Protease-activated receptor-2 activation exaggerates TRPV1-mediated cough in guinea pigs


Protease-activated receptor-2 (PAR2) activation plays a role in inflammation and sensitizes TRPV1 in cultured sensory neurons by a PKC-dependent pathway. Here, we have investigated whether PAR2 activation exaggerates TRPV1-dependent cough in guinea pigs and whether protein kinases are involved in the PAR2-induced cough modulation. Aerosolized PAR2 agonists (PAR2-activating peptide and trypsin) did not produce any cough per se. However, they potentiated citric acid- and resiniferatoxin-induced cough, an effect that was completely prevented by the TRPV1 receptor antagonist capsazepine. In contrast, cough induced by hypertonic saline, a stimulus that provokes cough in a TRPV1-independent manner, was not modified by aerosolized PAR2 agonists. The PKC inhibitor GF-109203X, the PKA inhibitor H-89, and the cyclooxygenase inhibitor indomethacin did not affect cough induced by PAR2 agonists. In conclusion, PAR2 stimulation exaggerates TRPV1-dependent cough by activation of diverse mechanisms, including PKC, PKA, and prostanoid release. PAR2 activation, by sensitizing TRPV1 in primary sensory neurons, may play a role in the exaggerated cough observed in certain airways inflammatory diseases such as asthma and chronic obstructive pulmonary disease.

citrlic acid; protein kinase; resiniferatoxin; hypertonic saline; indomethacin; transient receptor potential vanilloid 1

Cough is one of the most common presenting symptoms of diverse diseases characterized by airway inflammation such as asthma, postnasal drip, chronic bronchitis, and chronic obstructive pulmonary disease (COPD). A lowered threshold to citric acid- and capsaicin-induced cough has been demonstrated in patients with asthma or COPD (17, 20, 45). Inflammatory mediators such as bradykinin and prostaglandins have been shown to enhance cough in animal models (18, 19). There is evidence that citric acid, capsaicin, and resiniferatoxin (RTX), agents frequently used in provocative cough tests in experimental animals (24, 39) and in humans (3, 12, 26), act as agonists to the transient receptor potential vanilloid 1 (TRPV1). TRPV1 is an excitatory cation channel (6) selectively expressed in a subset of nociceptive primary sensory neurons with C and Aδ fibers. TRPV1 is activated by vanilloid molecules, noxious temperature, low extracellular pH, and various lipid derivatives (2, 21, 22, 38) and undergoes sensitization by several proinflammatory mediators, including prostaglandins, bradykinin (33), neurotrophins (27), and other agents.

Protease-activated receptor-2 (PAR2) belongs to a family of four G-protein-coupled receptors that are uniquely activated by tethered ligands, unmasked by the proteolytic cleavage of the NH2-terminal domain of the receptor by specific proteases (13, 28, 32). In addition to proteases such as trypsin and tryptase, PAR2 is activated by synthetic peptides that mimic the sequence of the tethered ligand PAR2-activating peptide (PAR2-AP) (32). PAR2 is expressed in sensory nerve terminals where its activation promotes neurogenic inflammation and hyperalgesia (36, 43). PAR2 stimulation in vitro and in vivo potentiates TRPV1-mediated nociceptive responses, and this effect requires the activation of PKC (1, 14). PAR2 is also expressed in a variety of cells, including endothelial, epithelial, and exocrine gland cells, fibroblasts, and other cells (32). Both protective and detrimental effects mediated by PAR2 activation have been reported in models of airway diseases (11, 34, 35). The observation of increased PAR2 expression in bronchial vessels of smokers with chronic bronchitis (29) and in the epithelium of asthmatic patients (23) suggests a role of PAR2 in these pathological conditions. Stimulation of PAR2 causes release of PGE2 and other prostanoids in human and rodents airway epithelial cells (10, 25) and induces cyclooxygenase 2 mRNA and protein expression in human airway smooth muscle cells (8). It has been shown that cyclooxygenase products (30) and anandamide (15) sensitize TRPV1-mediated responses by a PKA-dependent pathway.

The aim of the present study was to investigate whether PAR2 activation could increase the cough response induced by TRPV1 stimulation in a guinea pig model and to define the role of PKC and PKA in this modulation.

Address for reprint requests and other correspondence: M. Trevisani, Center of Excellence for the study of Inflammation, Dept. of Clinical & Experimental Medicine, Pharmacology Section, Univ. of Ferrara, 44100 Ferrara, Italy (e-mail: tvm@unife.it).
PAR2 AGONISTS EXACERBATE TRPV1-MEDIATED COUGH

MATERIALS AND METHODS

Animals. Male Dunkin-Hartley guinea pigs (250–350 g, Pampalonei, Pisa, Italy) were acclimatized in cages (24 ± 0.5°C) for 1 wk after delivery and were allowed free access to water and standard rodent diet. The study conformed to the Declaration of Helsinki, complied with the Italian guidelines, and was approved by the local ethical committee for animal studies.

Measurement of cough responses. After the period of acclimatization to laboratory conditions, animals were individually placed in a transparent Perspex box (20 × 10 × 10 cm, Vetrotecnica) ventilated with a constant airflow of 400 ml/min. All cough responses were elicited using a minultrasonic nebulizer (Ugo Basile). The particle size produced had an aerodynamic mass median diameter of 0.9 μm, and the output of the nebulizer was 0.4 ml/min. Cough was detected by means of a tie-clip microphone (Sony) and confirmed by observing the characteristic posture of the animal. The cough sounds were recorded, digitally stored, and counted by a blind observer (39).

Experimental protocol. All experiments started at 9:00 AM. To elicit cough, guinea pigs were exposed to an aerosol of citric acid (0.25 M), RTX (0.5 μM), hypertonic saline (7% sodium chloride, 1.2 M), or their vehicles [isotonic saline, 0.005% dimethyl sulfoxide (DMSO) and isotonic saline, respectively] for 10 min. The ability of citric acid to induce cough in a concentration-dependent manner was investigated. At the concentration of 0.25 M, citric acid-induced cough was practically abolished (≈75% of inhibition) by the pretreatment with the TRPV1 antagonist capsazepine (CPZ), and by another TRPV1 antagonist (iodoresiniferatoxin, 1 μM; data not shown). For this reason, citric acid 0.25 M was selected for producing a TRPV1-dependent cough in the present work. To evaluate the modulatory role of PAR2 activation, animals were exposed to an aerosol of SLIGRL-NH₂ (PAR2-AP, 100 μM), its biologically inactive reverse peptide LRGILS-NH₂ (PAR2-RP, 100 μM), or trypsin (10 μM) 10 min before the cough challenge. When used, soybean trypsin inhibitor (SBTI, 1 mg/ml) was dissolved in the trypsin solution.

To investigate the molecular mechanism of PAR2-induced modulation of cough, the effect of the PKC activator 12-O-tetradecanoylphorbol 13-acetate (TPA, 10 μM, aerosolized for 10 min before the cough challenge) was also studied. Furthermore, the TRPV1 antagonist CPZ (10 μM), the PKC blocker GF-109203X (GFX, 1 μM), the potent PKA inhibitor N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide dihydrochloride (H-89, 1 μM), the cyclooxygenase inhibitor indomethacin (5 μM), or their respective vehicles (0.1% DMSO in isotonic saline) were aerosolized per 10 min before the administration of the PAR2 agonists.

RESULTS

PAR2 agonists and citric acid- and RTX-induced cough. Aerosolized citric acid (0.25 M) and RTX (0.5 μM) produced a significantly higher number of coughs (10.3 ± 1.7, n = 12 and 12.0 ± 1.0, n = 10, respectively; P < 0.05, Fig. 1) compared with aerosolized isotonic saline (1.5 ± 1.0, n = 12). In contrast, aerosolized PAR2-AP (100 μM), PAR2-RP (100 μM), and trypsin (10 μM) induced only a small number of coughs (1.2 ± 1.0, 1.5 ± 0.5, and 1.2 ± 0.3, respectively, n = 8) that was not different from the cough induced by isotonic saline (1.4 ± 0.8, n = 12; P > 0.05) (data not shown). However, aerosolization of PAR2-AP and trypsin, for 10 min before the cough challenge, significantly enhanced citric acid-induced cough (66 and 38% of increase, respectively, n = 8) and RTX-induced cough (83 and 73% of increase, respectively, n = 8) (Fig. 1). On the other hand, aerosolized PAR2-RP did not modify the cough response induced by the two TRPV1 agonists. Finally, addition of SBTI (1 mg/ml) to the solution of the trypsin aerosol abrogated the potentiation effect of trypsin on both citric acid- and RTX-induced cough (Fig. 1).

![Fig. 1. Effect of aerosolized isotonic saline (IS, 0.9% sodium chloride), protease-activated receptor-2 (PAR2) activating peptide (PAR2-AP; 100 μM), PAR2 reverse peptide (PAR2-RP; 100 μM), and trypsin (TRY, 10 μM) on the number of coughs induced by citric acid (CA) and resiniferatoxin (RTX). Soybean trypsin inhibitor (SBTI; 1 mg/ml) was administered by aerosol together with trypsin. Each column is presented as mean (SE) of at least 8 experiments. *P < 0.05, ANOVA and Bonferroni’s test vs. respective isotonic saline; #P < 0.05, ANOVA and Bonferroni’s test vs. respective PAR2 agonist alone.](http://jap.physiology.org/)
**Effect of aerosolized CPZ on citric acid- and RTX-induced cough.** Aerosolized CPZ (10 μM, per 10 min) prevented the exaggerated cough response induced by both citric acid and RTX in guinea pigs pretreated with the PAR2-AP (100 μM, n = 8) or trypsin (10 μM, n = 8). Responses to citric acid and RTX after pretreatment with the PAR2-RP (100 μM, n = 8) was also abolished (Fig. 2). The extent of the inhibitory effect of CPZ encompassed both the direct protussive effect of the two TRPV1 agonists and the potentiating effect by PAR2-AP and trypsin of citric acid- and RTX-induced cough.

**PAR2 agonist-, citric acid-, and RTX-induced cough and modulation of PKC and PKA activity.** To elucidate the mechanism of PAR2-induced potentiation of TRPV1-mediated cough, we first investigated the role of PKC on citric acid and RTX tussive responses by testing the effect of a PKC activator. Aerosolization of TPA (10 μM) did not induce cough per se if compared with its vehicle (1.2 ± 0.8 and 1.1 ± 0.9, respectively, n = 8; P > 0.05). However, pretreatment with TPA for 10 min before the cough challenge significantly enhanced the cough response induced by citric acid (~67% of increase, n = 6; P < 0.05) (Fig. 3) and RTX (~57% of increase, n = 6; P < 0.05) (Fig. 4). We then investigated the involvement of PKC on PAR2-mediated potentiation of citric acid and RTX-induced tussive responses testing the effect of a PKC blocker. Aerosolization of GFX (1 μM) for 10 min before the cough challenge did not affect the cough response induced by citric acid (Fig. 3) and RTX (Fig. 4). Conversely, it prevented the potentiation of the cough responses to both citric acid and RTX produced by TPA. In the same manner, inhibition of PKC prevented the potentiation of the cough responses to citric acid and RTX produced by PAR2-AP and trypsin (Figs. 3 and 4).

The involvement of prostanoids and PKA in the PAR2-induced potentiation of TRPV1-dependent cough was investigated in another set of experiments. Exaggeration by aerosolized PAR2 agonists of the cough response induced by citric acid and RTX was completely prevented by indomethacin (5 μM, by aerosol) or compound H-89 (1 μM, by aerosol) (Table 1). Neither indomethacin nor compound H-89 did modify per se the number of coughs induced by citric acid and RTX (see Table 1).

**PAR2 agonists and hypertonic saline-induced cough.** Aerosolization of hypertonic saline (7%) produced a higher number of coughs (8.3 ± 1.0, n = 10) compared with isotonic saline (1.3 ± 0.7, n = 8; P < 0.05). Also in this case we investigated the role of PKC on hypertonic saline tussive response. The activation of PKC by pretreatment with TPA did not affect the hypertonic saline-induced cough (Fig. 5). Similarly, pretreatment with PAR2-AP or trypsin did not have any modulatory effect against hypertonic saline-induced cough (Fig. 5). Thus activation of PAR2 and PKC did not affect the TRPV1-independent hypertonic saline-induced cough.

**DISCUSSION**

The present findings show that stimulation of PAR2, obtained with two diverse PAR2 agonists, PAR2-AP and trypsin, did not activate the cough reflex but significantly enhanced cough induced by both citric acid and RTX in a guinea pig model. Involvement of PAR2 in the potentiation is further indicated by the observation that the PAR2-RP, which is unable to stimulate the receptor, or inactivated trypsin by the SBTI did not modify the cough response induced by the two TRPV1 agonists.
tussive agents. PAR2 has been shown to be expressed on sensory nerve endings and to induce release of neuropeptides (34, 36). However, its activation is reported to not generate action potential in airways sensory neurons (5). This neurophysiological feature may be the underlying factor of the lack of tussive effect of PAR2 agonists observed in the present article. Trypsin induces bronchoconstriction but does not evoke action potential in sensory C fibers innervating the guinea pig trachea and bronchi (5). This observation further supports the hypothesis that absence of cough after PAR2 stimulation resides on its failure to activate action potential.

RTX is an ultrapotent agonist of the TRPV1 (37) and by direct channel activation causes cough (26). It is well known that acidity can activate both C fibers and rapid adapting receptors by stimulating various ion channels (40). Whereas elevated proton concentrations may cause cough via diverse mechanisms (4), the relatively low concentration of citric acid utilized in the present study has been shown to produce cough in guinea pigs in a manner almost entirely sensitive to the TRPV1 antagonists CPZ and ruthenium red (4, 24, 39). Here we confirmed that TRPV1 blockade abolished the cough responses produced by both RTX and a low concentration of citric acid. Thus, because cough induced by RTX and low concentration of citric acid appears to be a TRPV1-mediated response, it may be concluded that PAR2 activation exaggerates the TRPV1-mediated tussive response. Selectivity of PAR2 potentiation for cough mediated by TRPV1 is supported by experiments with hypertonic saline. We also confirmed previous evidence that hypertonic saline-induced cough in guinea pigs is not affected by CPZ (24, 39); hence it may be considered independent from TRPV1 activation. PAR2 agonists were unable to exaggerate hypertonic saline-induced cough. This further corroborates the hypothesis that PAR2 selectively sensitized the cough response mediated by TRPV1.

PAR2 is a G protein-coupled receptor activated by proteases released during trauma and inflammation, and it is widely expressed in a large variety of tissues and cell types where it exerts pleiotropic functions (32). PAR2 expressed in a subpopulation of capsaicin-sensitive primary sensory neurons promotes neurogenic inflammation and hyperalgesia (36, 42). In the airways, its activation has been associated with proinflammatory roles (34, 35). We found that PAR2 agonists enhanced TRPV1-mediated cough, suggesting that PAR2 stimulation potentiates effects produced by TRPV1. Various studies have proposed that activation of G protein-coupled receptors, including receptors for bradykinin or prostaglandins, results in TRPV1 sensitization caused by either activation of PKC or cAMP-dependent protein kinase (PKA) pathways (7, 9, 15, 30, 33). Recently it has been shown that PAR2 activation exaggerated TRPV1-mediated responses by a PKC-dependent mechanism in human embryonic kidney cells transfected with the human TRPV1 or in rat dorsal root ganglion neurons that constitutively express the channel (1). This evidence suggests that cough exaggeration is mediated by PAR2 and TRPV1 coexpressed in the same sensory nerve terminal. However,

Table 1. Effects of PAR2 agonists, the cyclooxygenase inhibitor indomethacin (5 μM), and the PKA blocker H-89 (1 μM) on citric acid- and resiniferoxin-induced cough in guinea pigs

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Vehicle</th>
<th>Indomethacin</th>
<th>H-89</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isotonic Saline</td>
<td>10.8±0.7</td>
<td>11.3±2.5</td>
<td>12.0±1.8</td>
</tr>
<tr>
<td>Trypsin</td>
<td>18.7±1.5*</td>
<td>8.4±1.3†</td>
<td>9.0±0.6†</td>
</tr>
<tr>
<td>SLIGRL-NH₂</td>
<td>22.0±1.5*</td>
<td>11.3±2.0†</td>
<td>8.0±1.5†</td>
</tr>
<tr>
<td>LRGILS-NH₂</td>
<td>11.3±1.2</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Resiniferoxin</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>11.2±1.7</td>
<td>10.0±1.5</td>
<td>10.3±1.3</td>
</tr>
<tr>
<td>Trypsin</td>
<td>19.6±2.0*</td>
<td>10.2±1.9†</td>
<td>9.0±2.2†</td>
</tr>
<tr>
<td>SLIGRL-NH₂</td>
<td>17.3±1.2*</td>
<td>9.5±1.2†</td>
<td>9.3±2.2†</td>
</tr>
<tr>
<td>LRGILS-NH₂</td>
<td>10.3±2.1</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Values are means ± SE of at least 8 experiments. H-89, N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide dichloride; NA, not applicable.
*P < 0.05; Bonferroni’s test vs. the respective vehicle; †P < 0.05; Bonferroni’s test vs. the respective protease-activated receptor-2 (PAR2) ligand.
The present results indicate PAR2 as an additional contributor such as asthma and COPD (20). Different mediators, including kinins, prostanooids, nerve growth factor, and others released during the inflammatory process, may contribute to the sensitization of TRPV1 that seems to occur in asthma and COPD. The present results indicate PAR2 as an additional contributor to this mechanism. Treatment of exaggerated and persistent cough that not infrequently accompanies or follows inflammatory and infectious airway diseases is a desirable objective. An ideal antitussive drug should limit this often unnecessary and disturbing symptom without affecting the ability of the subject to respond with cough to life-threatening injury in the airways. Thus the understanding of fine mechanisms involved in the exacerbation of the cough response, as for instance the isoforms of PKC and PKA, may be of importance for the design and development of novel efficacious antitussive treatments.

GRANTS

This work was in part supported by Associazione Ricerca Cura Asma, Padua, and Ministero dell’Istruzione, dell’Università e della Ricerca (Cofin 2002–2003), Rome, and by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-57480 and DK-43207.

REFERENCES


