Substance P and capsaicin release prostaglandin E₂ from rat intrapulmonary bronchi

JOHN L. SZAREK, BEVERLY SPURLOCK, CARL A. GRUETTER, AND SALLY LEMKE
Department of Pharmacology, Marshall University School of Medicine, Huntington, West Virginia 25704-9388

Szarek, John L., Beverly Spurlock, Carl A. Gruetter, and Sally Lemke. Substance P and capsaicin release prostaglandin E₂ from rat intrapulmonary bronchi. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L1006–L1012, 1998.—We hypothesized that substance P and capsaicin would cause the release of prostaglandin E₂ (PGE₂) from intrapulmonary bronchi isolated from Sprague-Dawley rats. Substance P (1 µM) caused the release of PGE₂, measured with enzyme immunoassay, from the isolated airway segments; PGE₂ release was inhibited by the neurokinin (NK)₁-receptor antagonist, RP-67580, by inhibition of cyclooxygenase and by removal of the epithelium. The release of PGE₂ caused by capsaicin (1 µM) was similar in magnitude to that caused by substance P. The capsaicin-induced release of PGE₂ was inhibited by desensitization of sensory nerves with capsaicin and by RP-67580, medofenamate, and epithelial denudation. We conclude that activation of NK₁ receptors on epithelium causes release of PGE₂, which most likely represents the ultimate mediator of airway smooth muscle relaxation, produced by exogenous neuropeptides and by activation of the sensory nerve inhibitory system. Epithelial damage, such as that seen in asthmatic airways, would disrupt this protective system in the lungs, which could contribute to the development of airway disease.

Endogenously released tachykinins, and those cited above, we hypothesized that endogenous tachykinins released in response to capsaicin would be the ultimate mediator of relaxation responses. We have termed this inhibitory pathway the sensory nerve inhibitory system.

The results of these previous studies provide pharmacological evidence for a role of an inhibitory PG in mediating the response. A more definitive approach to confirm our proposed mechanism by which the sensory nerve inhibitory system relaxes airway smooth muscle would be to measure release of PGs from the airways in response to exogenous and endogenous sensory neuropeptides. Thus, on the basis of our previous study and the results of others cited above, we hypothesized that substance P and capsaicin would cause the release of PGE₂ from isolated airway segments. To test this hypothesis, we examined the effects of C-fiber desensitization, NK₁-receptor antagonism, cyclooxygenase inhibition, and epithelial removal on release of the PG by substance P and by activation of the sensory nerve inhibitory system. In a previous study, Devillier and co-workers (6) showed that substance P caused the release of PGE₂ from epithelium-intact tracheal segments of rats. However, in their study, they did not examine the effects of C-fiber desensitization, NK₁-receptor antagonism, or inhibition of cyclooxygenase on the substance P-induced release of the prostanooid. Furthermore, the effects of endogenously released tachykinins on PGE₂ release have not been previously examined. The results of our study corroborate and extend the results of this earlier study and elucidate the mechanism whereby...
exogenous and endogenous sensory neuropeptides cause the release of PGE₂.

METHODS

Preparation of airway segments. Male Sprague-Dawley rats (270–330 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg) and exanguinated, and the lungs and a portion of the trachea were removed and placed in aerated physiological salt solution (PSS; in mM: 117.9 NaCl, 4.7 KCl, 1.5 CaCl₂, 2H₂O, 1.2 KH₂PO₄, 1.1 MgCl₂·6H₂O, 25 NaHCO₃, and 5.6 glucose) at room temperature. Cylindrical segments of primary intrapulmonary bronchi comparable to those used in a previous study (35) by our laboratory were isolated from right caudal or left lobes with a dissecting microscope. In some segments, the epithelium was denuded by rotating the isolated segment on a roughened 18-gauge needle that had been inserted into the lumen. This procedure denudes the epithelium lining, which was confirmed histologically in a previous study (35) by our laboratory. Epithelium-intact and -denuded segments were cut open parallel to the lumen, and each segment was placed into a well of a 12-well tissue culture plate containing 1 ml of PSS.

Mediator release from airway segments. The airways were incubated for 1 h at 37°C in a metabolic shaker under an air-5% CO₂ atmosphere. The airway segments were washed with fresh medium after 30 min and at the end of the incubation. The washes were accomplished by carefully removing the medium and replacing it with 1 ml of fresh medium; this procedure was repeated three times in succession. To assess the effects of NK₁-receptor blockade, cyclooxygenase inhibition, and C-fiber desensitization, we incubated epithelium-intact airways with RP-67580 (10 µM), meclofenamate (10 µM), or capsaicin (10 µM), respectively, during the last 30 min of the 1-h incubation period. After the last wash at the end of the 1-h incubation, RP-67580 and meclofenamate were added afresh; capsaicin was not added after the wash. We also examined the effects of inhibition of neutral endopeptidase on capsaicin-induced release of PGE₂. In these experiments, phosphoramidon (10 µM) was added only after the last wash at the end of the 1-h incubation. Incubation of tissues with vehicle for inhibitors did not affect the release of PGE₂ in response to capsaicin or substance P.

After the last wash and the addition of inhibitors as appropriate, the airway segments were allowed to equilibrate briefly for an additional 15 min. In a previous study (35) by our laboratory, the airways were contracted with bethanechol to facilitate study of relaxation responses. Thus, to mimic the previous study as closely as possible, we added bethanechol (3 µM) after the 15-min incubation. Ten minutes after the bethanechol was added, 500 µl of buffer were removed; basal levels of PGE₂ were measured in this sample. In preliminary studies, we found that bethanechol did not affect the basal release of PGE₂ (P = 0.956). Subsequently, vehicle, substance P (1 µM), or capsaicin (1 µM) was added to the remaining 500 µl, and the airways were incubated for an additional 6 min. The bronchi were then removed, and the buffer was collected and divided into aliquots. All samples were placed into microcentrifuge tubes that were then sealed and stored at −20°C until analyzed for PGE₂ levels by enzyme immunoassay (EIA). The airway segments were then blotted and weighed. With the use of an EIA kit for PGE₂ (Cayman Chemical, Ann Arbor, MI), the levels of PG were determined in the thawed samples according to the manufacturer’s instructions.

Data representation and statistical analysis. The levels of PGE₂ measured by EIA were normalized to milligrams of airway tissue and divided by the appropriate incubation interval (10 or 6 min) to obtain a value for net mediator release (in pg·mg⁻¹·min⁻¹). Release of PG is expressed as the ratio of stimulated to basal levels (S/B, where S equals PGE₂ release measured after challenge with substance P or capsaicin and B equals basal release of the PGE₂ measured in samples acquired just before addition of one of the stimuli) (36).

A total of 67 animals was used in preliminary studies and in the studies described herein. In these experiments, four airway segments were isolated from each animal; one segment served as a control and the three remaining segments were treated as described. Comparison among the means of the treatment groups was accomplished with ANOVA. Because it was decided a priori which means would be compared after the experiment was completed, contrasts were defined between these means in the ANOVA (33). In all analyses, P < 0.05 was considered significantly different. Statistical analyses were accomplished with Crunch Statistical Package, version 4 (Crunch Software, Oakland CA, 1991). Data are expressed as means ± SE. The number of preparations (n) reported represent the number of animals from which airway segments were isolated.

Reagents. Most drugs and reagents were purchased from Sigma (St. Louis, MO). The NK₁-selective receptor antagonist RP-67580 was kindly provided by Dr. C. Garret (Rhône-Poulenc Rorer, Vitry-sur-Seine cedex, France). Stock solutions for most of the drugs were prepared in water on the day of the experiment. Stock solutions of capsaicin were prepared in DMSO and diluted with PSS as appropriate. Substance P was dissolved in water, divided into aliquots, and stored frozen at −20°C. On the day of the experiment, an aliquot was removed from the freezer, allowed to thaw at room temperature and diluted in PSS as appropriate. Stock solutions of the tachykinin-receptor antagonist were prepared in 50% polyethylene glycol 200 in saline. Control segments in each group were incubated with vehicle.

RESULTS

Release of PGE₂ from airway segments by substance P. Basal release of PGE₂ was measured in the medium surrounding the airways just before the addition of substance P (Fig. 1). Basal release of PGE₂ in meclofenamate-treated airways was significantly reduced relative to control segments (P = 0.018). Incubation of airway segments with substance P (1 µM) increased the release of PGE₂ measured in the medium surrounding the airways segments as evidenced by the increase in the S/B (Fig. 1). In a functional study, Szarek et al. (35) found that inhibition of cyclooxygenase, antagonizing NK₁ receptors, or removal of the epithelium blocked relaxation responses caused by substance P. Thus we examined the effects of these interventions on substance P-induced release of PGE₂. Each of these interventions blocked the release of PGE₂ (Fig. 1).

Release of PGE₂ from airway segments by capsaicin. The same interventions as above (cyclooxygenase inhibition, NK₁-receptor blockade, and epithelium removal) blocked capsaicin-induced relaxation responses in a functional study (35). In addition, desensitization of C fibers by capsaicin blocked relaxation responses evoked by the sensory nerve inhibitory system (35). Thus similar experiments were undertaken to examine the effects of these interventions on capsaicin-induced release of PGE₂.
In our initial experiments, we assessed the effects of neutral endopeptidase inhibition and C-fiber desensitization on capsaicin-induced release of PGE2. We also assessed the effects of the capsaicin vehicle. The results are summarized in Fig. 2. In these experiments, basal levels of the PG were not different among the groups (P = 0.063; Fig. 2). The DMSO vehicle did not cause appreciable release of PGE2. Capsaicin evoked release of PGE2 from the isolated airway segments; the magnitude of the release was comparable to that caused by substance P. Desensitization of C fibers by prior exposure to capsaicin blocked the release of PGE2 caused by subsequent exposure to capsaicin. Phosphoramidon did not enhance capsaicin-induced release of PGE2. Although inhibition of neutral endopeptidase did not affect the release of PGE2 caused by capsaicin, we included phosphoramidon in the medium in subsequent experiments.

The results in Fig. 3 show the effects of cyclooxygenase inhibition, NK1-receptor antagonism, and epithelium removal on capsaicin-induced release of PGE2 from airway segments; each of the interventions blocked release of PGE2. In these experiments, basal release of PGE2 in meclofenamate-treated airways was significantly reduced relative to control segments (P = 0.002).

**DISCUSSION**

In this study, we found that activation of the sensory nerve inhibitory system caused release of PGE2 from isolated airway segments, a finding heretofore unreported. Activation of this system was confirmed by the results of experiments in which desensitization of C fibers with capsaicin abolished release of PGE2 in response to activation of the inhibitory system. The response was dependent on activation of NK1 receptors on the epithelium because blockade of these receptors blocked release of PGE2 from the segments. The fact that denuding the epithelium abolished PGE2 release in response to activation of the sensory nerve inhibitory system implicates the epithelium as the cellular source of PGE2. Although not measured directly in this study, substance P may be one neuropeptide mediator in this system for two reasons: 1) substance P caused release of PGE2 from the airway segments and 2) the same inhibitors that blocked the effects of activation of the sensory nerve inhibitory system also blocked the substance P-induced release of PGE2. The results of the present study confirm our proposed mechanism (35): activation of the sensory nerve inhibitory system re-

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**Fig. 1.** Effects of meclofenamate (Mec; 10 µM), RP-67580 (RP; 10 µM), and epithelium removal (–E) on substance P (SP; 1 µM)-induced release of prostaglandin E2 (PGE2) from intrapulmonary bronchi. Data are ratios of PGE2 release after stimulus to basal release before challenge with stimulus (S/B). Basal levels of PGE2 in each group before addition of SP were (in pg·mg⁻¹·min⁻¹) SP, 4.83 ± 0.51; Mec, 2.11 ± 0.62; RP, 3.56 ± 0.37; and –E, 6.48 ± 1.40. Each point is mean ± SE of responses of 8 preparations. Each of the inhibitors and removal of epithelium inhibited SP-induced release of PGE2 from airways (*P ≤ 0.009 compared with SP alone).

**Fig. 2.** Effects of capsaicin desensitization (Des; 10 µM) and phosphoramidon (Phos; 10 µM) on capsaicin (Cap; 1 µM)-induced release of PGE2 from intrapulmonary bronchi. Basal levels of PGE2 in each group before addition of vehicle (Veh) or Cap were (in pg·mg⁻¹·min⁻¹) Veh, 4.07 ± 0.62; Cap, 5.94 ± 1.36; Des, 3.38 ± 0.67; and Phos, 7.28 ± 1.43. Each point is mean ± SE of responses of 9 preparations. Challenge of segments with Cap Veh did not cause release of PGE2. Capsaicin evoked release of PGE2 from the isolated airway segments; the magnitude of the release was comparable to that caused by substance P. Desensitization of C fibers by prior exposure to capsaicin blocked the release of PGE2 caused by subsequent exposure to capsaicin. Phosphoramidon did not enhance capsaicin-induced release of PGE2. Although inhibition of neutral endopeptidase did not affect the release of PGE2 caused by capsaicin, we included phosphoramidon in the medium in subsequent experiments.

**Fig. 3.** Effects of Mec (10 µM), RP (10 µM), and –E on Cap (1 µM)-induced release of PGE2 from intrapulmonary bronchi. Basal levels of PGE2 in each group before addition of Cap were (in pg·mg⁻¹·min⁻¹) Cap, 8.11 ± 2.43; Mec, 0.87 ± 0.39; RP, 5.28 ± 1.02; and –E, 3.99 ± 0.53. Each point is mean ± SE of responses of 6 preparations. Each of the inhibitors and removal of epithelium blocked Cap-induced release of PGE2 from airways (*P ≤ 0.005 compared with Cap alone).
leases sensory neuropeptides that activate NK₁ receptors on the epithelium, causing the release of PGE₂, the ultimate mediator of smooth muscle relaxation (Fig. 4).

The experiments in this study were designed to mimic the experimental conditions in our laboratory's functional study (35) with respect to increased smooth muscle tone by incubating the airway segments with bethanechol. Under these conditions, substance P evoked the release of PGE₂ from the isolated airway segments. In our functional study (35), epithelium removal, NK₁-receptor antagonist, and inhibition of cyclooxygenase abolished or markedly inhibited relaxation responses in contracted airways in response to substance P. Thus we examined the effects of these interventions on release of PGE₂ caused by substance P. Corroborating the results of the study by Devillier and co-workers (6), we showed that substance P-induced release of PGE₂ was abolished in segments that had been denuded of epithelium. Our results extend those of this previous study and show that blocking NK₁ receptors and inhibition of cyclooxygenase also abolished substance P-induced release of the prostanoid. Although other tachykinin receptors may be involved in releasing PGE₂, we are convinced that the release of PGE₂ by substance P was mediated by the activation of NK₁ receptors for several reasons. First, RP-67580 is highly specific for rat NK₁ receptors (38). Garret and co-workers (13) showed that this nonpeptide antagonist in concentrations up to 10 µM (the concentration of RP-67580 used in the present study) did not displace the binding of NKA or senktide to NK₂ or NK₃ receptors, respectively. Moreover, in a previous functional study (35), the NK₂-receptor antagonist SR-48698 did not affect substance P-induced relaxation responses. We conclude from our observations that substance P activates NK₁ receptors on the epithelium followed by activation of cyclooxygenase and release of PGE₂.

We found nearly identical results when capsaicin was used to activate C fibers within the airway segments. Removal of the epithelium, NK₁-receptor antagonism, and inhibition of cyclooxygenase each blocked release of PGE₂ evoked by capsaicin. The fact that capsaicin-induced release of the prostanoid was inhibited by desensitization of C fibers confirmed that PGE₂ release was dependent on activation of the sensory nerve inhibitory system. The finding that phosphoramidon did not affect capsaicin-induced release of PGE₂ reflects the fact that the concentration of capsaicin used in this study produces a near-maximal response that cannot be potentiated by inhibition of neutral endopeptidase. These results are the first to show that endogenous neuropeptides released from C fibers cause the release of PGE₂ and that the mechanism is identical to that used by exogenous substance P.

Of the several prostanooids that have been shown to relax airway smooth muscle, we focused on PGE₂ in this study for several reasons: 1) PGE₂ is the predominant prostanooid released from normal and transformed rat tracheal epithelial cells in culture (7, 40); 2) Devillier and co-workers (6) found that substance P did not evoke release of prostacyclin, thromboxane, or PGF₂α; and 3) PGE₂ relaxes airway segments in a concentration-dependent manner, whereas prostanoids do not exhibit inhibitory effects in rat airways (unpublished data). Pertaining to this latter finding, ~10 nM PGE₂ produced a relaxation response that was comparable to that observed in a previous study (35) by our laboratory. In response to 1 µM substance P or capsaicin, the calculated concentration of PGE₂ in the medium after the addition of the same concentration of substance P and capsaicin in the present study was ~0.7 nM. This difference could be accounted for by release of another inhibitory PG that was not measured in this study. Alternatively, the difference between the amount of PGE₂ released and an equieffective concentration of exogenous PGE₂ can be attributed to the close apposition of the C fibers, epithelium, and smooth muscle in the airway wall that leads to higher local concentrations than those measured in the medium (Fig. 4).

Several studies have examined tachykinin-receptor distribution and C-fiber distribution in the airways of the rat. Using 125I-Bolton-Hunter-labeled substance P, Sertl and co-workers (31) showed that receptors for substance P were localized predominantly to the epithelial cells of rat bronchi similar to those used in the present study. The results of our study suggest that these receptors were most likely NK₁ receptors because the NK₁-selective antagonist RP-67580 blocked release of PGE₂ in response to substance P. Using immunohistochemistry, investigators (10, 20) have localized the NK₁ receptors predominantly to the luminal surface of the epithelial cells. Immunohistochemical techniques have been utilized to locate the distribution of substance P-containing nerves within the airways of the rat. Baluk and co-workers (1) showed that, of the single substance P-immunoreactive axons within the airway wall, 85% of these were in the epithelium. These axons passed between and beneath the epithelial cells in the airway (1). In another study, McDonald and co-workers (25) found that the substance P-immunoreactive nerves were within 0.1 µm of the epithelial cells. Thus endogenous neuropeptides released from activated C fibers have to traverse only a short distance before encountering epithelial NK₁ receptors. Although the actions of the released neuropeptide would be expected to be
mediator release from rat airways

short-lived because of abundant neutral endopeptidase within the epithelium (30), the proximity of the nerves to the epithelial receptors ensures the activation of the NK1 receptors and subsequent release of PG before degradation of the neuropeptide. Given the location of the C fibers in relation to the epithelium, it is possible that in the process of denuding the epithelium, we removed the nerves. To address this issue, we tried to measure substance P release from epithelium-intact and -denuded airway segments. After repeated attempts, we were not able to measure substance P release from the segments. We attribute this result to the small size of the airway segments. This contention is supported by results of a study by Hua and Yaksh (17). Using an isolated perfused rat trachea, they measured the release of substance P, NKA, and calcitonin gene-related peptide (CGRP) in response to capsaicin. They were able to measure release of substance P in only two of six experiments in response to 1 μM capsaicin. Their data also showed that capsaicin caused ~10-fold more CGRP release than NKA and substance P. Many investigators have used CGRP release as an index of sensory nerve activation in the airways. In the discussion of their paper, Hua and Yaksh (17) mention that removing the epithelium did not alter basal secretion or capsaicin-evoked release of CGRP. Assuming that our process of removing the epithelium from bronchi is similar to removing it from the trachea, this result suggests that the sensory nerves are not removed in the process of denuding the airway segments. Thus, in the experiments in which denuding the epithelium abolished the capsaicin-induced release of PGE2, we conclude that this was due to removal of the cellular source of PG. In a previous study (35), our laboratory showed that NKA mimicked the effects of substance P in producing relaxation responses, and these responses were blocked by inhibition of cyclooxygenase. Although there have been no quantitative studies of the distribution of NKA-immunoreactive nerves in the rat airways, there is evidence that NKA and substance P are present in the same axons (16). Moreover, as mentioned above, substance P, NKA, and CGRP are released from rat airways in response to capsaicin (17). Thus either one or all of these peptides may have contributed to the release of PGE2 caused by capsaicin in the present study. However, a role for CGRP is precluded on the basis of the results of a functional study (35) by our laboratory. In that study, blocking CGRP receptors inhibited CGRP-induced relaxation but did not affect relaxation evoked by activation of the sensory nerve inhibitory system. We cannot assign a principal role for either endogenous substance P or NKA in mediating release of PGE2 because both of these neuropeptides bind to and activate NK1 receptors nonselectively (29). Thus endogenous substance P, NKA, or both contribute to the release of PGE2 after the activation of the sensory nerve inhibitory system.

Inhibitory PGs represent epithelium-derived relaxing factors that modulate contractions in the airways (2). In our study, we focused on one of these, PGE2. In addition to the direct relaxant effects of PGE2 on airway smooth muscle, PGE2 inhibits release of acetylcholine from parasympathetic nerve endings (32). PGE2 also has anti-inflammatory activity. It inhibits release of mediators from lung mast cells (28) and inhibits cellular responses in other inflammatory cells such as eosinophils (14) and macrophages (5). Substance P and other tachykinins are generally considered proinflammatory and mediate neurogenic inflammation. These effects include bronchoconstriction (19), airway hyperresponsiveness to other bronchoactive stimuli (39), augmentation of release of acetylcholine from cholinergic nerve endings (37), vasodilation (24), and increased vascular permeability (1). A hypothetical scheme of the role of these autacoids in modulating airway responses begins to emerge. Specifically, substance P promotes inflammatory responses in the airways, whereas PGE2 exerts a dampening effect. Hence the opposing roles in modulating airway function may serve to protect the lungs from excessive neurogenic inflammatory activity.

Results of our study may have relevance to the development of human lung disease; several previous studies suggest that a sensory nerve inhibitory system exists in the human lung. Activation of C fibers by aerosolized capsaicin has been shown to causebronchodilation in humans (21, 22). Intravenous infusion of substance P has also been shown to cause bronchodilation in humans (9); however, this effect may have been a consequence of the vasodilator response caused by the tachykinin. When administered as an aerosol, substance P in concentrations up to 1 μmol/l was devoid of bronchomotor effects in normal human volunteers (18). In a previous study (35), our laboratory also did not see any bronchomotor effects of substance P in animals unless we first induced bronchoconstriction. Results of a recent in vitro study (4) showed that substance P caused a biphasic response in isolated human airways, transient relaxation followed by contraction. In this same study, a selective NK1-receptor agonist, [Sar9,Met(O2)11]substance P, evoked relaxation of isolated human bronchi (4). Capsaicin has been shown to cause no effect (26), contraction only (15), and contraction and/or relaxation (4) in isolated human airways. In a recent study (8), investigators showed that very high concentrations of capsaicin caused relaxation responses in human bronchi but that these occurred independently of the release of endogenous tachykinins. Thus whether this system exists in human airways cannot be stated with certainty.

In support of the notion that a sensory nerve inhibitory system does exist in human airways, results of studies from other investigators showed that the neural and cellular components exist for such a system and that the inhibitory pathway is similar to that which we showed in the rat (Fig. 4). Substance P-immunoreactive nerves exist in the epithelial basement membrane of human airways and have branches extending into the epithelium (27); NK1 receptors are localized to the epithelium of the airways predominantly on the luminal side (10); and, as mentioned above, NK1-receptor activation relaxes isolated human bronchi (4). The
presence of a similar system in human airways suggests that studies in the rat are relevant to human airway function.

Epithelial damage is a prominent histological feature of asthmatic airways. Thus our results suggest that such damage to the epithelium would remove the dampening effects of PGE2 in the airways, resulting in exaggerated effects of C-fiber activation. This may contribute to the development of airway diseases such as asthma. In support of this contention, inhaled substance P causes bronchoconstriction in asthmatic subjects (3, 12). Moreover, we have shown that acute ablation of C-fiber function in isolated airways enhanced electrical field stimulation-induced contractions in the airway segments and that replacement of substance P inhibited these contractions (34).

In conclusion, our results show that PGE2 released from the epithelium likely represents the ultimate mediator responsible for relaxation of smooth muscle in response to activation of the sensory nerve inhibitory system.

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REFERENCES


