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Mechanisms underlying the relaxation induced by isokaempferide from *Amburana cearensis* in the guinea-pig isolated trachea

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Abstract

The present study examines possible mechanisms by which the flavonoid isokaempferide (IKPF; 5,7,4′-trihydroxy-3-methoxyflavone) from *Amburana cearensis*, a Brazilian medicinal plant popularly used as bronchodilator, induces relaxation of guinea-pig isolated trachea. In the trachea (with intact epithelium) contracted by carbachol, IKPF (1–1000 μM) caused a graded relaxation, and the epithelium removal increased the sensitivity of the airway smooth muscle to IKPF (EC50, in intact tissue: 77.4 [54.8–109.2] μM; in denuded epithelium: 15.0 [11.3–20.1] μM). The IKPF-induced relaxation was inhibited in 41% by the nitric oxide (NO) synthase inhibitor L-NAME (100 μM); in 31% and 50% by the soluble guanylate cyclase (sGC) inhibitor ODQ (3 and 33 μM); by propranolol (31%) and also by capsaicin (37%). In the trachea pre-contracted by 40 mM KCl the pre-incubation with glibenclamide (33 μM) or iberiotoxin (IbTX, 0.1 μM), selective K^+ channel inhibitors, inhibited the IKPF-induced relaxation by 39% and 38%, respectively. On the other hand, 4-aminopyridine (100 μM), a nonselective K^+ channel antagonist, did not significantly influence the effect of IKPF, while IbTX induced a rightward displacement of the IKPF concentration–response curve. However, in muscle pre-contracted with 120 mM KCl the relaxant effect of IKPF was significantly reduced and not affected by glibenclamide. In conclusion, these results indicate a direct and epithelium-independent relaxant effect of IKPF on smooth muscle fibers. Although this IKPF relaxant action seems to be multi-mediated, it occurs via both Ca^{2+} and ATP-sensitive K^+ channels, but some other possible mechanisms unrelated to K^+ channels cannot be excluded.

Keywords: Isokaempferide; *Amburana cearensis*; Guinea-pig trachea; K^+ channels; Soluble guanylate cyclase

Introduction

One of the most important cell types involved in the pathology of airways is the smooth muscle cell. A potentially important feature of the smooth muscle is their ability to alter their phenotype in response to different stimuli (Hirst et al., 2000). This cell type is implicated in the development of asthma for two reasons. Firstly, it is an effector of contractile stimuli, and secondly, due to hyperplasia and or hypertrophy, it is responsible for the increase of airway wall mass (Wills-Karp, 1997). Asthma is a complex clinical disease characterized by airway obstruction, inflammation and hyper-responsiveness to a variety of pharmacological stimuli such as histamine, methacoline as well as physical stimuli as the exercise and cold air (Hargreave et al., 1981; Bharadwaj and Agrawal, 2004).

Amburana cearensis (Fabaceae) is a medicinal plant common to the Brazilian Northeastern "caatinga" (savannah), and popularly used in respiratory tract diseases including asthma (Braga, 1976). Several compounds such as 3,4-dihydroxybenzoic acid (protocatechuic acid), a mixture of glucosylated β-sitosterol and stigmasterol, coumarin, four flavonoids

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(isokaempferide-IKPF, kaempferol, afrormosin, and 4′-methoxyfisetin) and the phenol glucosides, amburosides A and B, were isolated from the trunk bark of this plant (Bravo et al., 1999; Canuto and Silveira, 2000).

5,7,4′-Trihydroxy-3-methoxyflavone or IKPF has been also isolated from other species as *Genista ephedroides*, *Combretum quadrangulare* and *Dracocephalum subcapitatum* (Pistelli et al., 1998; Banskota et al., 2000a, Saeidnia et al., 2005). It has antibacterial and antiviral activities (Wang et al., 1989; De Meyer et al., 1991) and also presents a strong inhibitory effect on the TNF-alpha-induced cell death (Banskota et al., 2000b). In another work, IKPF was shown to inhibit the sea urchin egg development, as well as the growth of tumor cell lines (Costa-Lotufo et al., 2003).

In previous studies (Leal et al., 2000, 2003), we showed that hydroalcoholic extract, coumarin and the flavonoid fraction (with IKPF as the main constituent) from *A. cearensis* have anti-inflammatory activity and were able to relax the isolated guinea-pig tracheal smooth muscle pre-contracted by carbachol, histamine or KCl. Despite the fact that IKPF is a known molecule, its effect on the smooth muscle has not been investigated yet. The objective of the present study is to expand our previous findings, emphasizing whether IKPF causes relaxation in the guinea-pig isolated trachea and to characterize some of the putative pharmacological mechanisms responsible for such effects.

Materials and methods

Drugs

Carbachol (CCh), theophylline, propranolol hydrochloride, 4-aminopyridine, iberiotoxin (IbTX), glibenclamide, L-N^G-nitro-arginine methyl ester (L-NAME), 1H-[1,2,4]oxadiazolol [4,3-*a*] quinoxalin-1-one (ODQ), indomethacin, phentolamine and capsaicin were purchased from Sigma Chemical Co. (St. Louis, MO). All other drugs were of analytical grade.

Plant material and isolation of isokaempferide (IKPF)

Trunk barks of *A. cearensis* were collected at Quixeramobim County, Ceará State. Voucher specimens (nos. 837 and 847) were deposited at the Prisco Bezerra Herbarium, Federal University of Ceará, and authenticated by Dr. Afrânio G. Fernandes, Department of Biology. The powdered trunk bark (3.3 kg) was submitted to extraction with ethanol (EtOH) and rotoevaporated yielding a dark brown residue, designated ACCE. ACCE was dissolved in H_2O and partitioned with EtOAc to yield ACCEAq and ACCEA. ACCEA was dissolved in MeOH and partitioned with hexane to yield ACCEAM and ACCEAH.

ACCEAM was chromatographed on a silica gel column, and eluted with CH_2Cl_2 followed by CH_2Cl_2/EtOAc 1:1, EtOAc and finally MeOH to yield, ACCEAM/C, ACCEAM/CA, ACCEAM/A and ACCEAM/M, respectively. ACCEAM/CA was chromatographed on a silica gel column and fractions were collected by elution with CH_2Cl_2 (1), CH_2Cl_2/EtOAc 9:1 (2–3); CH_2Cl_2/EtOAc 8:2 (4–13); CH_2Cl_2/AcOEt 7:3 (14–17), CH_2Cl_2/AcOEt 5:5 (18–20), EtOAc (21–24) and finally MeOH (−25). TLC analysis led to the pooled fraction ACCEAM/CA (6–7; 1.1 g). ACCEAM/CA was chromatographed over a Sephadex LH-20® column and 20 fractions of 8 mL were collected. TLC analysis allowed to pool fractions 11–15 (427 mg) that were again submitted to filtration over Sephadex LH-20®, affording 18 MeOH fractions of 8 mL. TLC analysis led to the pooled fractions 2–10, consisting of a yellowish powder identified as isokaempferide (139.5 mg, 0.004%) by spectroscopy methods. The physical–chemical characteristics found for isokaempferide agree with earlier studies (Sim, 1969; Ganzera et al., 1998; Canuto and Silveira, 2000).

Isolated guinea pig trachea

Guinea-pigs from the Animal House of the Federal University of Ceará were maintained in cages under a 12 h/12 h light/dark cycle for 2–3 days before use. Animals (300–400 g) from both sexes were anaesthetized with ethyl ether and killed by cervical dislocation. The trachea was rapidly removed and, after freed from connective tissues, was cut into pieces (3–4 mm wide) and mounted in a 7 mL organ chamber, containing Krebs–Henseleit solution of the following composition (mM): NaCl 119.0, $NaHCO_3$ 25.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.1, KH_2PO_4 1.2, glucose 11.0. All experiments were carried out in the presence of indomethacin (3 μM), to prevent the fading of neural response as a result of endogenous prostaglandin production (Miura et al., 1997; Vaali et al., 1998). In some experiments phentolamine (10 μM) and propranolol (1 μM) were added to inhibit alpha and beta adrenergic response (Vaali et al., 1998; Kesler et al., 2002). The solution was maintained at 37 °C, pH 7.4, gassed with 95% O_2–5% CO_2 during the experiments. Tracheal rings were submitted to 1 g tension and isometric responses were measured by means of a F-60 force displacement transducer, and recorded on a polygraph (Narco Bio Systems Inc., USA). Tissues were allowed to equilibrate for at least 60 min before drug addition, and during this time the buffer solution was renewed every 15 min. In the case of experiments performed with the denuded trachea, the epithelial layer was gently removed with a cotton-tipped applicator, and the denudation was assessed by histological analysis (light microscopy, haematoxylin–eosin staining) and by the contractile response to bradykinin (1 μM) (Folkerts and Nijkamp, 1998).

Experimental procedure

After the equilibrium period of at least 60 min, tissues with or without epithelium were pre-contracted with carbachol (CCh, 30 or 100 μM, 70–90% maximal contraction) or KCl (40 mM (49% maximal contraction) or 120 mM). When the contraction became stable (usually after 10 min), tissues were exposed to IKPF (10–1000 μM), which was added to the bath by the cumulative method of Van Rosum (1963). All relaxations were expressed as a percentage of CCh

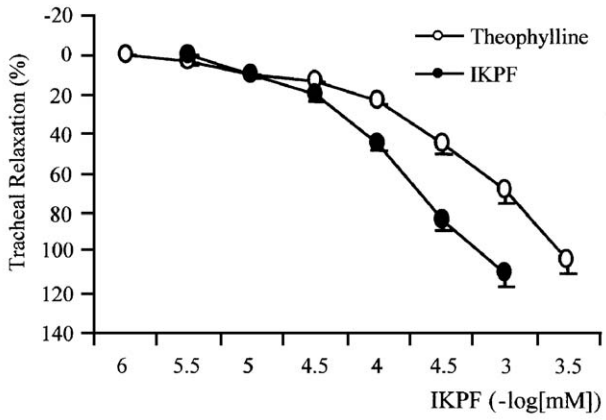

Fig. 1. Relaxant concentration–response curves of isokaempferide (IKPF) or theophylline on the carbachol-contracted guinea-pig trachea. Each point represents the mean±S.E.M. of 6 experiments. In some points of the figure, the S.E.M. does not appear for being very close to the symbols.

or KCl-induced maximal contractile response. Theophylline, a nonselective inhibitor of phosphodiesterase, was used as standard.

To investigate possible mechanisms responsible for the myorelaxant action induced by IKPF, the guinea-pig trachea, with or without epithelium, was pretreated with one of the following drugs: L-NAME (nitric oxide synthase competitive antagonist, 100 μM), ODQ (selective inhibitor of the soluble guanylate cyclase-sGC, 3 and 33 μM), propranolol (β-adrenergic blocker, 1 μM), capsaicin (an excitatory and desensitizing agent on a subset of primary afferent sensory neurons, 3 μM), 4-aminopyridine (4-AP, a selective blocker of K^+ channels, 100 μM), iberiotoxin (IbTX, a selective blocker of large Ca^{2+}-sensitive K^+ channels, 0.1 μM) and glibenclamide (a selective blocker of ATP-sensitive K^+ channels, 33 μM) for 20 min prior to the addition of CCh or KCl. Experiments were performed according to the Guide for Care and Use of Laboratory Animals, from the US Health and Human Services Department. The manuscript protocol was approved by our Institutional Ethics Committee. Indomethacin and phentolamine was dissolved in absolute ethanol, the final concentration of ethanol in the baths being 0.004%. Other reagents were dissolved in distilled water or Tween 80 (final concentration not more than 0.05%). IKPF was dissolved in an aqueous solution containing 4% Tween 80 before use, and the final concentration of Tween 80 did not exceed 0.24%.

Statistical analysis

Data are presented as mean±S.E.M. of 6 to 8 experiments. EC50 values (i.e., the concentration of IKPF at which 50% of the maximal response was observed) were calculated by interpolation from semi-logarithmic plots, and expressed as geometric means (95% confidence interval). Statistical analyses of the results were carried out by unpaired Student's *t*-test (GraphPad Prism program, USA), comparing each IKPF concentration effect in the presence or absence of antagonists. The level of significance was set to $p<0.05$.

Results

Relaxations induced by IKPF (10–1000 μM) and by theophylline (3–3000 μM), used as standard, in the CCh pre-contracted trachea were well reproducible, with no evidence of tachyphylaxis when experiments were carried out at 60–120 min intervals between curves (Fig. 1). The IKPF (10–1000 μM) elicited a relaxation of the spontaneous tone of the trachea in part of the experiments with a maximum effect corresponding to 6.15±2.92% ($n=7$) of the amplitude of the contraction induced by 40 mM KCl. The control (aqueous solution of Tween 80) did not interfere with the spontaneous tone. In the guinea-pig trachea pre-contracted by CCh or KCl, the maximum relaxation induced by Tween 80 was 8.38±1.23% or 7.39±1.07%, respectively. IKPF exhibited more potent relaxing effect on the contraction induced by KCl (IKPF EC50: 15.5 [11.3–21.0]) than after CCh (IKPF EC50: 107.3 [80.8–142.4]). The removal of the epithelium significantly increased the sensitivity of the airway smooth muscle to IKPF (EC50 values, in intact tissue: 77.4 [54.8–109.2] μM in denuded epithelium: 15.0 [11.3–20.1] μM). The maximal relaxation induced by IKPF in the guinea-pig trachea with denuded epithelium and pre-contracted by CCh (E_{max}: 122.6±9.97%) was not significantly different as related to IKPF-induced relaxation in the trachea with intact tissue (E_{max}: 98.0±7.42%) (Fig. 2).

The role of the nitric oxide pathway was also investigated by incubating the guinea-pig trachea with L-NAME (100 μM). In the intact tissue, the maximal relaxation induced by IKPF in the absence of L-NAME was 112% after 100 μM carbachol, while

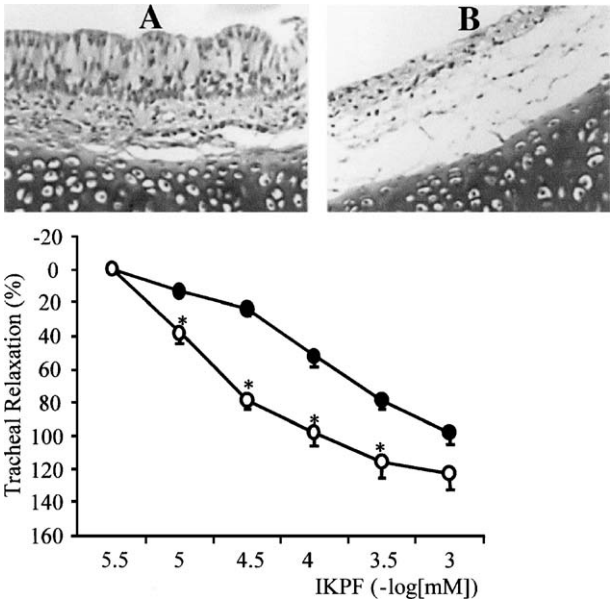

Fig. 2. Hematoxylin–eosin stained histological sections of guinea-pig trachea displayed before (A) and after (B) removal of the epithelial layer (upper). Relaxant concentration–response curves of isokaempferide (IKPF) on the carbachol-contracted guinea-pig trachea with intact (●) or denuded epithelium (○). Each point represents the mean±S.E.M. of 6–8 experiments. *$p<0.05$ for comparing IKPF concentrations (Student's *t*-test). In some points of the figure, the S.E.M. does not appear for being very close to the symbols.

in the presence of L-NAME, the maximal relaxing effect of IKPF was 66%. We also found that, in tissues with denuded epithelium, the incubation with L-NAME also affected the maximal relaxation to IKPF (E_{max} in the absence and in the presence of L-NAME were 136.5±7.9% and 96.0±5.4%, respectively) (Fig. 3).

In the intact tissue, ODQ (3 and 33 μM) prevented the relaxing effects of IKPF in a concentration-dependent manner, after the CCh contraction (Fig. 4). The IKPF relaxation in the absence of ODQ was 96.6±4.3%; and, in the presence of 3 and 33 μM ODQ, relaxations were 66.6±8.7% and 48.5±6.7%, respectively.

Glibenclamide (33 μM) significantly inhibited (39.0%) maximal relaxations induced by IKPF (1–300 μM), in the KCl (40 mM) pre-contracted trachea. Likewise, pre-incubation of tracheal rings with IbTX (0.1 μM) or 4-AP (100 μM) inhibited the IKPF-induced relaxation in 38.0% or 20.0%, respectively. Furthermore, IbTX caused a rightward displacement (about 10 fold) of the IKPF concentration–response curve (Fig. 5A). However, in preparations contracted with 120 mM KCl, a concentration that is presumed to induce a maximum depolarization of the cell membrane, the relaxation induced by IKPF was significantly reduced from 34.7% to 100% when related to the muscle pre-contracted by 40 mM KCl. In addition, 120 mM

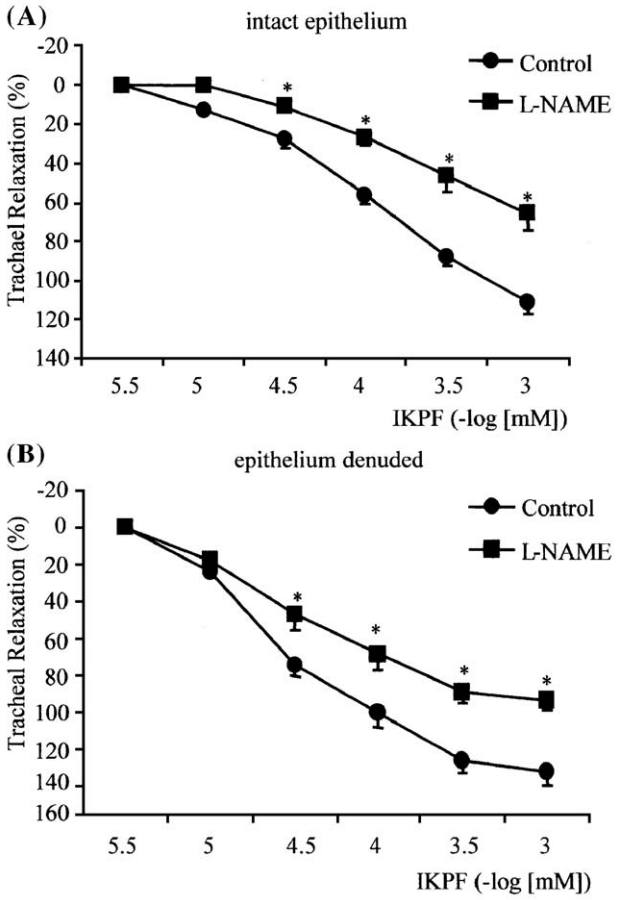

Fig. 3. Relaxant concentration–response curves of isokaempferide (IKPF) on the carbachol-contracted guinea-pig trachea, in the absence (control, ●) or in the presence of L-NAME 100 μM (■). Each point represents the mean±S.E.M of 6–8 experiments. *$p<0.05$ for comparing IKPF concentrations (Student's t-test). In some points of the figure, the S.E.M. does not appear for being very close to the symbols.

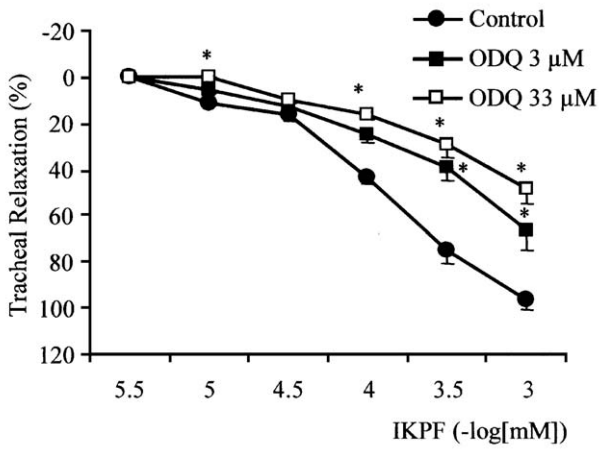

Fig. 4. Relaxant concentration–response curves of isokaempferide (IKPF) on the carbachol-contracted guinea-pig trachea, in the absence (control, ●) or in the presence of ODQ 3 (■) and 33 μM (□). Each point represents the mean± S.E.M of 6 experiments. *$p<0.05$ for comparing IKPF concentrations (Student's t-test). In some points of the figure, the S.E.M. does not appear for being very close to the symbols.

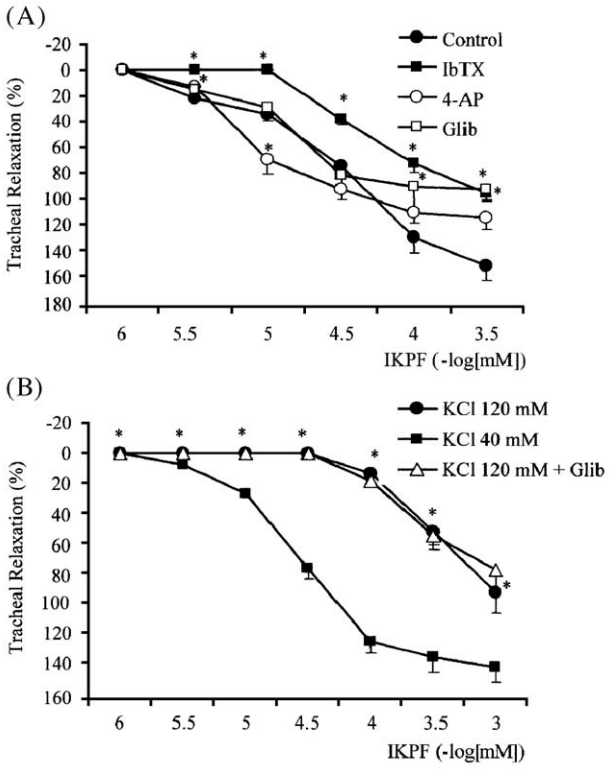

Fig. 5. Relaxant concentration–response curves of isokaempferide (IKPF) on the KCl (40 (A) or 120 mM (B)-contracted guinea-pig trachea, in the absence (control, ●) or in the presence of iberiotoxin, IbTx 0.1 μM (■), 4-aminopyridine, 4-AP 100 μM (○) or glibenclamide, Glib 33 μM (△)). Each point represents the mean±S.E.M of 5–8 experiments. *$p<0.05$ for comparing IKPF concentrations, in the absence or presence of K^+ channel blockers (Student's t-test). In some points of the figure, the S.E.M. does not appear for being very close to the symbols.

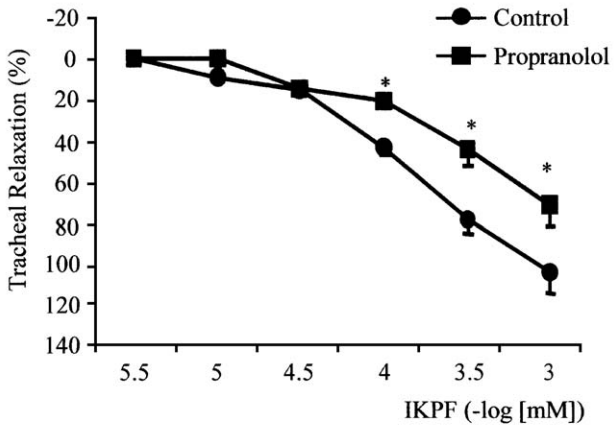

Fig. 6. Relaxant concentration–response curves of isokaempferide (IKPF) on the carbachol-contracted guinea-pig trachea, in the absence (control, ●) or in the presence of propranolol 1 μM (■). Each point represents the mean±S.E.M. of 6 experiments. *$p<0.05$ for comparing IKPF concentrations (Student's *t*-test). In some points of the figure, the S.E.M. does not appear for being very close to the symbols.

KCl induced a rightward displacement of the IKPF concentration–response curve. Glibenclamide (33 μM) did not modify the muscle response to IKPF in the trachea contracted by 120 mM KCl (Fig. 5B).

The pre-incubation of tracheal rings with propranolol (1 μM) produced a 31% inhibition of IKPF-induced relaxation (Fig. 6). Pretreatment of the preparations with capsaicin (3 μM), an excitatory and desensitizing agent on a subset of primary afferent sensory neurons, significantly inhibited by 37% the IKPF-mediated relaxation of the guinea-pig trachea (Fig. 7).

Discussion

In the present study, IKPF (5,7,4′-trihydroxy-3-methoxyflavone) relaxed in a concentration-dependent manner the guinea-pig trachea pre-contracted by carbachol or KCl. IKPF exhibited a more potent relaxing effect in the presence of KCl than in the presence of carbachol, and was able to completely revert the tone induced by KCl (40 mM), indicating a direct effect of IKPF on the smooth muscle. In addition, IKPF was more potent to induce a relaxant effect, as compared to theophylline, a nonselective inhibitor of cyclic nucleotide phosphodiesterase. These findings suggest that IKPF is, at least in part, responsible for the relaxant effect of the flavonoid fraction from *A. cearensis*, as shown in an earlier study (Leal et al., 2003).

IKPF elicited relaxations of KCl or carbachol-induced contractions, in magnitudes that went even below the baseline tension. This was unexpected since our experiments were conducted in the presence of indomethacin, in order to abolish the eicosanoid-dependent basal tension (Miura et al., 1997; Vaali et al., 1998).

As IKPF was able to cause a significantly greater relaxant effect in epithelium-denuded than that in epithelium-intact trachea, this suggests that the IKPF effect is an epithelium-independent one. Previous observations (Holroyde, 1986) showed that the supersensitivity to 5-HT, histamine, adenosine, isoprenaline, and minimally to KCl, produced by the epithelium removal of guinea-pig trachea, was not due to the absence of a relaxant factor, but rather to the withdrawal of a permeability barrier. Hence, the increase of smooth muscle contractile response to IKPF after removal of the epithelium could be due to its greater concentration at the level of target cells.

Nitric oxide (NO) is generally accepted to play an important role in the regulation of the airway function under physiological and pathological conditions, such as asthma (Spicuzza et al., 2002). Several NO-related compounds, such as sodium nitroprusside and endogenous NO, activate sGC, elevate cyclic GMP and relax airway smooth muscle (Katsuki and Murad, 1977). NO can be released in airway tissues, either in the epithelium or in sensory neurons (Vaali et al., 2000). At least part of the relaxant effect of IKPF involves NO release from neurons, since in intact or epithelium-denuded trachea the pretreatment with the nitric oxide synthase inhibitor, L-NAME, significantly decreased IKPF-induced relaxation. In addition, the tracheal relaxant effect of IKPF was significantly attenuated, but not completely inhibited, by the pretreatment with the sGC inhibitor, ODQ. Taken together, these results suggest that IKPF-induced relaxation of the guinea-pig trachea is mediated by the activation of NO/sGC/GMPc pathways, hence we know that the accumulation of cyclic GMP can activate the expression of cyclic GMP-dependent protein kinase I (PKG), stimulating K^+ outflow and leading to smooth muscle relaxation.

It has been reported that NO is capable of stimulating Ca^{2+}-activated K^+ channels in smooth muscles (Bolotina et al., 1994), including those in the airways (Abderrahmane et al., 1998), causing membrane hyperpolarization, a decrease in calcium influx, and muscle relaxation (Lincoln and Cornwell, 1991). Agents that increase K_{Ca} activity during or after events that increase intracellular Ca^{2+} concentration would be expected to reduce the intracellular excitability and could direct

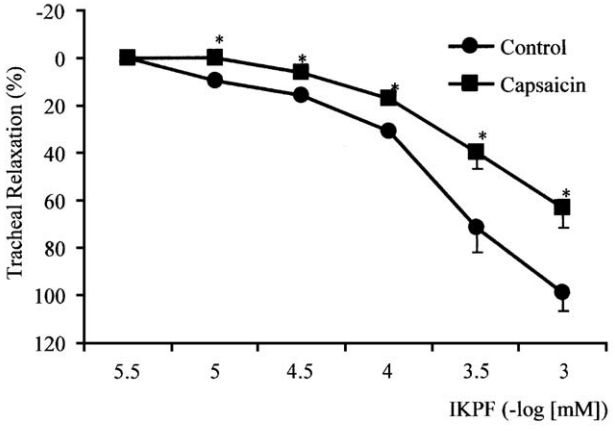

Fig. 7. Relaxant concentration–response curves of isokaempferide (IKPF) on the carbachol-contracted guinea-pig trachea, in the absence (control, ●) or presence of capsaicin 3 μM (■). Each point represents the mean±S.E.M. of 6 experiments. *$p<0.05$ for comparing IKPF concentrations (Student's *t*-test). In some points of the figure, the S.E.M. does not appear for being very close to the symbols.

or indirectly reduce neurotransmitter release (Gribkoff et al., 1996).

To investigate whether K^+ channels are also involved in the trachea relaxation response to IKPF, the preparation was pre-treated with K^+ channel blockers. Glibenclamide as well as IbTx were able to reduce significantly the relaxant effect of IKPF. Thus, both ATP-sensitive as well as large-conductance Ca^+-activated K^+ channels appear to be a molecular target for the relaxant activity of IKPF. However, the relaxant effect of IKPF (from 10^{-5} to 10^{-3} M) was significantly inhibited in the muscle pre-contracted by KCl 120 mM and, under this condition, glibenclamide did not interfere with the IKPF response. Similarly, Vaali et al. (1998) showed that in KCl-constricted guinea-pig trachea (40 mM KCl), iberiotoxin (IbTX) inhibited the relaxant effect of the nitric oxide donors, 3-morpholino-sydnonimine (SIN-1) and *S*-nitroso-*N*-acetylpenicillamine (SNAP). A previous study (Nielson-Kudsk, 1996) demonstrated that the prototype K^+ channel opener cromakalim relaxed contractions induced by 20–30 mM KCl, but had no effect against contraction induced by 124 mM KCl, while pinacidil relaxed the muscle in all these concentrations. In the present study, the tracheal tone induced by 40 mM KCl possibly still allows the K^+ efflux induced by K^+ channel openers. Taken together these results suggest that the relaxant effect of the IKPF is mediated via K^+ channels. However, some other possible mechanisms unrelated to K^+ channels might be involved.

The involvement of β-adrenoceptors in the relaxant action induced by IKPF in the guinea-pig trachea was drawn from the fact that the non-selective β-receptor antagonist, propranolol, inhibited the relaxing effect of IKPF. However, further studies are necessary for determining the precise participation of adrenoceptors in the relaxant effect of IKPF.

When excitatory C-fibers are stimulated, several neuropeptides are released from their nerve endings and exert various respiratory actions (Holzer, 1988). We showed that, in the trachea depleted of neuropeptides by capsaicin pretreatment, the IKPF relaxant activity was reduced. It has been reported that K^+ channels show inhibitory modulations on the activation of C-fibers (Ichinose and Barnes, 1990; Stretton et al., 1992). Since our work demonstrated that IKPF is an activator of K^+ channels, it is reasonable to suggest that, at least part of the relaxant effect of IKPF, may be caused by the inhibition of Ca^{2+} influx and the release of neuropeptides from sensory nerves, via the opening of K^+ channels.

Conclusion

In conclusion, our study provides pharmacological evidence that the relaxation of the guinea-pig isolated trachea, induced by IKPF, is a direct and an epithelium-independent phenomenon, resulting from several intracellular actions through a common pathway, the opening of Ca^{2+} and ATP-sensitive K^+ channels. However, an additional independent K^+ channel mechanism for the IKPF action seems to be involved. These results further indicate that IKPF may be responsible, at least in part, for the bronchodilator activity of *A. cearensis*.

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