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Role of Ca^{2+}-activated K^+ channel in epithelium-dependent relaxation of human bronchial smooth muscle

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1 To elucidate whether K^+ channels play a role in the action of epithelium-dependent bronchodilatation, we studied responses in human bronchial strips in the presence of indomethacin and N^G-nitro-L-arginine methylester under isometric conditions, *in vitro*.

2 Mechanical removal of the epithelium increased the contractile responses to acetylcholine; the pD_2 values increased from 5.0 ± 0.2 to 5.9 ± 0.3 ($P < 0.001$). This potentiation was abolished by iberiotoxin but not by apamin or glibenclamide.

3 In cascade bioassay, application of the bathing medium from dispersed, bronchial epithelial cells to epithelium-denuded bronchial strips decreased acetylcholine-induced contraction by $44 \pm 6\%$. This effect was reduced to $10 \pm 3\%$ ($P < 0.01$) when the epithelial cells were pretreated with iberiotoxin, and to $4 \pm 1\%$ ($P < 0.001$) when the epithelial cells were incubated with Ca^{2+}-free medium containing [1,2-*bis* (2) aminophenoxy] ethane *N,N,N′,N′*-tetraacetic acid-acetomethoxy ester.

4 In contrast, the bronchodilator effect of the medium bathing epithelial cells was not altered by the direct addition of iberiotoxin to epithelium-denuded tissues.

5 These results suggest that the Ca^{2+}-activated K^+ channel may play a role in the synthesis and/or release of smooth muscle relaxing factor, which is neither nitric oxide nor a cyclo-oxygenase product, from airway epithelial cells.

Keywords: Epithelium-derived relaxing factor; airway smooth muscle; acetylcholine; airway hyperreactivity; asthma

Introduction

A major clinical feature of asthma is exaggerated responsiveness of the airways to physical, chemical, and pharmacologic stimuli (Boushey 1980). It has been shown that epithelial destruction associated with airway inflammation can be frequently observed at all levels of the airways in asthmatic patients (Laitinen *et al*., 1985). Mechanical removal of epithelial cells in isolated airways increases the contractile responses to pharmacological stimuli in various species including man (Flavahan *et al*., 1985; Raeburn *et al*., 1986; Aizawa *et al*., 1988), suggesting a role of epithelial cells in the regulation of bronchomotor tone. Indeed, airway epithelial cells are capable of reducing airway smooth muscle contraction through release of inhibitory prostaglandins (Barnett *et al*., 1988) and epithelium-derived relaxing factor (EpDRF) (Flavahan *et al*., 1985; Barnes *et al*., 1985). The chemical nature of EpDRF has yet to be elucidated, but it appears to be unrelated to nitrovasodilators such as nitric oxide or eicosanoids (Fernandes & Goldie, 1990; Munakata *et al*., 1990).

There are several types of K^+ channels in airway tissues (Black & Barnes, 1990), and activation of K^+ channels causes hyperpolarization or repolarization of the cells, which may in turn modify a variety of airway functions, such as bronchoconstriction (Miura *et al*., 1992), release of neurotransmitters from autonomic nerves (Stretton *et al*., 1992; Tagaya *et al*., 1995) and electrolyte secretion from epithelial cells (McCann & Welsh, 1990). Recent evidence suggests that a Ca^{2+}-activated K^+ channel mediates the release of endothelium-derived relaxing factor (EDRF) and, hence, contributes to vascular tone in the rabbit aorta (Demirel *et al*., 1994; Hutcheson & Griffin, 1994). Therefore, it is possible that K^+ channels may also be involved in the action of EpDRF in airway smooth muscle preparations. To test this hypothesis, we studied the effects of K^+ channel blockers on acetylcholine-induced contraction of epithelium-intact and epithelium-denuded human bronchial strips under isometric conditions *in vitro*. Moreover, to determine the role of Ca^{2+}-activated K^+ channels in the release of EpDRF, by means of cascade bioassay, we assessed the contractile responses of epithelium-denuded bronchial tissues in the bathing medium from bronchial epithelial cells in the absence and presence of iberiotoxin, a specific blocker of Ca^{2+}-activated K^+ channels (maxi-K^+ channel) (Galvez *et al*., 1990).

Methods

Preparation of bronchial strips

Human lung tissues were obtained from 26 patients at thoracotomies performed because of carcinoma. After surgical removal, macroscopically normal lung tissues were rapidly immersed in Krebs-Henseleit solution (composition in mM: NaCl 118, KCl 5.9, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25.5 and D-glucose 5.6). Cartilaginous bronchi, 2 to 4 mm in internal diameter, were then dissected free of parenchyma, fat and surrounding connective tissues and cut helically at a 45° pitch to obtain bronchial strips measuring 2 to 3 mm in width and ~15 mm in length. Between two and eight strips were dissected from each specimen and mounted in 7 ml organ chambers containing Krebs-Henseleit solution maintained at 37°C and continuously aerated with a gas mixture of 95% O_2–5% CO_2 to obtain a pH of 7.4, a PCO_2 of 38 mmHg, and a PO_2 of >500 mmHg. Contractile responses were continuously measured isometrically with a force-displacement transducer (Nihon Kohden, TB-652T, Tokyo, Japan) and were recorded on a pen recorder (Nihon Kohden, WT-685G). In some experiments, the epithelial cells were removed by passage of a moistened cotton-wrapped piper cleaner through the bronchial lumen, and the absence of epithelial layer was confirmed after the experiment by staining tissues with Masson's trichrome.

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The tissues were allowed to equilibrate in the baths for 60 min, during which time they were washed with Krebs-Henseleit solution every 15 min and the resting tension was adjusted to 1 g. Our preliminary studies on the relationship between resting tension and active tension of human bronchial strips showed the maximal response with 1 g of resting tension. A contractile response was measured as the difference between peak developed tension and resting tension. It has been shown that airway smooth muscle and epithelium release prostaglandin E_2, which can decrease bronchoconstrictor responses (Walters *et al*., 1984). To avoid this possibility, indomethacin (3×10^{-6} M, Sigma Chemical, Co., St Louis, MO, U.S.A.) was present in the chamber throughout the experiments. Moreover, to avoid a possible contribution of nitric oxide that is released from airway epithelial cells (Tamaoki *et al*., 1995), N^G-nitro-L-arginine methylester (L-NAME, 10^{-3} M, Sigma), an inhibitor of nitric oxide synthase (Rees *et al*., 1990), was also added.

Effect of epithelial removal on acetylcholine-induced contraction

To determine whether alterations in K^+ channel activity are involved in the action of EpDRF, the effect of epithelial removal on acetylcholine-induced contraction was assessed in the absence and presence of various K^+ channel blockers. Paired bronchial strips from the same patient were used; one was epithelium-intact and the other was epithelium-denuded. Acetylcholine (10^{-8} to 10^{-3} M, Sigma) was cumulatively added to the chamber in half-molar increments at 5 min intervals or 2 min after a stable plateau was achieved, whichever was the longer period, and the contractile response to each concentration was determined. With other tissue pairs, the concentration-response curves for acetylcholine were likewise constructed in the presence of the following K^+ channel blockers: iberiotoxin (10^{-7} M, Peptide Institute Inc., Osaka, Japan), a Ca^{2+}-activated K^+ channel (maxi-K^+ channel) blocker (Galvez *et al*., 1990); glibenclamide (10^{-5} M, Yamanouchi Pharmaceutical Co., Tokyo), an ATP-sensitive K^+ channel blocker (Cook & Hales, 1984); and apamin (10^{-7} M, Peptide Institute), a small conductance K^+ channel blocker (Banks *et al*., 1979). The concentrations of the blockers were chosen based on a previous study (Hamaguchi *et al*., 1992). To characterize the concentration-response curves, the pD_2 values were determined by linear regression analysis

Effect of iberiotoxin on EpDRF release from dispersed epithelial cells

Because only iberiotoxin inhibited the leftward shift of acetylcholine concentration-response curves induced by removal of the epithelium, we tested whether activation of Ca^{2+}-activated K^+ channels was involved in EpDRF action on smooth muscle cells, or in the process of synthesis and/or release of EpDRF by epithelial cells by use of a cascade bioassay. To prepare the isolated and dispersed epithelial cells, bronchial mucosa freed from cartilage and smooth muscle layers was cut into small pieces (1 to 2 mm^3) and bathed in phosphate-buffered saline. After the chopped mucosa had been rinsed, the tissues were incubated for 2.5 h at 37°C in phosphate-buffered saline containing 0.1% protease type XIV (Sigma), and then the cells were pelleted by centrifugation (200 *g*, 10 min). This procedure was repeated three times.

In our preliminary experiments, the density of epithelial cells in the medium needed to inhibit acetylcholine (10^{-5} M)-induced contraction was approximately 10^5 cells ml^{-1}. Thus, to obtain a sufficient number of epithelial cells, we used cultured cells rather than freshly isolated cells. The dispersed epithelial cells were cultured in Ham's nutrient F12 medium containing 10 $\mu g\ ml^{-1}$ insulin, 5 $\mu g\ ml^{-1}$ transferrin, 25 ng ml^{-1} epidermal growth factor, 7.5 $\mu g\ ml^{-1}$ endothelial cell growth supplement, 50 u ml^{-1} penicillin, 50 $\mu g\ ml^{-1}$ streptomycin and 50 $\mu g\ ml^{-1}$ gentamicin at 37°C in a CO_2 incubator (95% air-5% CO_2). After 72 h, when the cultured cells became almost confluent, they were redissociated with protease, washed three times with phosphate-buffered saline. Cells were then resuspended at a density of 2×10^5 cells ml^{-1} in 10 ml of oxygenated Krebs-Henseleit solution containing indomethacin (3×10^{-6} M) and L-NAME (10^{-3} M), and incubated for 15 min with or without iberiotoxin (10^{-7} M). This preparation of cells consisted of 99% epithelial cells and 1%

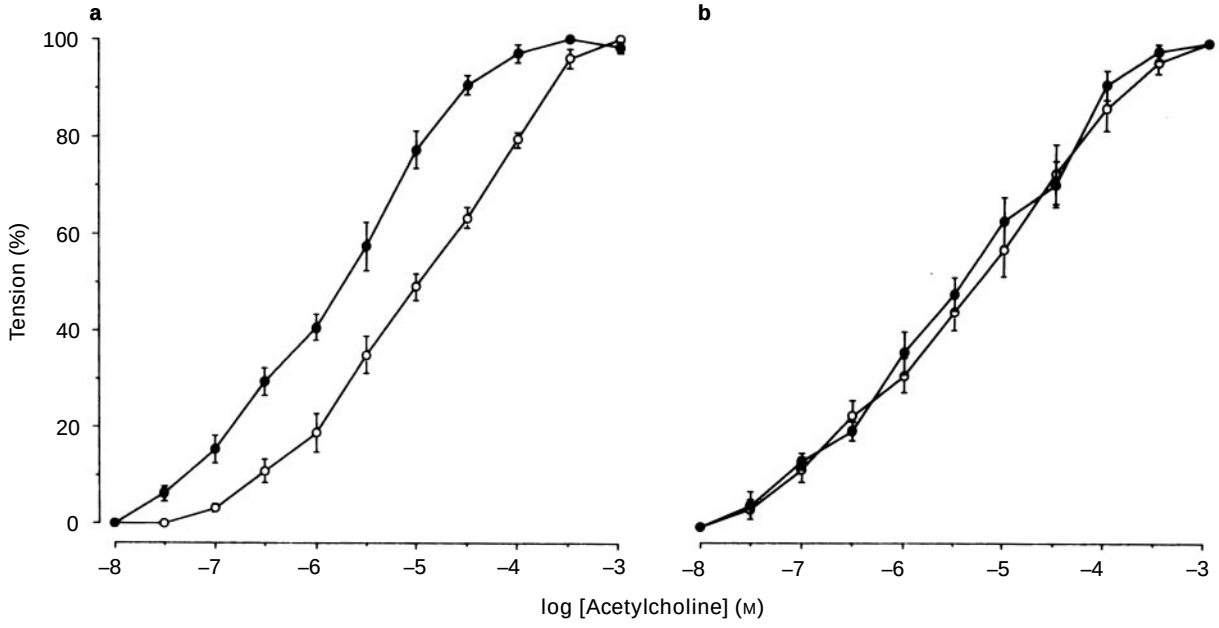

Figure 1 Effect of epithelial removal on contractile responses of human bronchial strips to acetylcholine in the absence (a) and presence (b) of iberiotoxin (10^{-7} M). Cumulative concentration-response curves for acetylcholine were generated in the epithelium-intact tissues (○) and in the tissues in which the epithelial cells had been mechanically removed (●). Responses are expressed as % of contractile response of epithelium-intact tissue to 10^{-3} M acetylcholine. Data are means, $n = 12$ for each point; vertical lines show s.e.

fibroblasts and other nonepithelial cells, as determined by immunohistochemical staining for cytokeratin. The bronchial strips in which the epithelial cells had been mechanically removed were mounted in organ chambers and, after equilibration, the contractile responses to acetylcholine (10^{-5} M) were determined. The tissues were then washed with fresh Krebs-Henseleit solution, and the medium bathing the epithelial cells was filtered through a polycarbonate filter (pore size 8 μm, Millipore Co., Tokyo), and continuously applied to the organ chambers for 3 min by a peristaltic pump at a rate of 0.5 ml min^{-1}, while the second responses to acetylcholine were measured. In separate experiments, iberiotoxin was directly added to the epithelium-denuded tissues, the medium of epithelial cells incubated in the absence of iberiotoxin was then applied, and the contractile responses to acetylcholine measured. In addition, to assess the involvement of Ca^{2+} mobilization in the release of EpDRF, dispersed epithelial cells were incubated for 15 min in Ca^{2+}-free medium containing [1,2-bis (2) aminophenoxy] ethane *N,N,N′,N′*-tetraacetic acid-acetomethoxy ester (BAPTA-AM, 5×10^{-5} M, Sigma), an intracellular Ca^{2+} chelating agent, and then the effect of the medium of epithelial cells on acetylcholine-induced contraction was likewise determined.

Statistics

All values are expressed as means ± s.e. Statistical analysis was performed by Student's *t* test or Newman-Keuls multiple comparison test. Statistical significance was accepted at a *P* value of less than 0.05.

Results

Addition of K^+ channel blockers to the chamber at concentrations used in the present study *per se* did not alter the resting tension of human epithelium-intact or epithelium-denuded bronchial strips. As demonstrated in Figure 1, mechanical removal of the epithelium increased the contractile responses to acetylcholine, causing a leftward displacement of the concentration-response curves, so that the pD_2 value increased from 5.0 ± 0.2 to 5.9 ± 0.3 ($P < 0.001$, $n = 12$). This potentiation of acetylcholine-induced contraction produced by epithelial removal was abolished in the presence of iberiotoxin, the pD_2 value being 5.7 ± 0.3 in epithelium-intact tissues and 5.9 ± 0.2 in epithelium-denuded tissues ($n = 12$, respectively), but the potentiation was still observed in the presence of glibenclamide or apamin (Table 1).

In cascade bioassay experiments, the second contractile responses of epithelium-denuded bronchial tissues to acetylcholine (10^{-5} M) after application of medium that did not bathe dispersed epithelial cells were not significantly different from the first responses (Figure 2). In contrast, application of the medium bathing epithelial cells decreased the second contractile response to acetylcholine by 44 ± 6% ($n = 11$). This inhibition was not significantly altered by the direct addition of iberiotoxin to the organ chamber containing epithelium-denuded strips, but was reduced to 10 ± 3% ($n = 9$, $P < 0.01$) when dispersed epithelial cells were pretreated with iberiotoxin, and to 4 ± 1% ($n = 9$, $P < 0.001$) when the epithelial cells were incubated with Ca^{2+}-free medium containing BAPTA-AM.

Discussion

Our *in vitro* studies demonstrate that Ca^{2+}-activated K^+ channels may be involved in the epithelium-dependent inhibition of human bronchial smooth muscle contraction. This conclusion is based on the findings that mechanical removal of the epithelium potentiated the contractile responses of bronchial strips to acetylcholine, causing a leftward displacement of the concentration-response curves, as has been previously shown in dog (Flavahan *et al*., 1985), guinea-pig (Goldie *et al*., 1986), rabbit (Raeburn *et al*., 1986) and human airways (Aizawa *et al*., 1988), and that this effect was abolished by pretreatment of tissues with iberiotoxin, a selective blocker of Ca^{2+}-activated K^+ channel (Galvez *et al*., 1990). We also found that blockade of ATP-sensitive K^+ channels and small conductance K^+ channels with glibenclamide and apamin, respectively (Banks *et al*., 1979; Cook & Hales, 1984), had no effect on the potentiation of the contractile responses induced by removal of the epithelium. These results exclude the possible involvement of these K^+ channel subtypes. In contrast to our findings, Shikada and Tanaka (1995) recently showed that

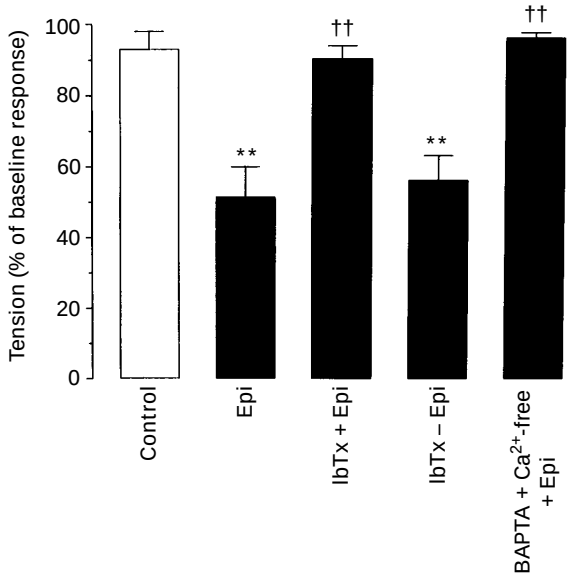

Figure 2 Effects of pharmacological blocking agents on epithelium-dependent inhibition of acetylcholine-induced contractions. After the effect of acetylcholine (10^{-5} M) on epithelium-denuded bronchial strips had been determined, the strips were incubated in medium from dispersed bronchial epithelial cells that had been incubated in the absence (Epi) or presence of iberiotoxin (10^{-7} M) (IbTx + Epi), or in Ca^{2+}-free medium containing BAPTA-AM (BAPTA + Ca^{2+}-free + Epi), and the response to acetylcholine was repeated. In some experiments, iberiotoxin was added directly to bronchial strips (IbTx − Epi). Responses are expressed as % of the initial response to acetylcholine in the absence of medium from epithelial cells. Data are means ± s.e., $n = 9-11$ for each column. **$P < 0.01$, significantly different from the response to the medium that did not bathe epithelial cells (Control). ††$P < 0.01$, significantly different from the response to the epithelial cell bathing medium.

Table 1 Effect of epithelial removal on contractile responses of human bronchial strips to acetylcholine

	Without blocker		*With blocker*	
	Epithelium-intact	*Epithelium-denuded*	*Epithelium-intact*	*Epithelium-denuded*
Iberiotoxin	5.0 ± 0.2	5.9 ± 0.3***	5.7 ± 0.3	5.9 ± 0.2
Glibenclamide	5.1 ± 0.3	5.7 ± 0.4**	5.1 ± 0.2	5.8 ± 0.3**
Apamin	5.2 ± 0.3	5.8 ± 0.2**	5.1 ± 0.2	5.7 ± 0.4**

Data are expressed as means ± s.e. of pD_2 values in epithelium-intact and epithelium-denuded rabbit bronchial strips. **$P < 0.01$, ***$P < 0.001$, significantly different from values for epithelium-intact tissues; $n = 12$ for each group.

the relaxant responses of guinea-pig trachea to ATP-sensitive K^+ channel openers, including cromakalim and NIP-121, were reduced by rubbing of the epithelial cells, suggesting a role of ATP-sensitive K^+ channels in the epithelium-dependent bronchodilatation. The reason for this discrepancy is unknown, but it could be due to species heterogeneity, regional differences or differences in experimental conditions.

It is likely that airway epithelial cells release inhibitory factors that partially counteract contraction of airway smooth muscle cells induced by bronchoconstrictor substances. Firstly, airway epithelial cells can synthesize and release cyclo-oxygenase metabolites of arachidonic acid, among which prostaglandin E_2 is a major product and has been shown to inhibit contraction of airway smooth muscle via pre- and postjunctional mechanisms (Orehek *et al*., 1973; Walters *et al*., 1984). Flavahan and colleagues (1985) and Butler and colleagues (1987) suggested that the epithelium-dependent inhibitory response was at least partially mediated by cyclo-oxygenase products. However, because our experiments were conducted in the presence of indomethacin at a concentration sufficient to inhibit airway cyclo-oxygenase activity (Yamaguchi *et al*., 1976), the potentiated responses to acetylcholine in the preparations denuded of their epithelial cells may not be attributed to the loss of inhibitory prostaglandins. In support of this, Barnes and colleagues (1985) were unable to block the effects of epithelial removal with indomethacin or mepacrine and concluded that the epithelial product was unlikely to be an arachidonic acid metabolite. Secondly, nitric oxide can be generated from L-arginine via nitric oxide synthase and released from airway epithelial cells (Tamaoki *et al*., 1995), which could in turn counteract acetylcholine-induced contraction (Buga *et al*., 1989). This possibility is also unlikely, because the nitric oxide synthase inhibitor L-NAME was present throughout the experiments. Therefore, the effect of epithelial removal observed in the present study appears to be related to EpDRF, a compound which is neither an arachidonic acid metabolite nor nitric oxide (Fernandes *et al*., 1990; Munakata *et al*., 1990).

It has been shown that Ca^{2+}-activated K^+ channels are present on both airway smooth muscle cells (McCann & Welsh, 1986) and epithelial cells (McCann & Welsh, 1990). The increase in intracellular Ca^{2+} concentration activates Ca^{2+}-activated K^+ channels, which subsequently causes membrane hyperpolarization/repolarization of the cells, thereby regulating airway smooth muscle tone and electrolyte transport. The inhibitory effect of iberiotoxin on the potentiation of the contractile responses to acetylcholine, induced by removal of the epithelium, may be due to EpDRF directly increasing the K^+ permeability of tracheal smooth muscle cells, i.e., EpDRF acts as an opener of sarcolemmal Ca^{2+}-activated K^+ channel, or the release of EpDRF from epithelial cells may require activation of epithelial Ca^{2+}-activated K^+ channels, or both. The cascade bioassay experiment allowed for separation of the synthesis/release process of EpDRF with incubation of the donor dispersed epithelial cells in the presence of iberiotoxin and the effect of EpDRF on smooth muscle cells with direct addition of iberiotoxin to the recipient epithelium-denuded bronchial strips. In this study, application of the medium bathing the dispersed epithelial cells decreased the contractile responses to acetylcholine, implying a release of EpDRF from the epithelial cells. This bronchodilator effect of EpDRF was not affected by the direct addition of iberiotoxin to the recipient strips, but was greatly inhibited when the donor epithelial cells were preincubated with iberiotoxin. These results suggest that the effect of iberiotoxin is not associated with the airway smooth muscle Ca^{2+}-activated K^+ channel, but is more likely due to an inhibition of the synthesis and/or release of EpDRF from epithelial cells by blocking the epithelial Ca^{2+}-activated K^+ channel.

Consistent with our findings, the secretory process in several exocrine glands is thought to be related to the stimulation of Ca^{2+}-activated K^+ channels (Peterson & Maruyama, 1984), and recent studies have shown that certain vasodilators, such as bradykinin and acetylcholine, stimulate the release of EDRF via the opening of endothelial Ca^{2+}-activated K^+ channels (Groschner *et al*., 1992; Demirel *et al*., 1994).

In the present experiments, to determine further the participation of Ca^{2+}-activated K^+ channels in airway epithelium, we assessed the requirement of Ca^{2+} mobilization. When the dispersed epithelial cells were incubated in Ca^{2+}-free solution containing the intracellular Ca^{2+} chelating agent BAPTA-AM to deplete intracellular Ca^{2+} stores, application of the medium did not alter the acetylcholine-induced contraction of the recipient tissues. It can thus be speculated that the elevation of Ca^{2+} levels in epithelial cells may be an essential step for the activation of pathways leading to the synthesis and/or release of EpDRF. However, because the chemical nature of EpDRF is not clear, signal transduction between the Ca^{2+}-activated K^+ channel-mediated increase in plasma membrane K^+ permeability and the EpDRF synthesis/release remains unknown.

Recent studies have shown that K^+ channels play a role in the regulation of various physiological functions in the airways, such as airway smooth muscle tone (Miura *et al*., 1992), microvascular permeability (Martin & Advenier, 1993), release of acetylcholine and tachykinins from cholinergic and non-adrenergic non-cholinergic nerves, respectively (Stretton *et al*., 1992; Tagaya *et al*., 1995), and Cl secretion by epithelial cells (McCann & Welsh, 1990). Our findings provide evidence that the activation of Ca^{2+}-activated K^+ channels in airway epithelial cells is a principal mechanism for stimulating the synthesis/release of EpDRF. In contrast, the activation of Ca^{2+}-activated K^+ channels located on the airway smooth muscle cells appears to play a less important role in the epithelium-mediated bronchodilatation.

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