{2+} concentration were studied in capsaicin (1 μM)-sensitive neurones dissociated from jugular ganglia. BK (1 μM) caused a transient increase in intracellular free Ca^{2+} in 18 of 31 capsaicin-sensitive jugular ganglion neurones. The peak rise in Ca^{2+} averaged 18 ± 2% of that obtained with ionomycin (1 μM). Pretreatment with the B_2 receptor selective antagonist, HOE140 (1 μM), significantly inhibited the BK-induced Ca^{2+} increase. After 5 min pretreatment of HOE 140, 7/38 capsaicin-sensitive cells responded to BK ($P < 0.01$ *versus* control, χ^2 test), and the peak response (4.6 ± 0.5%, $n = 7$) was significantly smaller than the control response ($P < 0.001$ *versus* control, *t* test). Treating the neurones with NFA (100 μM) did not inhibit the intracellular Ca^{2+} increase induced by BK (Fig. 4). In NFA-treated neurones, BK caused an intracellular free Ca^{2+} increase in 18/33 capsaicin-sensitive jugular neurones, with peak responses that averaged 26 ± 6% of the ionomycin peak ($P = 0.7$, Mann–Whitney rank sum test). In addition, the capsaicin-induced intracellular Ca^{2+} increase was not changed by NFA treatment. The peak rises were 74 ± 9% ($n = 18$) and 72 ± 8% ($n = 18$) of the ionomycin peak in control and NFA-treated neurones, respectively.

Although both NFA and NPPB partially inhibited the BK-induced action potential discharge in the tracheal C-fibres, in most fibres, neither antagonist abolished the

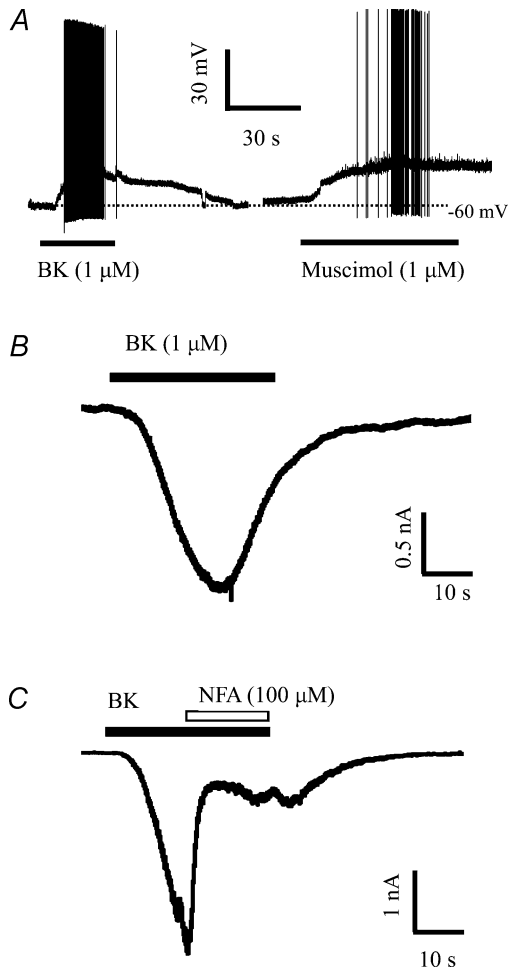

Figure 3. BK response in isolated jugular capsaicin-sensitive neurones in gramicidin-perforated patch-clamp recordings
A, BK and muscimol (selective $GABA_A$ receptor agonist) evoked membrane depolarization and action potential discharge in current-clamp mode. *B*, BK (1 μM, for 30 s) produced a large inward current in airway labelled capsaicin-sensitive neurones (representative of 12 neurones). *C*, rapid reversal of the BK-induced current by NFA (100 μM for 15 s) applied 15 s after BK (1 μM, for 30 s) application (representative of 5 neurones).

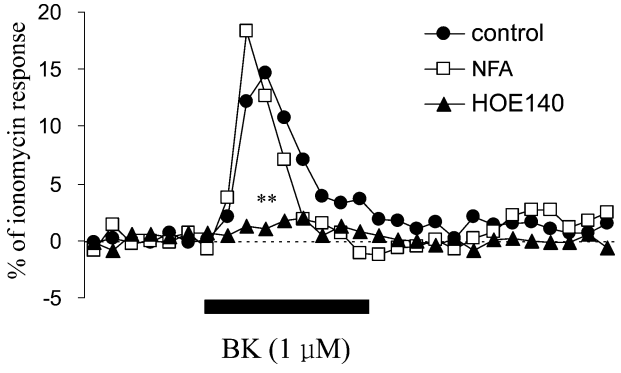

Figure 4. The effect of NFA on the intracellular Ca^{2+} increase induced by BK (1 μM, 1 min) in dissociated capsaicin-sensitive jugular neurones
NFA did not affect the intracellular free Ca^{2+} increases induced by BK. The peak values of the normalized BK-induced $[Ca^{2+}]_i$ increase (relative to the response induced by 1 μM ionomycin) were 18 ± 2 and 26 ± 6% in control ($n = 18$) and NFA ($n = 18$), respectively. HOE140 (1 μM, selective B_2 receptor antagonist), however, inhibited the BK-induced $[Ca^{2+}]_i$ increase (**$P < 0.01$, *t* test, $n = 6$). The data were acquired every 6 s.

response. In a previous study, we reported similar results using the nonselective TRPV1 antagonists capsazepine and ruthenium red (Carr *et al.* 2003). We next addressed the hypothesis that both TRPV1 and Cl^- channels contribute to the overall BK response in an additive fashion. A selective TRPV1 antagonist, I-RTX, as expected, partially inhibited the BK response in a fashion similar to NFA. In the presence of I-RTX (1 μM) the BK response averaged 38 ± 9% of the first BK response ($P < 0.05$, Fig. 5*A*).

The combination of I-RTX and NFA essentially abolished the response to BK in 5/5 fibres. Overall, the response to BK in the presence of I-RTX and NFA was 4 ± 3% of the first response (Fig. 5*A*). The combination of I-RTX and NFA did not significantly inhibit the action potential discharge evoked by citric acid ($P > 0.1$, Fig. 5*B*).

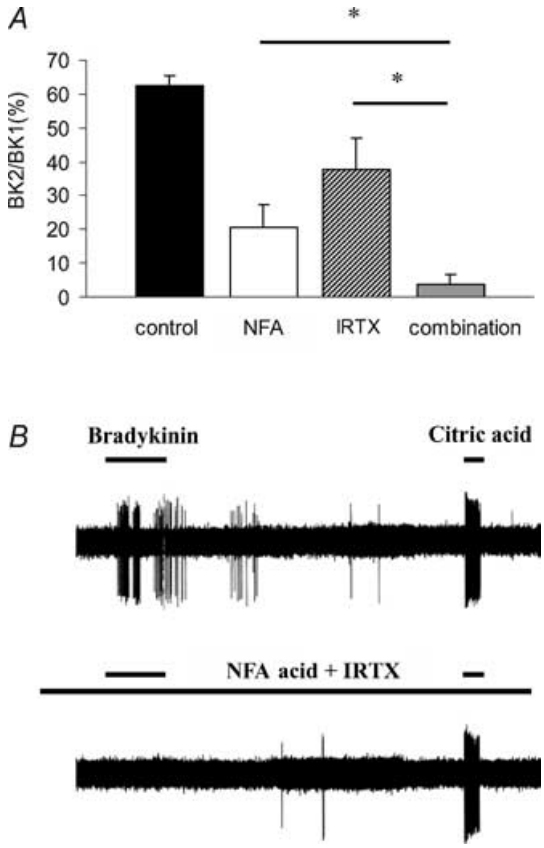

Figure 5. The effect of the combination of CLCA blocker (niflumic acid) and TRPV1 inhibitor (I-RTX) on bradykinin-induced action potential responses in guinea pig airway jugular C-fibers

A, the combination of niflumic acid (100 mM) and I-RTX (1 mM) virtually abolished the bradykinin-induced response. It was different from both niflumic acid alone (n = 5) and I-RTX alone (n = 5; *$P <$ 0.05, Student-Newman-Keul's test for multiple comparisons), which indicates that both activation pathways were independent to each other. *B,* representative trace of extracellular recording in combination treatment group. The response to bradykinin was completely blocked by the combination treatment but the response to citric acid (10 mM, 200 ml) was preserved.

Discussion

The results support the hypothesis that BK B_2 receptor activation causes a generator potential that can lead to action potential discharge in vagal airway C-fibre terminals by the opening of Ca^{2+}-activated Cl^- channels and TRPV1. When both of these ion channels are pharmacologically blocked, BK is essentially incapable of evoking an action potential discharge in the airway vagal C-fibres.

BK B_2 receptors are typically Gq-coupled receptors that are coupled to phospholipase C activation, resulting in products of phospholipid metabolism that induce activation of protein kinase C (PKC) and increases in intracellular Ca^{2+} (Wilk-Blaszczak *et al.* 1994). Studies at the level of the cell soma have elegantly demonstrated that signalling events subsequent to BK B_2 receptor activation can lead to the opening of TRPV1 in primary sensory neurones. This may occur either through PKC reactions (Premkumar & Ahern, 2000), indirectly through the production of lipoxygenase products of arachidonic acid (Hwang *et al.* 2000), or through the removal of the inhibitory action of PtdIns(4,5)P2 on TRPV1 (Chuang *et al.* 2001). A role for TRPV1 in BK-induced activation of C-fibre terminals has been suggested by pharmacological studies in isolated airway preparations and in whole-animal studies on nociception (Shin *et al.* 2002; Carr *et al.* 2003; Ferreira *et al.* 2004). How much TRPV1 contributes to the overall BK-induced C-fibre activation is not clear. In our previous studies on BK-induced action potential discharge in airway C-fibres using nonselective inhibitors such as capsazepine and ruthenium red, it appeared as if TRPV1 could account for as much as 80% of the total response (Carr *et al.* 2003). It is likely, however, that some of the inhibitory effects of large concentrations of capsazepine and ruthenium red observed in these studies were due to nonselective effects of these compounds. The results presented here using I-RTX, a TRPV1 antagonist much more selective than capsazepine (Wahl *et al.* 2001; Undem & Kollarik, 2002), indicate that TRPV1 contributes to, on average, less than half of the overall BK response in airway C-fibres. In similar types of *ex vivo* studies using TRPV1 knockout mice, the response of vagal airway C-fibres to BK was 65% of the overall response observed in the wild-type mouse (Kollarik & Undem, 2004).

The question addressed in the present study relates to what mechanism(s) other than TRPV1 account for BK B_2 receptor-mediated action potential discharge in airway C-fibre afferent nerves. Our data support the conclusion that activation of a Cl^- current is the dominant non-TRPV1 mechanism by which the terminal membranes are depolarized to action potential threshold. This conclusion is supported by pharmacological and electrophysiological evidence

NFA was used as a tool to block Cl^- channels. It could be argued that NFA inhibited the response to BK through some unknown action independently of Cl^- channels. Although this must remain a potential explanation, several results argue against this interpretation. First, the effect of NFA was selective for B_2 (G-protein-coupled) receptor-induced action potential discharge, having no effect on capsaicin- or citric-acid-induced responses. We have previously revealed that in this same experimental design, a rapid drop in pH activates the C-fibre mainly by gating certain types of acid sensing ion channels (Kollarik & Undem, 2002). Second, the data indicate that the inhibitory effect of NFA occurred distal to BK-induced signalling events in the neurones leading to increases in intracellular free Ca^{2+}. Third, the effect of NFA was mimicked by a second structurally unrelated arylaminobenzoate Cl^- channel inhibitor, NPPB (Jentsch *et al.* 2002).

The conclusion that the opening of Cl^- channels contributes to the C-fibre terminal membrane depolarization and action potential discharge is also supported by whole-cell patch-clamp studies of the somal membrane of guinea pig vagal sensory neurones (Oh & Weinreich, 2004; and present study). When evaluated under current clamp conditions, BK evoked the expected membrane depolarization and action potential discharge. In voltage-clamp experiments, BK evoked a large inward current that was associated with a decrease in membrane impedance. Oh & Weinreich (2004) concluded that these BK-induced events were found to be due, in large part, to the opening of Cl^- channels. Their conclusion was based not only on pharmacological studies (the current was inhibited by NFA), but also by studies of reversal potential and ion substitution studies.

Oh & Weinreich (2004) evaluated guinea pig nodose ganglion neurones, whereas the C-fibres in the guinea pig trachea are derived from jugular ganglion neurones. Schlichter *et al.* (1989) provided evidence that the extent to which CLCA is expressed in trigeminal sensory neurones may be dependent on whether the neurones are embryologically derived from the neural crest or placodes. This is interesting in the present context, as the jugular C-fibres innervating the guinea pig trachea are derived from the neural crest and are phenotypically distinct from the placodal-derived nodose C-fibres (Riccio *et al.* 1996; Undem *et al.* 2004). Although we thought it would be unproductive to repeat the detailed analysis of BK-induced currents presented by Oh & Weinreich (2004), our findings that BK evoked a large NFA-sensitive current in jugular neurones, suggests that functional Cl^- channels are present in both placodal and neural crest derived vagal sensory neurones. Although our data provide little information on the nature of the Cl^- channels involved, the finding that BAPTA-AM inhibited this current is consistent with the hypothesis that BK may activate chloride currents through Ca^{2+}-dependent mechanisms.

It is known that adult primary sensory nerves concentrate intracellular Cl^- such that the equilibrium potential for Cl^- (E_{Cl}) is more positive than the resting membrane potential (Alvarez-Leefmans *et al.* 1988; Rohrbough & Spitzer, 1996). That the $GABA_A$ receptor agonist muscimol was capable of evoking action potential discharge in tracheal-specific jugular ganglion neurones indicates that the reversal potential for Cl^- in these cells is in fact more positive than the action potential threshold. The large intracellular Cl^- concentration in primary afferent nerves can be explained by the effect of the electrically silent movement of Na^+, K^+ and Cl^- across the membrane via the activity of a Na^+–K^+–$2Cl^-$ cotransporter (NKCC) (Haas & Forbush, 1998). There are two isoforms of NKCC termed NKCC1 and NKCC2, with the former expressed in primary afferent neurones (Plotkin *et al.* 1997; Delpire, 2000). The Cl^- current reversal potential is shifted to more negative values in neurones isolated from dorsal root ganglia of NKCC1 knockout mice (Sung *et al.* 2000).

The hypothesis that Cl^- efflux is a major contributor to BK-induced action potentials in vagal afferent C-fibre terminals is analogous to odorant receptor potentials in the dendrites of olfactory neurones. Recent studies indicate that dendrites of olfactory sensory nerves concentrate Cl^- through the action of NKCC1 (Kaneko *et al.* 2004; Reisert *et al.* 2005). Odorants interact with G-protein-coupled receptors that lead to the opening of cAMP-gated cation channels. The consequential increase in cytosolic Ca^{2+} opens Cl^- channels leading to Cl^- efflux and membrane depolarization (Kurahashi & Yau, 1993). Our results indirectly support the hypothesis that vagal afferent C-fibre nerve terminals also concentrate Cl^- ions. If this is the case, any mechanism by which Cl^- channels are opened (e.g. increasing intracellular Ca^{2+} via G-protein-coupled receptors, or via ligand-gated Cl^- channels) can be expected to depolarize the terminal membranes and, if the stimulus is adequate, to evoke action potential discharge.

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