A Role for TRPV1 in Bradykinin-Induced Excitation of Vagal Airway Afferent Nerve Terminals

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ABSTRACT

Using single-unit extracellular recording techniques, we have examined the role of the vanilloid receptor-1 (VR1 aka TRPV1) in bradykinin-induced activation of vagal afferent C-fiber receptive fields in guinea pig isolated airways. Of 17 airway C-fibers tested, 14 responded to bradykinin and capsain, 2 fibers responded to neither capsain nor bradykinin, and 1 fiber responded to capsain but not bradykinin. Thus, every bradykinin-responsive C-fiber was also responsive to capsain. Bradykinin (200 μL of 0.3 μM solution) evoked a burst of approximately 130 action potentials in C-fibers. In the presence of the TRPV1 antagonist capsazepine (10 μM), bradykinin evoked 83 ± 9% (n = 6; P < 0.01) fewer action potentials. Similarly, the TRPV1 blocker, ruthenium red (10 μM), inhibited the number of bradykinin-evoked action potentials by 75 ± 10% (n = 4; P < 0.05). In the presence of 5,8,11,14-eicosatetraynoic acid (10 μM), an inhibitor of lipoxygenase and cyclooxygenase enzymes, the number of bradykinin-induced action potentials was reduced by 76 ± 10% (n = 6; P < 0.05). Similarly, a combination of the 12-lipoxygenase inhibitor, baicalein (10 μM) and the 5-lipoxygenase inhibitor ZD2138 [6-[3-fluoro-5-[4-methoxy-3,4,5,6-tetrahydro-2H-pyranyl-4-yl]phenoxy-methyl]-1-methyl-2-quinolone] (10 μM) caused significant inhibition of bradykinin-induced responses. Our data suggest a role for lipoxygenase products in bradykinin B2 receptor-induced activation of TRPV1 in the peripheral terminals of afferent C-fibers within guinea pig trachea.

The pharmacological activation of primary afferent neurons often involves the opening of ligand-gated ion channels such as 5-hydroxytryptamine3 receptors, P2X purinoceptors, nicotinic acetylcholine receptors, and vanilloid receptor 1 (TRPV1), the first cloned capsaicin receptor (Wood and Docherty, 1997). Upon agonist binding to these channels, their ion pore opens, allowing the influx of cations, resulting in a membrane depolarization of sufficient magnitude to initiate action potentials. Bradykinin, an endogenous metabolite of the kallikrein-kinin system often associated with inflammation, also directly activates nociceptive-like afferent neurons, but does so via metabotropic G protein-coupled bradykinin B2 receptors (McGuirk and Dolphin, 1992; Fox et al., 1993; Bevan, 1996; Kajekar et al., 1999; Maubach and Grundy, 1999). The ionic mechanism coupling bradykinin B2 receptors to initiation of action potentials in the peripheral terminals of vagal afferent neurons is unknown.

Several studies have provided data suggesting that at least some of the effects of bradykinin are secondary to the mobilization of arachidonic acid in afferent neurons. Bradykinin B2 receptor stimulation evokes the release of arachidonic acid in afferent neurons (Burgess et al., 1989; Gammon et al., 1989; Allen et al., 1992) and depolarizes the membrane potential of vagal (Undem and Weinreich, 1993; Kajekar et al., 1999) and dorsal root ganglion (Burgess et al., 1989; McGuirk and Dolphin, 1992) neuron cell bodies. In addition, bradykinin inhibits a calcium-dependent potassium current responsible for an afterspike hyperpolarization in nodose ganglion neurons (Weinreich et al., 1995). These effects are likely to be mediated in part by arachidonic acid metabolites derived from phospholipids in neuronal membranes inasmuch as the effect on the afterspike-hyperpolarization in acutely isolated nodose neurons was abolished by the cyclooxygenase inhibitor, indomethacin (Weinreich et al., 1995) and the lipoxygenase inhibitor, norhydroguaiaretic acid inhibited bradykinin-induced trains of action potentials in rat cultured dorsal root ganglion neurons (McGuirk and Dolphin, 1992).

Recent studies have found that agonist binding to B2 receptors can activate an intracellular signaling cascade leading to the opening of TRPV1 (Premkumar and Ahern, 2000; Chuang et al., 2001), and that 5-, 12-, and 15-lipoxygenase products may act as endogenous TRPV1 agonists (Hwang et al., 2000, Shin et al., 2002). The extent to which lipoxygenase products contribute to bradykinin B2 receptor-mediated ac...
tivation of TRPV1 in the peripheral terminals of vagal afferent neurons is unknown. In this study, we provide data suggesting that the activation of guinea pig tracheal C-fibers by bradykinin is, at least in part, dependent on the activation of TRPV1 and that this coupling involves the generation of lipoygenase metabolites that have agonist activity at TRPV1.

**Materials and Methods**

**Tissue Preparation.** Guinea pigs were killed by CO₂ inhalation and exsanguinated. The trachea/bronchus was prepared, as previously described (Riccio et al., 1996), for extracellular recording of action potentials from jugular vagal afferent nerve fibers that have defined receptive fields in the airway wall. The airways with intact right-side extrinsic vagal innervation (including nodose and jugular ganglia) were removed and placed in a dissecting dish containing Krebs’ bicarbonate buffer solution (KBS) gassed with 95% O₂-5% CO₂ and composed of 118 mM NaCl, 5.4 mM KCl, 1.0 mM NaH₂PO₄, 1.2 mM MgSO₄, 1.9 mM CaCl₂, 25.0 mM NaHCO₃, 11.1 mM dextrose. Connective tissue was trimmed away, leaving the trachea, larynx, and right mainstem bronchus with intact nerves (vagus, superior laryngeal, and recurrent laryngeal), including nodose and jugular ganglia. A longitudinal cut was made through the ventral surface of the larynx, trachea, and bronchi, and the airways were then pinned, as a flat sheet with the mucosal side up, to a Silgard-lined Perspex chamber. The right nodose and jugular ganglia, along with the rostral most vagus and superior laryngeal nerves, were gently pulled through a small hole into an adjacent compartment of the same chamber for recording of single fiber activity with 3 mM sodium chloride-filled glass microelectrodes as described previously (Riccio et al., 1996). The tissues in both compartments were superfused with KBS, and the temperature was maintained at 37°C with a flow rate of 6 to 8 ml min⁻¹. Conduction velocities were calculated by electrically stimulating the receptive field and measuring the distance traveled along the nerve pathway divided by the time between the shock artifact and the recorded action potential. Only mechanically sensitive neurons were studied; these fibers had little or no activity at rest; if spontaneous activity exceeded 1 action potential s⁻¹, the fiber was not studied further.

**Application of Agonists, Antagonists, and Inhibitors.** Responses of identified airway afferent C- or Aδ-fiber receptive fields to bradykinin or capsaicin were determined by application of 200 μl (over 3 s) of buffer containing a 0.3 μM solution of either capsaicin or bradykinin directly to the receptive field located in the isolated trachea/bronchus and recording the total number of evoked action potentials. In experiments with inhibitors/antagonists, 200 μl of buffer containing 0.3 μM bradykinin was applied to receptive fields before and 20 min after addition of the inhibitor/antagonist of interest to the buffer supplying the tracheal compartment of the Plexiglas chamber. For capsaicin studies we used an unpaired design because of the established desensitizing effects of capsaicin. To determine the influence of inhibitors/antagonists on capsaicin-evoked responses, the tracheal compartment was perfused with either KBS containing vehicle or the inhibitor/antagonist of interest for 20 min before application of capsaicin.

**Data Analysis.** The responses of afferent fibers were presented as the total number of action potentials recorded following application of bradykinin or capsaicin to receptive fields in the airway. Data obtained with drug were compared with vehicle control using analysis of variance, followed by a Student’s nonpaired t test.

**Drugs and Chemicals.** Bradykinin (Peninsula Laboratories, Belmont, CA), ruthenium red, and DesArg⁹-bradykinin (Sigma-Aldrich, St. Louis, MO) were diluted in distilled water. Indomethacin (Sigma-Aldrich), capsaicin (Sigma-Aldrich), and 5,8,11,14-eicosatetraynoic acid (ETYA) (Sigma-Aldrich) were diluted in ethanol. Arachidonyl trifluoromethyl ketone (AACOCF₃) and RHC 80267 (Calbiochem, San Diego, CA), baicalein (Cayman Chemical, Ann Arbor, MI), and ZD2138 (Zeneca, Wilmington, DE) were diluted in dimethyl sulfoxide. Further dilutions to final concentration were made in KBS on the day of use.

**Results**

**Concordance of Responsivity of Afferent Fibers to Capsaicin and Bradykinin.** The peripheral terminals of vagal afferent fibers whose cell bodies resided in the jugular ganglion and whose receptive fields were identified in the trachea or primary bronchus were studied. These nerve terminals were derived from axons that conducted action potentials in the C or Aδ range and were used to estimate the proportion of fibers that were responsive to bradykinin and capsaicin, bradykinin only, capsaicin only, or neither bradykinin nor capsaicin. Of 17 Aδ fibers studied, nine responded with action potential discharge to both bradykinin and capsaicin, seven responded to neither capsaicin nor bradykinin, and one fiber responded to capsaicin, but not bradykinin. Among the 17 C-fibers studied, 14 responded to both capsaicin and bradykinin, two fibers responded to neither agonist, and one fiber responded to capsaicin but not bradykinin. Thus, we found no fiber that responded to bradykinin, but not capsaicin. All subsequent studies were carried out on C-fibers, and indomethacin (3 μM) was added to the buffer solution at the beginning of the experiment.

**Capsazepine and Ruthenium Red.** To evaluate the role of TRPV1 in bradykinin-induced action potential discharge in airway C-fiber endings, responses to bradykinin (200 μl of a 0.3 μM solution) were recorded before and after a 20-min incubation with either the TRPV1 antagonist capsazepine (10 μM) or the TRPV1 channel blocker ruthenium red (10 μM). These experiments were confounded somewhat by a modest tachyphylaxis noted upon two consecutive exposures of bradykinin in the presence of vehicle alone. Bradykinin (0.3 μM) evoked 97 ± 14 and 77 ± 13 action potentials before and after vehicle treatment (P < 0.05, n = 14). In the presence of capsazepine, bradykinin evoked 83 ± 9% (n = 6; P < 0.01) fewer action potentials. Similarly, in the presence of ruthenium red, bradykinin evoked 75 ± 10% (n = 4; P < 0.05) fewer action potentials. The effect of either capsazepine or ruthenium red was significantly greater than that observed with vehicle alone (P < 0.01) (Fig. 1). In contrast, neither capsazepine (10 μM) nor ruthenium red (10 μM) reduced the mechanical sensitivity of airway C-fiber receptive fields (data not shown).

**Lipoxygenase Inhibitors.** Because lipoxygenase products of arachidonic acid may act as endogenous TRPV1 agonists (Hwang et al., 2000), we examined the influence of inhibitors of arachidonic acid metabolism on bradykinin-induced action potential discharge in guinea pig airway C-fibers. ETYA, an inhibitor of lipoxygenase and cyclooxygenase enzymes, caused significant inhibition of bradykinin-induced responses in C-fibers. In the presence of ETYA (10 μM), the number of bradykinin-induced action potentials was reduced by 76 ± 10% (n = 6; P < 0.05, relative to vehicle) (Fig. 2). Similarly, the 12-lipoxygenase inhibitor, baicalein (10 μM), caused significant inhibition of bradykinin-induced responses. In four of five C-fibers tested, baicalein caused a 60 ± 6% (P < 0.05) inhibition of bradykinin-induced responses. In the remaining C-fiber, application of bradykinin
ETYA, this fiber still responded to capsaicin (200 μM) and after perfusion with buffer containing ETYA (B). In the presence of ETYA (200 μM), bradykinin-evoked responses of a C-fiber before (A) and after perfusion with buffer containing capsaicin (B). The horizontal bar represents 60 s.

Fig. 2. Influence of ETYA on bradykinin-induced activation of C-fibers in guinea pig isolated trachea. Bradykinin (200 μl of a 0.3 μM solution) was applied before (Control) and after a 20-min perfusion with buffer containing ETYA (10 μM, n = 6). Data are expressed as the total number of action potentials evoked by bradykinin. An asterisk (*) denotes a significant decrease in the treatment group relative to the paired control. Insets show typical traces of bradykinin-evoked responses of a C-fiber before (A) and after perfusion with buffer containing ETYA (B). The horizontal bar represents 60 s.

**Discussion**

We provide data suggesting that the activation of guinea pig tracheal afferent nerve terminals by bradykinin is at least partially dependent on the activation of TRPV1. Our findings are consistent with the hypothesis that the activation of bradykinin B2 receptors in peripheral terminals of afferent neurons is coupled to the generation of lipoxygenase metabolites that have agonist activity at TRPV1. Furthermore, this appeared to be a means by which bradykinin evokes action potentials in the peripheral terminals of vagal afferent C-fibers that innervate guinea pig airways.

Bradykinin directly excites primary afferent neurons, including those with receptive fields in the airways, via metabotropic G protein-coupled bradykinin B2 receptors (McGuirk and Dolphin, 1992; Fox et al., 1993; Bevan, 1996; Kajekar et al., 1999; Maubach and Grundy, 1999). However, the molecular mechanisms ultimately responsible for a bradykinin B2 receptor-mediated membrane depolarization of sufficient magnitude to initiate action potential in the peripheral endings of primary vagal afferent nerve endings is unknown. In this study, we found a near-perfect concordance with respect to a given airway afferent fiber response to capsaicin and bradykinin. This led us to hypothesize that TRPV1 plays a role in bradykinin B2 receptor-mediated ac-
tion potential discharge in the peripheral terminal vagal afferent fibers that innervate guinea pig airways.

Consistent with this hypothesis, the TRPV1 antagonist capsazepine markedly inhibited bradykinin-induced action potential discharge in C-fibers. Capsazepine is selective for TRPV1 but may not be specific in its action. However, three additional lines of evidence favor the hypothesis that TRPV1 activation contributed to bradykinin-induced action potential discharge. First, the TRPV1 channel blocker ruthenium red also caused marked inhibition of bradykinin-induced action potential discharge. Second, recent studies from other laboratories demonstrated that the TRPV1 channel in membrane patches of afferent neuron cell bodies and transfected cells can be signaled to open by the activation of bradykinin B2 receptors (Premkumar and Ahern, 2000; Chuang et al., 2001). Finally, although capsazepine and ruthenium red markedly reduced responses to bradykinin, they had no influence on the mechanical responsiveness of primary afferent nerve receptive fields, indicating that these compounds did not have a nonselective inhibitory action on neuronal excitability. Combined, these findings favor the idea that the activation of TRPV1 plays a significant role in bradykinin B2 receptor-evoked action potential discharge in the peripheral terminals of afferent neurons within guinea pig airways.

The binding of bradykinin to B2 receptors in afferent neurons stimulates a variety of intracellular events (Bevan, 1996), including the mobilization of arachidonic acid (Burgess et al., 1989; Gammon et al., 1989; Allen et al., 1992). We have previously found that guinea pig airway C-fiber responses to bradykinin were not inhibited by indomethacin (Kajekar et al., 1999), indicating that cyclooxygenase products are not primarily responsible for the excitatory action of bradykinin, although they play a role in bradykinin-induced sensitization of afferent neurons in other tissues (Dray et al., 1992; Weinreich et al., 1995; Maubach and Grundy, 1999). In contrast, lipoxygenase products may play a central role in bradykinin-induced activation of guinea pig airway C-fibers inasmuch as responses to bradykinin were markedly attenuated in the presence of ETYA, an inhibitor of cyclooxygenase and lipoxygenase enzymes (Tobias and Hamilton, 1979). Indeed, the finding that several 5-, 12-, and 15-lipoxygenase products directly activate TRPV1 in isolated membrane patches of afferent neurons (Hwang et al., 2000) is consistent with our current finding that TRPV1 appears to mediate the excitatory action of bradykinin, and that this activation is sensitive to inhibition with ETYA but not indomethacin.

It is not known which lipoxygenase product may be playing a role in bradykinin B2 receptor-mediated activation of TRPV1. Our finding that the 12-lipoxygenase inhibitor baikaline was a more consistent inhibitor than the 5-lipoxygenase-selective inhibitor, ZD2138, suggests a more dominant role for 12-lipoxygenase products. These data should be cautiously interpreted, however, because these drugs are selective but not specific in their actions. It is unlikely that ETYA or baikaline acted as TRPV1 antagonist and/or channel blocker, because capsaicin evoked a similar number of action potentials in the absence and presence of these inhibitors. This is consistent with the hypothesis that the inhibition seen with these compounds is due to their established roles as inhibitors of lipoxygenase enzymes and that the products of these enzymes play a dominant role in bradykinin B2 receptor-induced, TRPV1-mediated activation of C-fibers in guinea pig airways.

Phospholipase A2 is considered to be the enzyme primarily responsible for the mobilization of the lipoxygenase substrate, arachidonic acid. However, we found that bradykinin evoked a similar number of action potentials in the absence and presence of the cytosolic phospholipase A2 inhibitor, AACOCF3. Similarly, Dray et al. (1992) found that the phospholipase A2 inhibitor, mepacrine, had little effect on bradykinin-induced responses of nociceptors in the neonatal rat tail. These findings may reflect a phospholipase A2-independent pathway for arachidonic acid release in the peripheral terminals of afferent neurons. Such a pathway is present in afferent neuron cell bodies, where bradykinin-induced arachidonic release occurred predominantly by the sequential actions of an sn-1 diacylglycerol lipase and a monoaclglycerol lipase, rather than by a phospholipase A2-mediated hydrolysis of phospholipids (Allen et al., 1992). The diacylglycerol lipase inhibitor RHC 80267 did have an apparent inhibitory effect in four of the six fibers studied; however, in two fibers, no effect was noted, and overall, there was not a significant difference in the average number of action potentials evoked by bradykinin. A difficulty in interpreting the negative results with these drugs is the lack of a suitable positive control. It is not possible to know whether sufficient concentrations of the lipase inhibitors were reached within the nerve terminals to have the putative inhibitory effects on the enzymes in question. Therefore, the biochemical pathways involved in the production of the TRPV1 agonists remain unknown.

Studies of membrane patches from afferent neuron cell bodies isolated from dorsal root ganglia have found that bradykinin B2 receptor-mediated modulation of TRPV1 may occur through a direct action of protein kinase C on TRPV1 (Premkumar and Ahern, 2000) and the phospholipase C-mediated release of TRPV1 from tonic phosphatidylinositol 4,5-bisphosphate-mediated inhibition (Chuang et al., 2001). In contrast, our current findings suggest a role for lipoxygenase products in bradykinin B2 receptor-mediated activation of TRPV1 in airway vagal afferent C-fiber terminals. Recently, Shin et al. (2002) published an elegant series of experiments showing that capsazepine, or the nonselective lipoxygenase inhibitor, nordihydropregualarin, inhibited bradykinin-induced action potential discharge in an in vitro rat skin-nerve preparation and in cultured rat dorsal root ganglion neurons. They also showed that bradykinin stimulated the production of 12-lipoxygenase products of arachidonic acid in cultured neurons isolated from dorsal root ganglia. Moreover, they found that the hyperalgesia caused by bradykinin in vivo was inhibited by baikaline. Thus, the results presented here are in support of their hypothesis in somatosensory neurons, that bradykinin activates nociceptors, at least in part, through a TRPV1- and lipoxygenase-dependent mechanism.

References