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Mechanisms underlying the relaxation response induced by bradykinin in the epithelium-intact guinea-pig trachea *in vitro*

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1 In this study, we investigated some of the signalling pathways involved in bradykinin (BK)-induced relaxation in epithelium-intact strips of the guinea-pig trachea (GPT + E). BK induced time- and concentration-dependent relaxation of GPT + E. Similar responses were observed for prostaglandin E_2 (PGE_2) or the combination of subthreshold concentrations of BK plus PGE_2.

2 The nonselective cyclooxygenase (COX) inhibitors indomethacin or pyroxicam, or the selective COX-2 inhibitors DFU, NS 398 or rofecoxib, but not the selective COX-1 inhibitor SC 560, all abolished BK-induced relaxation.

3 The tyrosine kinase inhibitors herbimycin A and AG 490 also abolished BK-induced relaxation in GPT + E.

4 The nonselective nitric oxide synthase (NOS) inhibitor 7-NINA concentration-dependently inhibited BK effects.

5 BK-induced relaxation was prevented by the selective antagonists for EP_3 (L 826266), but not by EP_1 (SC 19221), EP_1/EP_2 (AH 6809) or EP_4 (L 161982) receptor antagonists.

6 Otherwise, the selective inhibitors of protein kinases A, G and C, mitogen-activated protein kinases, phospholipases C and A_2, nuclear factor-κB or potassium channels all failed to significantly interfere with BK-mediated relaxation.

7 BK caused a marked increase in PGE_2 levels, an effect that was prevented by NS 398, HOE 140 or AG 490.

8 COX-2 expression did not differ in preparations with or without epithelium, and it was not changed by BK stimulation. However, incubation with BK significantly increased the endothelial NOS (eNOS) and neuronal NOS (nNOS) expression, independent of the epithelium integrity.

9 Our results indicate that BK-induced relaxation in GPT + E depends on prostanoids (probably PGE_2 acting *via* EP_3 receptors) and NO release and seems to involve complex interactions between kinin B_2 receptors, COX-2, nNOS, eNOS and tyrosine kinases.
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Abbreviations: AA, arachidonic acid; BK_{Ca}, high-conductance Ca^{2+}-activated potassium channel; COX, cyclooxygenase; EGFR, epidermal growth factor receptor; EGFR-Trk, epidermal growth factor receptor-related tyrosine kinase; eNOS, endothelial nitric oxide synthase; EP, prostaglandin E_2 receptors; GPT, guinea-pig trachea; GPT−E, epithelium-denuded guinea-pig trachea; GPT + E, epithelium-intact guinea-pig trachea; IK1, intermediate conductance Ca^{2+}-activated potassium channel; iNOS, inducible nitric oxide synthase; Kv, voltage-gated potassium channels; LPS, bacterial lipopolysaccharide; MAP kinase, mitogen-activated protein kinase; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; PKA, protein kinase A; PKC, protein kinase C; PLA_2, phospholipase A_2; Trk, tyrosine kinase

Introduction

Bradykinin (BK) is a nonapeptide (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) formed locally in body fluids and tissues from the plasma precursor kininogen during the inflammatory process (Farmer & Burch, 1991; Barnes, 1992). Among other effects, BK is known to elicit contraction or relaxation in vascular and nonvascular smooth muscles, to increase vascular permeability and to participate in blood pressure control, in addition to causing pain and inflammation (see, for review, Regoli & Barabé, 1980; Hall, 1992; Marceau *et al*., 1998; Calixto *et al*., 2000).

In the airways, there is accumulating evidence to indicate that kinins play an important role in the control of bronchoalveolar function (Barnes *et al*., 1988; Farmer, 1992). Pharmacological and molecular studies now indicate that most BK actions in the guinea-pig airways are mediated by the

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activation of B_2 receptors (Proud *et al*., 1993; Schlemper & Calixto, 1994) and the subsequent release of prostanoids and nitric oxide (NO) (Bramley *et al*., 1989; Schlemper & Calixto, 1994; 1995; Figini *et al*., 1996; Van Heuven-Nolsen *et al*., 1997; Mazzuco *et al*., 2000). On the other hand, BK-induced contraction in denuded guinea-pig trachea (GPT−E) is dependent on prostanoid production and on the release of calcium from intracellular sources, an effect that is largely sensitive to ryanodine, and it seems to be associated with the activation of protein kinase C (PKC) (Calixto, 1995; Schlemper & Calixto, 2002).

Prostaglandin E_2 (PGE_2) exerts important pathophysiological roles in the airways. The release of arachidonic acid (AA) is an important event preceding the production of prostanoids. Cyclooxygenase (COX, prostaglandin endoperoxide synthase) is the rate-limiting enzyme responsible for the conversion of AA to prostanoids and thromboxane A_2. Prostanoid production is mediated by two isoforms of COX: COX-1 produces physiological levels of prostanoids and is constitutively expressed under normal conditions, whereas COX-2 is not usually expressed, but may be upregulated in many tissues following inflammatory stimulation. Curiously, as first reported by Charette *et al*. (1995), prostanoid release in the isolated guinea-pig trachea (GPT) is dependent on the activation of a constitutive COX-2 enzyme. In the same study, the authors also suggested that the inhibition of intrinsic tone with indomethacin or with NS 398 was associated with the blockade of the constitutive COX-2 enzyme (Charette *et al*., 1995).

BK has been reported to cause AA release by increasing cytosolic phospholipase A_2 (Tanaka *et al*., 1995; Pyne *et al*., 1997), with subsequent prostanoid formation in the airways. In fact, previous reports have shown that prostanoids may be involved in BK-induced bronchoconstriction, since COX inhibitors, such as indomethacin, significantly attenuate BK-induced bronchoconstriction (Ichinose *et al*., 1990).

In the present study, we have used functional, biochemical and molecular techniques to investigate some of the signal transduction mechanisms through which BK induces relaxation in epithelium-intact GPT (GPT + E) *in vitro*, with special attention towards determining the involvement of PGE_2 production, prostaglandin E_2 receptors (EP) and signalling pathways in this process.

Methods

Functional experiments

All manipulation, anaesthesia and surgical procedures were carried out according to the guidelines for animal experimentation of the American Association for Laboratory Animal Science (Trowning Manual Series) (Stark & Ostrow, 1991). Guinea-pigs of both sexes (200–350 g) were killed by an overdose of pentobarbital, and the tracheas were rapidly removed and carefully dissected from adhering fat and connective tissues. Usually, eight transverse rings (3–4 mm wide) were obtained from each animal. The rings were opened and strips of 8–10 mm in length with intact epithelium (except where otherwise indicated) were suspended in individual 5 ml jacketed organ baths containing Krebs–Henseleit solution (composition, mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.1, KH_2PO_4 1.1 and glucose 11) maintained at 37°C, pH 7.2 and gassed with 95% O_2/5% CO_2. Preparations were allowed to equilibrate for at least 120 min before drug addition, under a resting tension of 1 g, during which the bath solution was replaced every 15 min. Isometric tension changes were recorded by means of an F-60 force transducer on a physiograph (Narco Biosystem). The epithelium integrity was assessed by the ability of BK (100 nM) to induce relaxation. Epithelium was considered viable when BK caused a relaxation response of up to 80% (approximately 300–400 mg) in preparations under spontaneous tonus (Schlemper & Calixto, 1994). In some cases, when the tonus was found reduced under the basal line (established at 1 g of tension during the time equilibration), low concentrations of carbachol (0.01–0.1 μM) were added to the organ baths in order to correct this parameter. This procedure was performed to maintain a similar tonus in all studied preparations.

Following the equilibration period, noncumulative complete concentration–response curves were obtained by the addition of single concentrations of BK (0.03–300 nM) for 5–10 min. All experiments with BK were carried out in the presence of captopril (3 μM) to avoid degradation of the peptide by the action of kininase II. For the purpose of comparison, in a separate series of experiments, a similar relaxant noncumulative concentration–response curve was obtained for PGE_2 (0.03–300 nM). We have used different preparations for each concentration of BK or PGE_2. In another group of experiments, in order to verify whether BK and PGE_2 could interact in a synergistic manner to produce relaxation in the GPT + E, relaxant responses were evaluated following the incubation of combined subthreshold concentrations of BK (0.01 nM) plus PGE_2 (0.01 nM). The experimental protocol was the same as described before.

Influence of several classes of drugs

To test the role played by prostanoid synthesis in BK-induced relaxation in GPT, single relaxation responses to BK (100 nM) were obtained in the absence or presence of the nonselective COX inhibitors indomethacin (1–10 nM) and pyroxicam (0.1–10 nM), the selective COX-2 blockers DFU (0.1–3 μM), NS 398 (0.1–3 μM) or rofecoxib (0.01–3 μM), or the selective COX-1 inhibitor SC 560 (100 nM). In other groups of experiments, to test the relevance of nitric oxide synthase (NOS) activation for the relaxant response induced by BK in the GPT, preparations were incubated with the NOS inhibitor 7-NINA (0.1–10 μM). These drugs were incubated with the preparations 30 min beforehand. The range of concentrations used for the COX-1 and COX-2 inhibitors was selected on the basis of previous studies or pilot experiments (Riendeau *et al*., 1997; Waskewich *et al*., 2002).

To assess the relative contribution of EP receptors to PGE_2 in BK-induced relaxation in the GPT, after obtaining a stable relaxant response to BK (100 nM), preparations were incubated for 20 min beforehand with SC 19220 (10 μM, a selective EP_1 receptor antagonist; Coleman & Kennedy, 1985), AH 6809 (10 μM, a nonselective EP_1/EP_2 receptor antagonist; Woodward *et al*., 1995), L 826266 (30 μM, a selective EP_3 receptor antagonist; Kvirkvelia *et al*., 2002) or with L 161982 (30 μM, a selective EP_4 receptor antagonist; Kvirkvelia *et al*., 2002), and a new relaxant response to BK (100 nM) was obtained.

To gain further insights into the mechanisms through which BK induces relaxation in GPT + E, the effects of several classes of inhibitors were assessed in this preparation. The following drugs were evaluated: ODQ (a guanylate cyclase inhibitor, 1–10 μM), MDL-12,330 (an adenylate cyclase inhibitor, 10 μM), HA 1004 (a nonselective protein kinase A (PKA) and protein kinase G (PKG) inhibitor, 10 μM), KT 5720 (a PKA inhibitor, 1–3 μM), KT 5823 (a PKG inhibitor, 1 and 3 μM), PDTC (a nuclear factor-κB inhibitor, 10 μM), PD 98059 (an inhibitor of mitogen-activated protein kinase kinase 1/2, 10 μM), SB 203580 (a p38 mitogen-activated protein (MAP) kinase inhibitor, 10 μM), dexamethasone (a transcriptional protein synthesis inhibitor, 10 μM), beclomethasone (a transcriptional protein synthesis inhibitor, 10 μM), quinacrine (100 μM, a phospholipase A_2 (PLA_2) inhibitor), $PACOCF_3$ (a PLA_2 inhibitor, 10 μM), U73122 (a phospholipase C (PLC) inhibitor, 300 nM), GF 109203x (a PKC inhibitor, 3 μM), staurosporine (a PKC inhibitor, 100 nM), herbimycin A (a nonselective tyrosine kinase (Trk) inhibitor, 10 μM), AG 490 (a selective epidermal growth factor receptor (EGFR)-related Trk (EGFR-Trk) inhibitor, 30 μM) or wortmannin (a selective phosphatidylinositol-3 kinase inhibitor, 30 μM).

We next investigated the possible role of potassium channels or epithelium-derived hyperpolarizing factors in BK-induced relaxation in the GPT. After obtaining a stable relaxant response to BK (100 nM), preparations were incubated with one of the following potassium (K^+) channel blockers: 4-aminopyridine (a blocker of voltage-gated K^+ channels (Kv), 1 mM), apamin (a small-conductance Ca^{2+}-activated K^+ channel blocker, 100 nM), charybdotoxin (a high-(BK_{Ca}) or intermediate-(IK1) conductance Ca^{2+}-activated and voltage-gated K^+ channel blocker, 100 nM), iberiotoxin (a high-conductance Ca^{2+}-activated (BK_{Ca}) K^+ channel blocker, 100 nM), or apamin (100 nM) plus charybdotoxin (100 nM), after which a second relaxant response to BK (100 nM) was obtained.

All drugs were incubated with the preparations 30 min beforehand, except the selective EP receptor antagonists, which were incubated for 20 min. The choice of all concentrations and intervals for the preincubation of antagonists was based on literature data or on pilot experiments.

Prostaglandin release in response to BK

In order to investigate further the role of PGE_2 in BK-induced relaxation in the GPT, the levels of this prostanoid were measured after BK incubation, in the absence or in the presence of several antagonists. The protocol used was identical to that employed in functional studies, and samples (500 μl) of the Krebs–Henseleit nutritive solution were collected from the cubes with a micropipette, 10 min after BK (100 nM) addition. The enzyme inhibitors NS 398 (100 nM), SC 560 (10 nM), aminoguanidine (10 μM) or AG 490 (30 μM) were left in contact with the tissue for 30 min before BK (100 nM) stimulations. The selective kinin B_2 receptor antagonist HOE 140 (100 nM) was left in contact with the tissue for 20 min before BK stimulation. Samples were frozen in liquid nitrogen and stored at -70°C until assay. The PGE_2 levels were assessed using a specific enzyme immunoassay (EIA) according to the manufacturer's instructions (R&D Systems Inc., MN, U.S.A.). The results were expressed in picograms of PGE_2 per milligram of tissue weight.

Determination of COX-1, COX-2, nNOS, eNOS and iNOS expression in GPT – Western blotting studies

To determine whether the expression of COX-1, COX-2, neuronal NOS (nNOS), endothelial NOS (eNOS) or inducible NOS (iNOS) was related to the presence of epithelium, preparations were mounted with or without epithelium using the previously described procedures. Following the stabilization period, some of the tissues were stimulated with BK (100 nM) for 10 min in the presence of captopril (3 μM). After the incubation period (2 h), the tissues consisting exclusively of smooth muscle, without cartilage, were immediately frozen in liquid nitrogen.

The protein extracts were prepared in accordance with D'Acquisto *et al.* (1999), with some modifications. Tissues were homogenized in ice-cold 10 mM HEPES (pH 7.4), containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethyl sulphonyl fluoride (PMSF), 20 μg ml^{-1} aprotinin (trasylol), 2 mM sodium orthovanadate (Na_3VO_4), 1.5 μg ml^{-1} trypsin inhibitor, 7 μg ml^{-1} pepstatin, 5 μg ml^{-1} leupeptin and 0.5 mM dithiothreitol. The homogenates were chilled on ice for 15 min and then vigorously shaken for 15 min in the presence of 0.1% Nonidet P-40. The homogenates were centrifuged at 10,000 $\times$ *g* for 30 min, and the resulting supernatant collected was considered as the cytosolic fraction. This supernatant was stored at -70°C until use. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, CA, U.S.A.). The amount of protein used is 50 μg.

As a positive control for the upregulation of COX-2, some experiments were performed using muscle extracts obtained from bacterial lipopolysaccharide (LPS) (50 μg kg^{-1}, i.v., 24 h)-treated guinea-pigs.

Drugs

Indomethacin, pyroxicam, ibuprofen, 7-nitroindazole (7-NINA), pyrrolidine dithiocarbamate (PDTC), dexamethasone, beclomethasone, quinacrine, apamin, charybdotoxin, iberiotoxin, ouabain, 4-aminopyridine, 1-[2-([4-chlorophenyl]methoxy)-2-(2,4-dichlorophenyl) ethyl]-1*H*-imidazole (econazole), 9*S*-(9α, 10β, 11β, 13α)-2,3,10,11,12,13-hexahydro-10-methoxy-9-methyl-11-(methylamino)-epoxy-1*H*, 9*H*-diindolo[1,2,3-gh:3′,2′, 1′-1 m] pyrrolo[3,4-j] [1,7] benzodiazonin-1-one (staurosporine), LPS (from *Escherichia coli*; serotype 0111:B4), all from Sigma (St Louis, MO, U.S.A.); 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1*H*-pyrazole (SC 560), 8-chloro dibenz [b, f] [1,4]oxazepin-10(11*H*)-carboxy-(2-acetyl)hydrazide (SC 19220), 6-isopropoxy-9-oxaxanthene-2-carboxylic acid (AH 6809), *N*-[2-cyclohexyloxy-4-nitrophenyl]methanesulphonamide (NS 398) from Cayman Chemical (Ann Arbor, MI, U.S.A.), 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl) phenyl-2(5*H*) furanone (DFU), 4-(4′-methylsulphonylphenyl)-3-phenyl-2-(5*H*)-furanone (rofecoxib), L 826266 and L 161982, which were kindly provided by Merck Froost (Kirkland, Québec, Canada), 1*H*-[1,2,4]oxadiazolo[4,3-a]quinolaxin-1-one (ODQ), 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4one (PD 98059),1,1,1,-trifluoro-2-heptadecanone ($PACOCF_3$), 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione(U73122),2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl])maleimide (GF 109203x), (*E*)-2-cyano-3-(3,4-dihydrophenyl)-*N*-(phenyl-methyl)-2-propenamide (AG 490), (1*S*, 6br, 9a*S*, 11*R*, 11b*R*) 11-acetyloxy)-1,6b, 7, 8, 9a,

10, 11, 11b-octahydro-1-methoxymethyl)9a,11b-dimethyl-3*H*-furo(4,3,2-de]indeno[4,5,-h]-2-h]-2-benzopyran-3,6, 9-trione (wortmannin), 4-[5-(fluorophenyl)-2-[4-(methylsulphonyl) phenyl]-1*H*-imidazol-4-yl] pyridine hydrochloride (SB 203580), from Tocris Cookson Inc., MO, U.S.A.; (9*R*, 10*S*, 12*S*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9methyl-1-oxo-9,12-epoxy-1*H*-diindolol[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-il] [1,6] benzodiazocine-10-carboxylic acid, hexyl ester (KT 5720) and (9*R*, 10*S*, 12*S*)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1*H*-diindolol[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-] [1,6] benzodiazocine-10-carboxylic acid, methyl ester (KT 5823) from Calbiochem (San Diego, CA, U.S.A.); *cis*-*N*-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine monohydro chloride (MDL-12,330 A), *N*-(2-guanidinoethyl)-5-isoquinoline-sulphonamide hydrochloride (HA 1004) and herbimycin A (ansamycin antibiotic) from RBI (Natick, MA, U.S.A.).

Stock solutions of these drugs (1–100 mM) were prepared as follows: Indomethacin, pyroxicam, NS 398, dexamethasone, PDTC, PD 98059, ODQ, SC 560, $PACOCF_3$, U73122, SC 19220, AH 6809, staurosporine, AG 490 and DFU were dissolved in 50% absolute ethanol and rofecoxib was dissolved in pure methanol. KT 5720, KT 5823, L 826266, GF 109203x, herbimycin A, wortmannin and SB 203580 were dissolved in dimethylsulphoxide (50%). The BK B_2 receptor antagonist HOE 140 was dissolved in 0.2% bovine serum albumin plus phosphate-buffered saline (PBS). Apamin was dissolved in 0.05 M acetic acid. All other drugs were dissolved in PBS to the desired concentrations, just before use. All dilutions were made in PBS. The final concentration of ethanol and dimethylsulphoxide did not exceed 0.02%, which alone had no effect on the tonus of the preparation or on BK-mediated relaxation. KT 5720, KT 5827, rofecoxib and sodium nitroprusside were protected from light to avoid their photodegradation.

Statistical analysis

Data are presented as the mean ± s.e.m., except for the EC_{50} or IC_{50} (i.e. the concentration of drugs causing half-maximal relaxant response or the concentration of antagonist required to inhibit the agonist's response by 50% relative to the control response, respectively), where it is given as the geometric mean accompanied by its respective 95% confidence limit. The relaxant responses to BK and PGE_2 are expressed as absolute mg of tension. Statistical analysis was performed by means of unpaired Student's *t*-test or by analysis of variance followed by Dunnett's multicomparison test when appropriate. *P*-values less than 0.05 were considered as indicative of significance.

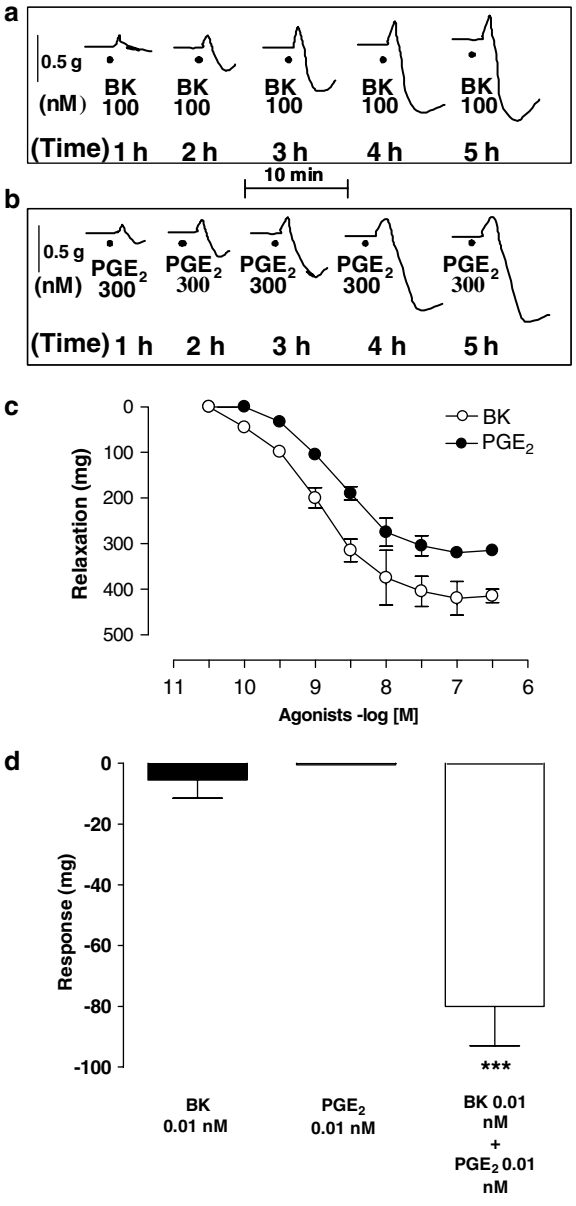

Figure 1 Typical records showing the time-dependent increase in BK (100 nM)- (a) and PGE_2 (300 nM)- (b) induced relaxation responses in the GPT + E. Relaxation concentration–response curves for BK and for PGE_2 in the GPT + E under spontaneous tonus following 2 h of time equilibration (c). Relaxant effect observed with the combination of subthreshold concentrations of BK (0.01 nM) and PGE_2 (0.01 nM) in the GPT + E after 2 h of time equilibration (d). Each point represents the mean of 6–7 experiments and the vertical lines indicate the s.e.m.

Results

Complete relaxation concentration–response curves for BK

The successive additions of a single concentration of BK (100 nM) with a 30 min interval between each addition caused a time-dependent (1–5 h) relaxation response, with a marked increase of BK-induced relaxation in GPT + E (Figure 1a). Likewise, the addition of PGE_2 (300 nM) also induced a relaxation response in the GPT + E, with a profile very similar to that observed for BK (Figure 1b). Addition of BK (0.03–300 nM) or PGE_2 (0.03–300 nM) to the bath 120 min after setting up the preparations caused a small, transient contraction followed by concentration-dependent relaxation with a slow onset. The calculated mean EC_{50} values accompanied by the 95% confidence limits were (nM) 18.8 (12.4–21.0) and 3.2 (2.7–3.4) and the maximal relaxation responses obtained were 387 ± 23 and 348 ± 33 mg of tension, for BK and PGE_2, respectively (Figure 1c). In this last protocol, in order to obtain the concentration–response curves, we used different preparations for each concentration of BK or PGE_2. Of note, the association of subthreshold concentrations of BK (0.01 nM)

plus PGE_2 (0.01 nM) resulted in a marked relaxation effect in the GPT + E (Figure 1d). The relaxation induced by either BK or PGE_2 was well reproducible with no evidence of tachyphylaxis. However, about 45% of the preparations failed to relax following addition of BK or PGE_2. This is probably related to epithelium injury and/or to seasonal bronchial hyper-reactivity (results not shown).

Analysis of the signalling pathways involved in BK-induced relaxation in GPT + E

The relevance of COX-1 and COX-2 activation to the relaxation responses induced by BK in the GPT + E was investigated by testing the effects of various COX inhibitors. The preincubation of the preparations with the nonselective COX inhibitors indomethacin (0.1–3 nM) or pyroxicam (0.1–3 μM) (Figure 2) or with the selective COX-2 inhibitors DFU (100–3000 nM), NS 398 (0.1–1000 nM) or rofecoxib (1–30 μM) caused a concentration-dependent inhibition of BK (100 nM)-induced relaxation in GPT + E (Figure 3). The calculated mean IC_{50} values (and 95% confidence limits) from these effects were as follows: indomethacin 8.16 (6.12–9.71 nM), pyroxicam 0.27 (0.17–0.45), DFU 0.37 (0.20–0.48) μM, NS 398 0.18 (0.11–0.23) μM and rofecoxib 3.0 (1.91–3.12) μM. All the COX inhibitors tested (except DFU) changed the BK-induced relaxation response to a sustained contraction, similar to that observed following the removal of the epithelium. On the other hand, the selective COX-1 inhibitor SC 560 (100 nM) had no significant effect on the relaxation caused by BK (results not shown).

Next, we investigated the involvement of NOS activation in the relaxation caused by BK in the GPT + E. The NOS inhibitor 7-NINA (0.1–3 μM) caused a concentration-dependent inhibition of BK-induced relaxation. The calculated mean IC_{50} (accompanied by the 95% confidence limit) was 0.4 (0.3–0.6) μM and the maximal inhibition was 83.1 ± 5.2% (Figure 4).

The results in Figure 5 show the effects of incubation with selective antagonists of PGE_2 receptors (EP) on BK-induced relaxation in GPT + E. The relaxation caused by BK (100 nM) was significantly inhibited by preincubation with the selective EP_3 receptor antagonist L 826266 (30 μM). However, the selective antagonists of EP_1 (SC 19220, 10 μM), EP_1/EP_2 (AH 6809, 10 μM) or EP_4 (L 161982, 30 μM) receptors did not significantly interfere with the BK-induced relaxation in the GPT + E. Notably, EP_3 and EP_4 antagonists also caused a marked reduction of tonus in the preparations (data not shown).

In another set of experiments designed to clarify further the mechanisms involved in the relaxation caused by BK in the GPT + E, the effects of several classes of inhibitors were tested in this preparation. All the following drugs failed to significantly inhibit the relaxation caused by BK in the GPT + E: ODQ (1–10 μM), KT 5720 (1–3 μM), KT 5823 (1 and 3 μM), PDTC (10 μM), PD 98059 (10 μM), SB 203580 (10 μM), dexamethasone (10 μM), beclomethasone (10 μM), MDL-12,330 (10 μM), $PACOCF_3$ (10 μM), quinacrine (100 μM), U73122 (300 nM), HA 1004 (10 μM), GF 109203x (3 μM), staurosporine (100 nM) or wortmannin (30 μM) (results not shown).

However, the relaxation response to BK (100 nM) was markedly inhibited by the nonselective Trk inhibitor herbimycin A (10 μM) (Figure 6a). In the same way, the selective

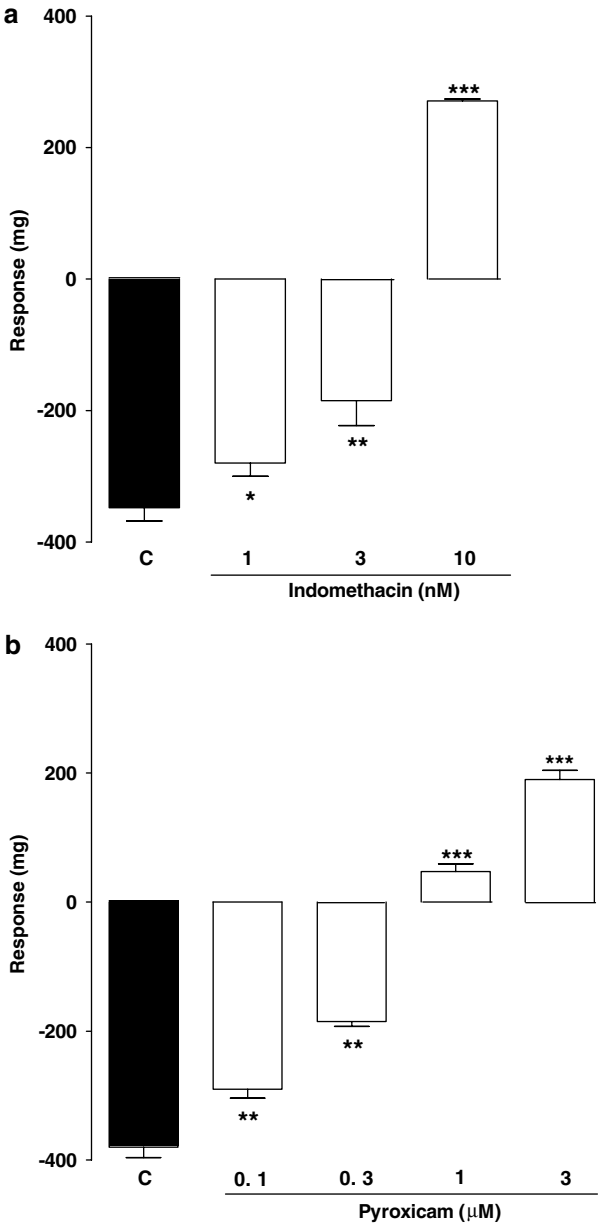

Figure 2 Inhibitory effect of the nonselective COX inhibitors indomethacin (a) and pyroxicam (b) on BK (100 nM)-induced relaxation in the GPT + E. Negative and positive values represent relaxation and contraction responses, respectively. The bars represent the mean of 5–7 experiments and the vertical lines indicate the s.e.m. Data differ significantly from control value, $*P<0.05$, $**P<0.01$.

EGFR-Trk inhibitor AG 490 (1–30 μM) inhibited the relaxation response caused by BK (100 nM) in a concentration-dependent manner (Figure 6b). The calculated mean IC_{50} value for AG 490 was 13.4 (10.8–21.1) μM. Both of the Trk inhibitors changed the BK relaxation response to a sustained contraction and produced a marked reduction in the tonus of the preparations (data not shown).

The possible role of potassium channels and/or epithelium-derived hyperpolarizant factor in BK-induced relaxation in the GPT was also investigated. The following potassium channel blockers did not display any significant effect on BK-induced

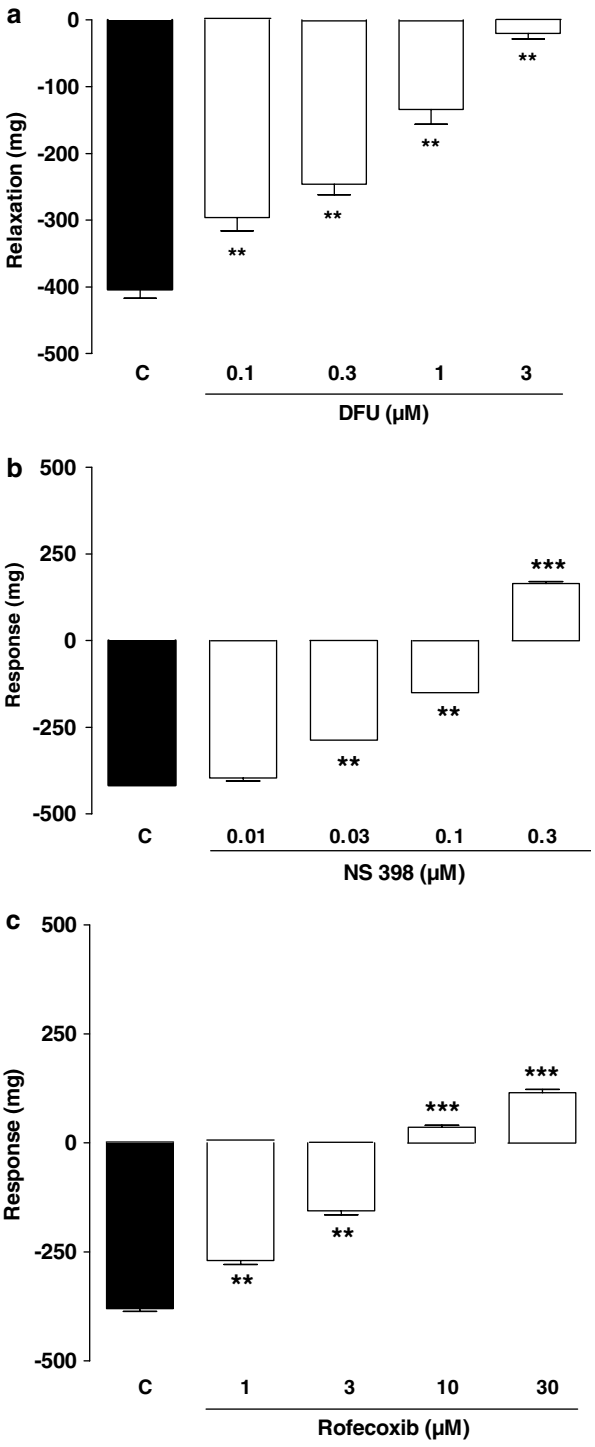

Figure 3 Inhibitory effect of selective COX-2 inhibitors DFU (a), NS 398 (b) and rofecoxib (c) on BK (100 nM)-induced relaxation in the GPT + E. Negative and positive values represent relaxation and contraction responses, respectively. The bars represent the mean of 7–8 experiments and the vertical lines indicate the s.e.m. Data differ significantly from control values, $^{**}P<0.01$, $^{***}P<0.001$.

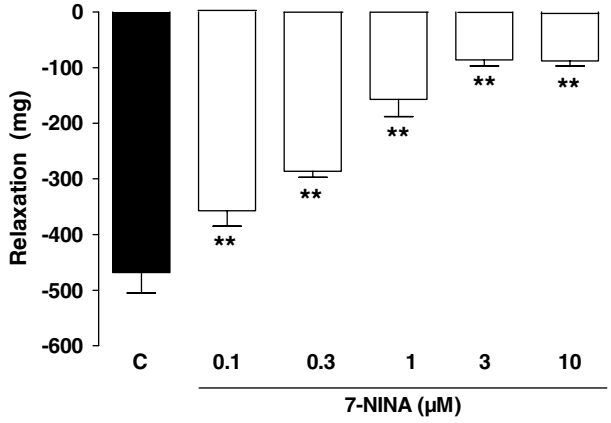

Figure 4 Inhibitory effect of the nonselective NO synthase inhibitor 7-NINA on BK (100 nM)-induced relaxation in the GPT + E. The bars represent the mean of 7–8 experiments and the vertical lines indicate the s.e.m. Data differ significantly from control values, $^{**}P<0.01$.

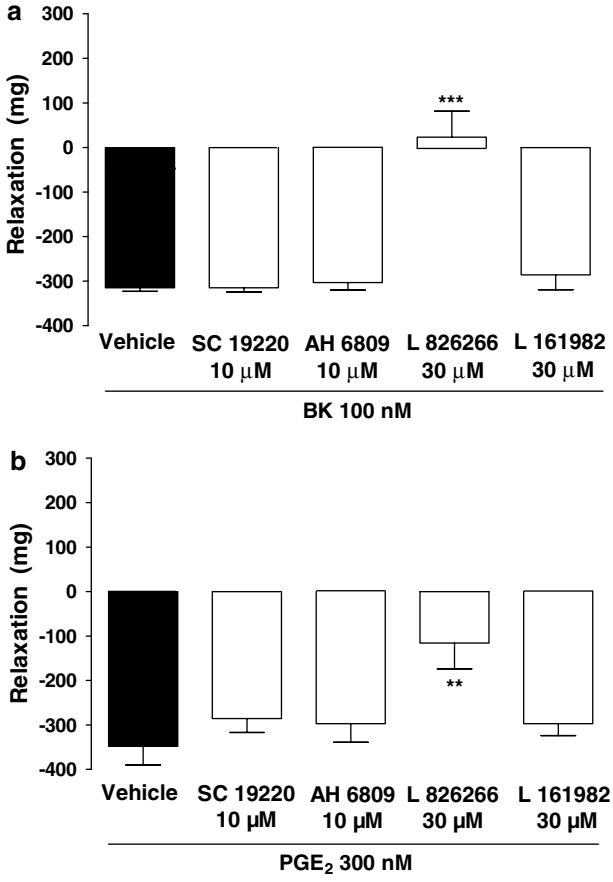

Figure 5 Effect of selective EP_1/EP_2 (SC 19220, 10 μM), EP_2 (AH 6809, 10 μM), EP_3 (L 826266, 30 μM) and EP_4 (L 161982, 30 μM) receptor antagonists on (a) BK (100 nM)- or (b) PGE_2 (300 nM)-induced relaxation in the GPT + E. Negative and positive values represent relaxation and contraction responses, respectively. The bars represent the mean of 7–8 experiments and the vertical lines indicate the s.e.m. Data differ significantly from control value, $^{**}P<0.01$, $^{***}P<0.001$.

relaxation in the GPT + E (results not shown): 4-aminopyridine (1 mM), apamin (100 nM), charybdotoxin (100 nM), iberiotoxin (100 nM) or the association of apamin (100 nM) plus charybdotoxin (100 nM).

It is worth to mention that all tested drugs, which affected BK-induced relaxant responses, did not significantly alter the relaxant response caused by the β-adrenergic agonist isoproterenol in the GPT + E (results not shown).

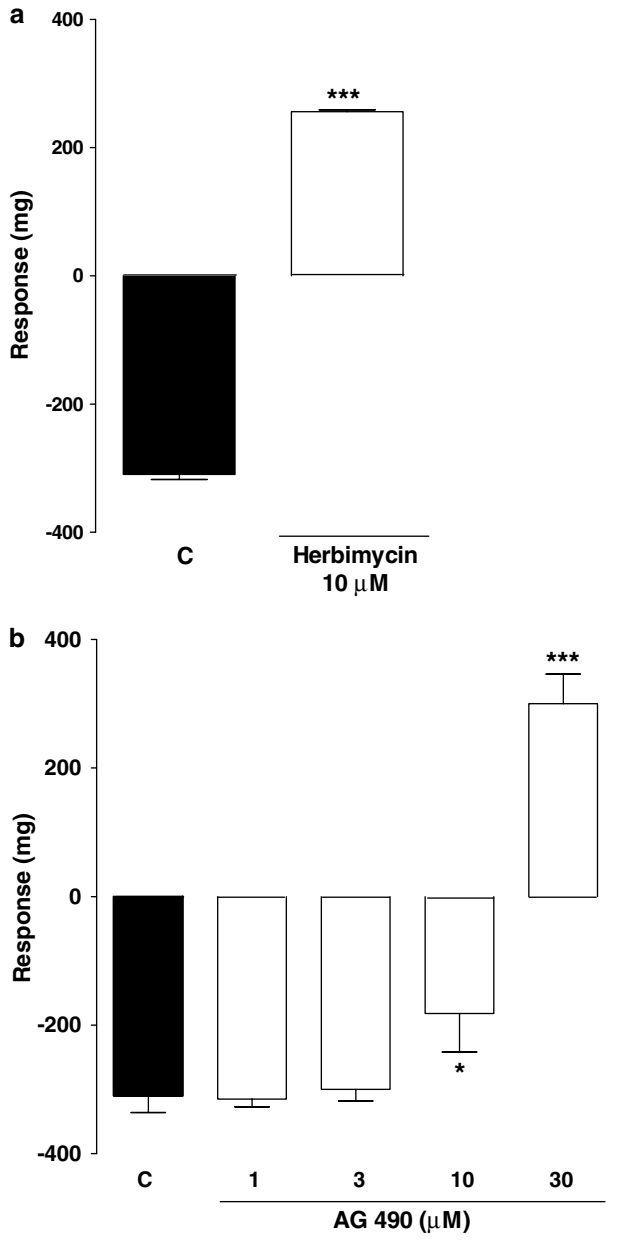

Figure 6 Relaxing effect of BK (100 nM) in the absence and presence of herbimycin A (a) and AG 490 (b) in the GPT + E. Negative and positive values represent relaxation and contraction responses, respectively. The bars represent the mean of 8–9 experiments and vertical lines are the s.e.m. Data differ significantly from control value, $*P<0.05$, $***P<0.01$.

BK-induced prostaglandin release in the GPT

The functional evidence indicating the importance of PGE_2 production for BK responses in the GPT + E was extended by measuring the levels of this prostanoid in the nutrient solution. Even in basal conditions, there was a substantial production of PGE_2 in the GPT + E. Following stimulation of this preparation with BK (100 nM) for 10 min, a marked increase in PGE_2 production was observed. The increase in PGE_2 in response to BK was significantly inhibited by selective COX-2 inhibitor NS 398 (100 nM) (82.5 ± 13.8%), the selective kinin B_2 receptor antagonist HOE 140 (100 nM) (83.7 ± 17.4%) or the selective

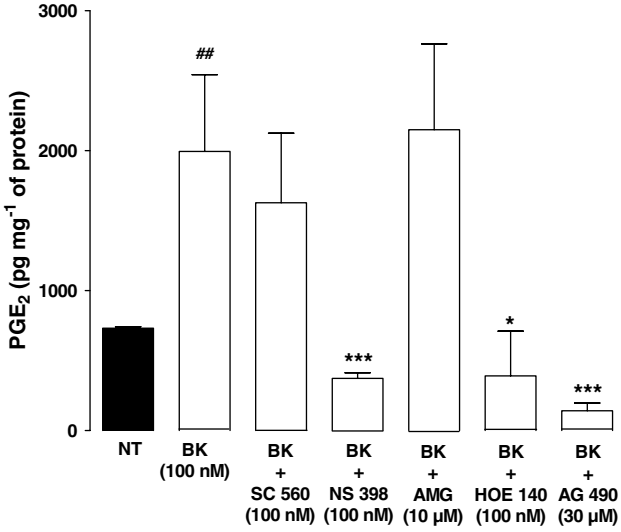

Figure 7 Effect of incubation with COX-1 (SC 560), COX-2 (NS 398), NO synthesis (aminoguanidine, AMG) and EGFR-Trk (AG 490) inhibitors or the selective B_2 receptor antagonist (HOE 140) on BK (100 nM)-induced PGE_2 generation in the GPT + E. PGE_2 concentrations were measured by ELISA. Each point represents the mean ± s.e.m. of three experiments. $\#P<0.05$ vs nontreated (NT) preparations, $**P<0.01$, $***P<0.001$ vs BK-stimulated GPT + E.

EGFR-Trk inhibitor AG 490 (10 μM) (95.2 ± 7.6%). On the other hand, neither the NOS inhibitor aminoguanidine (10 μM) nor the COX-1 inhibitor SC 560 (100 nM) succeeded in significantly affecting PGE_2 release caused by BK in the GPT + E (Figure 7).

Determination of the expression of COX-1, COX-2, eNOS, nNOS and iNOS in the GPT

Constitutively expressed COX-2, nNOS and eNOS (but not COX-1 and iNOS) proteins were found in the GPT with either intact or denuded epithelium (Figure 8a). The stimulation with BK (100 nM) did not significantly alter the COX-2 expression, suggesting that BK regulates PGE_2 release by post-transcriptional mechanisms. On the other hand, the addition of BK (100 nM) to the preparations resulted in a significant increase in nNOS and eNOS expression (38.3 ± 11.3 and 33.2 ± 8.9% increase, respectively) (Figure 8b).

Discussion

It has been well established that kinins, especially BK, are potent inducers of prostanoid release (Regoli & Barabé, 1980; Farmer & Burch, 1991; Farmer, 1992). BK has been reported to cause the activation of the 85-kDa cytosolic PLA_2 ($cPLA_2$) in a process greatly dependent on the rise in cytosolic free Ca^{2+}, which causes the release of AA from the cell membrane phospholipids, leading to prostanoid formation by the action of COX (Tanaka *et al*., 1995). BK has been found to be capable of stimulating the production of AA and PGE_2 in pulmonary A549 cells (Tokumoto *et al*., 1994; Saunders *et al*., 1999) and in human (Pang & Knox, 1997), bovine (Delamere *et al*., 1994) and guinea-pig (Farmer *et al*., 1991) airway smooth muscle cells.

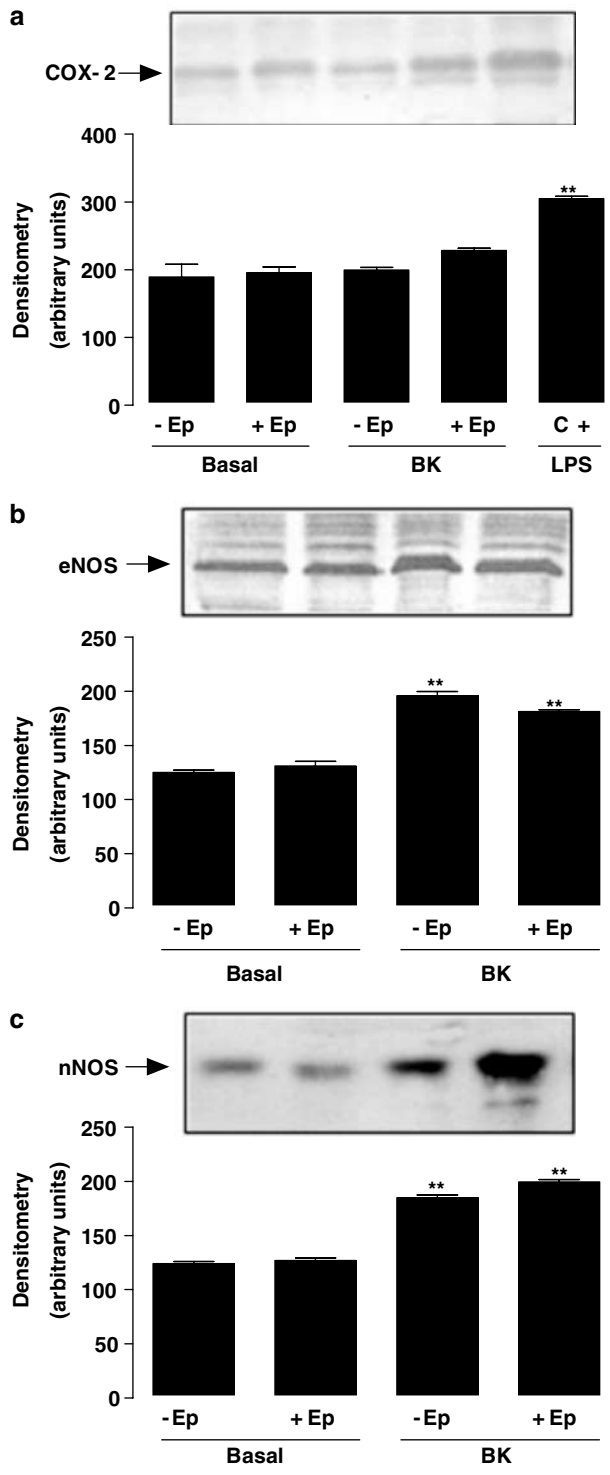

Figure 8 Representative Western blot showing the constitutive protein expression of COX-2 (a), eNOS (b) and nNOS (c) in the absence or presence of BK (100 nM; 10 min of incubation) in the GPT + E (+Ep) and GPT−E (−Ep). Results were normalized by arbitrarily setting the densitometry of the basal group and are expressed as the mean ± s.e.m. ($N = 3$). Data differ significantly from basal levels (−Ep and +Ep), $^{**}P < 0.01$.

The present study provides, by means of functional, biochemical and molecular approaches, new insights into the mechanisms involved in the relaxation response caused by BK in the GPT + E. Our data suggest that the response to BK involves complex mechanisms, mainly associated with the release of PGE_2. The increased expression of the constitutive COX-2 enzyme and both eNOS and nNOS appears to substantially modulate BK-mediated relaxation in the GPT + E. Moreover, we have found convincing evidence that BK-mediated relaxation in GPT + E is probably dependent on the activation of EGFR-Trk. Thus, the present findings contribute to understanding some of the physiological mechanisms underlying BK effects in the airways. As BK has been demonstrated to induce bronchoconstriction in asthmatic subjects (normally associated to the epithelial damage) (Ichinose *et al*., 1990; Polosa & Holgate, 1990), on the basis of the present results, it is possible to suggest a protective role for BK in the airways under normal conditions.

The airway epithelium is a physical barrier that protects sensory nerves and smooth muscle from stimulation by inhaled irritants. In addition, epithelial cells release mediators called epithelial-derived relaxing factors (EpDRFs) that can inhibit bronchoconstriction by relaxing the smooth muscle. Clear functional data for EpDRFs have been provided by experiments where different endogenous mediators have been found to induce the relaxation of trachea preparations with epithelium, but which cause a contraction in preparations lacking this layer. Recently, several pieces of evidence have suggested a protective role for the EpDRFs in the airways (Fernandes & Goldie 1990; Burgaud *et al*., 1993). Folkerts & Nijkamp (1998) have drawn attention to the pharmacological relevance of the putative EpDRFs, PGE_2 and NO to the modulation of airway tone under basal conditions *in vitro* and *in vivo*. Special attention has been given to the role of EpDRFs in the development of airway hyper-responsiveness in animal models and in patients with asthma. In fact, a previous publication of our group has indicated that the epithelium removal completely prevented BK-induced relaxation, changing the relaxant response to a sustained contraction (Schlemper & Calixto, 2002).

NO (or an NO-like substance) has been demonstrated to produce the relaxation of airway smooth muscle preparations obtained from several animal species (Hamad *et al*., 2003). NOS inhibition enhances agonist-induced increase in airway resistance in the guinea-pig, and this supports a role for EpDRFs in the control of bronchomotor tone (Nijkamp *et al*., 1993; Yoshihara *et al*., 1998). Previous studies have indicated that BK causes relaxation of the GPT + E through the activation of B_2 receptor and the subsequent release of NO and prostanoids (Schlemper & Calixto, 1994; 1995). Thus, BK, distinct from other agonists, seems to be capable of releasing inhibitory substances, which may be represented by PGE_2 and NO.

In various blood vessels, endothelium-dependent relaxation can be accompanied by the endothelium-dependent hyperpolarization of smooth muscle cells. This response can be partially or totally resistant to inhibitors of cyclooxygenases and NO synthases (Nagao & Vanhoutte, 1992). Several lines of evidence have suggested that the endothelium-dependent hyperpolarization results from the opening of K^+ channels in the smooth muscle plasmalemma. Thus, K^+ channels play a key role in the regulation of membrane potential and cell excitability. The most important K^+ channels in smooth muscle, including the airways, are ATP-dependent K^+ channels (activated by a fall in intracellular ATP and a rise in nucleotide diphosphates), BK_{Ca} (activated by a rise in

intracellular Ca^{2+}) and Kv (activated by depolarization) (Kotlikoff, 1993; Nielsen-Kudsk, 1996). In the present work, no pharmacological strategy used to block K^+ channels was able to interfere with BK-evoked relaxation in the GPT + E, thus discarding the possibility that in that preparation, EpDRFs is represented by one of the above-mentioned K^+ channels.

The present results indicate that BK-mediated relaxation in the GPT + E involves the release of PGE_2 *via* the activation of the constitutive COX-2 enzyme. In this way, several known selective COX-2 inhibitors, including NS 398, DFU and rofecoxib, as well as the nonselective COX-1 and COX-2 inhibitors indomethacin and pyroxicam, completely prevented BK-mediated relaxation in the GPT + E. On the other hand, the selective COX-1 inhibitor SC 560 had no effect on the relaxation effects of BK. We employed Western blotting technique to gain further insights into the role of the constitutive COX-2 enzyme in BK-mediated relaxation in GPT + E. Our data are in agreement with those from an earlier study conducted by Charette *et al.* (1995) regarding the presence of a constitutive COX-2 enzyme in this tissue. In contrast, the COX-1 enzyme was not detected. Interestingly, BK addition to GPT + E failed to cause any change in COX-2 expression, in conditions where LPS caused a marked increase in the expression of the enzyme.

Another striking aspect investigated in the present study was the involvement of NOS in the relaxation induced by BK in the GPT + E. Thus, the nonselective NOS inhibitor 7-NINA significantly prevented BK-induced relaxation in this preparation. In addition, Western blotting studies confirmed the presence of both constitutive eNOS and nNOS, while iNOS was not detected in the GPT + E. Notably, acute BK addition to the preparations produced a marked increase in the expression of both eNOS and nNOS. Our results also show that the effects of BK on the expression of eNOS and nNOS seem to be independent of the integrity of the tracheal epithelium. The reason why BK produced an increase in the expression of both eNOS and nNOS is presently unclear and requires further investigation. Interestingly, in our experiments, ODQ (a known guanylate cyclase) did not display any significant effect on BK-induced relaxant responses, indicating that cGMP-independent pathways are involved in the actions of NO. A similar event has been demonstrated previously in various systems, including the guinea-pig isolated bronchus (Janssen *et al.*, 2000; Mazzuco *et al.*, 2000; Mingone *et al.*, 2003; Soloviev *et al.*, 2004).

We next investigated which prostanoid receptor might be involved in BK-mediated relaxation in GPT + E, by using selective EP receptor antagonists. Our results show that the selective EP_3 receptor antagonist L 826266 consistently prevented BK-mediated relaxation in GPT + E. On the other hand, the selective antagonists for the EP_1/EP_2 (SC 19220), and the EP_2 (AH 6809) or EP_4 (L 161982) receptors, employed at appropriate concentrations, all failed to affect BK-mediated relaxation in this preparation. From this, it is possible to conclude that PGE_2 production is directly implicated in the relaxing effects of BK in the GPT + E. The effects of PGE_2 seem to be related to the activation of EP_3, but not EP_1, EP_2 or EP_4, receptors. These results further confirm the presence of the EP_3 receptor for PGE_2 in the GPT + E and indicate that this receptor plays a critical role in BK-mediated relaxation responses. The EP_3 exists in multiple isoforms, numbering eight subtypes in humans (Namba *et al.*, 1993; Pierce & Regan, 1998; Breyer *et al.*, 2001). Its isoforms are differentially expressed in tissues, interacting with all agonists, but they show quite different coupling types for intracellular second messengers. All isoforms couple to G_i, resulting in inhibition of adenylate cyclase, while some couple themselves to G_s-type proteins, activating the adenylate cyclase or G_q to activate PLC. Another type is the EP_{3B} isoform, which leads to G_{13} coupling, activation of small GTPase Rho, activation of Rho kinase and multiple physiological responses such as neurite retraction or smooth muscle contraction (Somlyo & Somlyo, 2000; Fukata *et al.*, 2001; Hatae *et al.*, 2002).

The importance of PGE_2 in the GPT + E was further confirmed by functional studies indicating that PGE_2 is able to induce a relaxation response very similar to that produced by the incubation of BK. Moreover, biochemical data showed that incubation of BK causes a marked increase in PGE_2 production in the GPT + E. In this case, the effects of BK are probably related to the activation of B_2 receptor and COX-2 stimulation, as the increased production of PGE_2 was markedly prevented by incubation with the selective B_2 receptor antagonist HOE 140 or the COX-2 inhibitor NS 398. Moreover, the involvement of COX-1 was discarded, since the incubation with the selective COX-1 inhibitor SC 560 had no effect on BK-evoked PGE_2 production. A new and interesting result of the present study was the synergistic interaction obtained by the association of low concentrations of BK and PGE_2 that alone failed to induce GPT + E relaxation, but when used in combination produced a striking synergistic relaxation effect. Such results further reinforce the notion that an interesting crosstalk between BK, PGE_2 and NO might be crucial for the relaxation responses in the GPT + E.

Another relevant new finding of the present study was that herbimycin A, a nonselective Trk inhibitor, and especially AG490, a selective EGFR-Trk inhibitor, consistently inhibited BK-mediated relaxation in GPT + E. Of note, the incubation with AG-490 was also highly effective in preventing the BK-induced increase of PGE_2 generation. Interestingly, the same drugs failed to inhibit the relaxation induced by PGE_2 (results not shown). These results strongly suggest, for the first time, that the EGFR-Trk might modulate BK actions *via* an interaction with B_2 receptor, with the subsequent activation of the constitutive COX-2 enzyme and PGE_2 release in the airways. Recently, in this regard, observations of the cooperative actions of BK and EGFR-Trk have been reported (Mukhin *et al.*, 2003; Hur *et al.*, 2004). Moreover, an important role for EGFR-Trk has been recently proposed in the airways and related pathologies (Wong & Leong, 2004).

Curiously, other relevant signalling pathways frequently involved in BK actions, such as K^+ channel activation, nuclear factor-κB, the protein kinases A, C and G, PLA_2 and C or MAP kinases, appear not to account for BK-mediated relaxation in the GPT + E. In this context, the relaxation caused by BK in the GPT + E appears to involve nonclassical transduction mechanisms, different from those usually linked to relaxation actions of BK *in vitro* (Chand *et al.*, 1987; Pyne *et al.*, 1997; Van Heuven-Nolsen *et al.*, 1997; Mazzuco *et al.*, 2000; Teoh & Man, 2000; Mukhin *et al.*, 2003).

In summary, the results of the present study provide consistent experimental support indicating that BK, like PGE_2, induces a concentration- and time-dependent relaxation

effect in the GPT + E *via* the activation of B_2 receptor with stimulation of the constitutive COX-2 enzyme and PGE_2 production. In addition, PGE_2 effects are probably related to the activation of EP_3 receptors. The present data also demonstrate that the release of NO, presumably through stimulation of both eNOS and nNOS, also largely accounts for the relaxing action of BK in this preparation. A new and relevant event for the mechanisms underlying BK-mediated relaxation in GPT + E is its ability to activate the EGFR-Trk, probably leading to the release of PGE_2, which can induce smooth muscle relaxation. Thus, PGE_2 and NO might represent EpDRF in this preparation or could induce the release of EpDRF. Collectively, the present results provide a comprehensive view regarding the possible mechanisms through which BK induces relaxation in the GPT + E. Such an action might account for the protective effect of BK in airway smooth muscle.

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