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The Relaxant Effect of Extract of *Phyllanthus urinaria* in the Guinea-pig Isolated Trachea. Evidence for Involvement of ATP-sensitive Potassium Channels

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Abstract

This study analyses the relaxation induced by the hydroalcoholic extract of stems, leaves and roots from *Phyllanthus urinaria* (Euphorbiaceae) in the guinea-pig trachea (GPT) pre-contracted by carbachol.

The hydroalcoholic extract of *P. urinaria* (0.1-10 mg mL^{-1}) caused a graded relaxation in GPT with or without epithelium, with mean EC50 values of 1.94 (1.41–2.67) and 2.00 (1.47–2.78) mg mL^{-1} and E_{max} of 717 mg (± 16) and 627 mg (± 12), respectively. The relaxation in response to hydroalcoholic extract, like that to cromakalim (EC50 3.57 (2.75–4.64 μM) in GPT without epithelium, was fully abolished in the presence of high KCl concentrations (80 mM), and was significantly attenuated by tetraethylammonium (10 or 30 mM) or glibenclamide (0.1 or 3 μM). However, the relaxation caused by the hydroalcoholic extract was unaffected by apamin (0.1 or 1.0 μM), nitro-L-arginine (L-NOARG, 100 μM), methylene blue (10 μM) or by calcitonin gene-related peptide (CGRP) (8–37) (a CGRP antagonist, 0.1 μM). Both propranolol (1 or 3 μM) and [D-p-Cl-Phe^6,Leu^{17}]VIP (a vasoactive intestinal peptide (VIP) receptor antagonist, 0.1 μM) produced a significant displacement to the right (about 2-fold) of the relaxation response to hydroalcoholic extract of *P. urinaria*.

Thus, the present results indicate that the ATP-activated potassium channels sensitive to glibenclamide, but not the small conductance calcium-activated potassium channels sensitive to apamin, largely contribute to the relaxation effect of the hydroalcoholic extract of *P. urinaria* in GPT. In addition, both β_2 and VIP-mediated responses seem to account, at least in part, for the relaxation effect of the hydroalcoholic extract, as its relaxant response was partially attenuated by both propranolol and VIP receptor antagonist.

The hydroalcoholic extract of several species of Phyllanthus, including *P. urinaria* as well as the extract of callus culture of Phyllanthus species, when given systemically to mice, caused dose-related antinociception, mainly against the neurogenic pain induced by formalin and capsaicin (Gorski et al 1993; Santos et al 1994, 1995 a, b). In addition, low concentrations of the hydroalcoholic extract (HE) of *P. urinaria* also produced concentration-dependent contractile response followed by relaxation in the guinea-pig isolated trachea and only contraction in guinea-pig urinary bladder (Dias et al 1995; Paulino et al 1995). These contractile responses were demonstrated to be largely dependent on the increase of extracellular calcium influx, an effect which was found to be insensitive to both L- and N-type calcium-channel antagonists. In addition, at least in guinea-pig trachea, the tachykinin pathway seems to have a role in the contraction of the HE of *P. urinaria*, evident by the fact that contraction induced by HE was selectively antagonized by either NK_1 or NK_2 selective receptor antagonists and by ruthenium red, a selective blocker of the cation channel coupled to vaniloid receptors (Paulino et al 1995).

In the present study, we have attempted to characterize, by the use of selective antagonists, some of the mechanisms underlying the relaxation caused by the HE of *P. urinaria* in the guinea-pig trachea in-vitro.

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Materials and Methods

Preparation of the crude extract

Botanical material was collected and classified by Dr Leila da Graça Amaral and Miss Mirian Ulyssea (Department of Botany, Federal University of Santa Catarina). The dried leaves, stems and roots of *Phyllanthus urinaria* were minced and extracted with 50% ethanol-water in the proportion of 1:3 (w/v), being stirred and macerated at room temperature (21 ± 3°C) for 15 days. The ethanol was evaporated and the extract was concentrated to the desired level and stored in the freezer at −20°C. The extract was dissolved in distilled water just before use.

Tissue preparations

Guinea-pigs (250–400 g) of both sexes were anaesthetized with ether and were killed by cervical dislocation. The trachea was rapidly removed, and after being freed from connective tissue, each trachea was cut into six transverse rings (3–4 mm wide), each containing 3 cartilages as described previously (Schlemper & Calixto 1994, 1995). The rings were opened (usually 6 strips of 8–10 mm in length were obtained from the same animal) and were suspended in individual 5-mL jacketed organ baths containing Krebs-Henseleit solution maintained at 37°C, pH 7.2, gassed with a mixture of 95% O_2 and 5% CO_2. The Krebs solution had the following composition (mM): NaCl 118.0, KCl 4.4, $MgSO_4$ 1.1, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.2, glucose 11.0. Tissues were allowed to equilibrate for at least 60 min before drug additions, during which time the fresh buffer solution was renewed every 15 min, under a

resting tension of 1 g. Isometric responses were measured by means of TRI-201 force displacement transducers and were recorded on a polygraph (Letica Scientific Instruments, Barcelona, Spain). In most experiments the epithelial layer of the trachea was gently removed with a cotton-tipped applicator. The integrity of the epithelium was assessed by the ability of bradykinin (100 nM) to induce relaxation (Schlemper & Calixto 1994). Tissues were considered to contain a viable epithelium when bradykinin caused a relaxation of over 80% (approximately 300–400 mg) in preparations under spontaneous tonus.

Experimental procedure

After the equilibration period of at least 60 min, preparations with or without epithelium were exposed to HE of *Phyllanthus urinaria* ($0 \cdot 1$–10 mg mL^{-1}), which was added to the bath by means of a cumulative method (Van Rossum 1963). Two to five complete cumulative concentration-response curves were obtained for the extract in each preparation, at 60-min intervals between curves.

To investigate the possible mechanisms responsible for the relaxant response induced by HE of *P. urinaria* in guinea-pig trachea, the preparations without epithelium were contracted by addition of carbachol (0.1 or 0.3 μM), previously incubated with one of the following drugs 15 to 30 min beforehand: tetraethylammonium (TEA, a non selective blocker of K^+ channels, 10 or 30 mM), apamin (a selective blocker of calcium-sensitive K^+ channels of low conductance, 0.1 or 1 μM), glibenclamide (a selective blocker of ATP-sensitive K^+ channels, 0.1 or 3 μM), propranolol (a β-adrenergic receptor antagonist, 1 or 3 μM), [D-p-Cl-Phe^6, Leu^{17}] VIP (a VIP receptor antagonist, 0.1 or 0.3 μM), CGRP (8–37) (a CGRP receptor antagonist, 0.1 μM), methylene blue (an inhibitor of soluble guanylate cyclase, 10 μM), L-NOARG (an NO synthase competitive antagonist, 100 μM). In addition, we have also investigated the ability of the HE of *P. urinaria* to elicit relaxation in preparations pre-contracted with different concentrations of KCl (20, 40 or 80 mM). In order to correct for any spontaneous or agonist-induced changes in the responsiveness to preparations, parallel control experiments were always carried out in presence of the vehicles used to dilute each antagonist.

Drugs

The drugs used were obtained from the following sources: tetraethylammonium chloride, apamin, glibenclamide, noradrenaline bitartrate, propranolol chloride, methylene blue, L-N^G-nitroarginine (L-NOARG), vasoactive intestinal peptide (VIP) porcine, [D-p-Cl-Phe^6, Leu^{17}] VIP porcine, calcitonin gene-related peptide (CGRP) human, CGRP (8–37) human, carbamylcholine chloride (carbachol), cromakalim ([±]-*trans*-6-cyano-3,4-dihydro-2,2-dimethyl-4-[2oxopyrrolidin-1-yl]-2*H*-1-benzopyran-3-ol), EGTA (ethylene glycol-bis (β-aminoethyl ether]N,N,N′,N′-tetraacetic acid), PBS (phos-

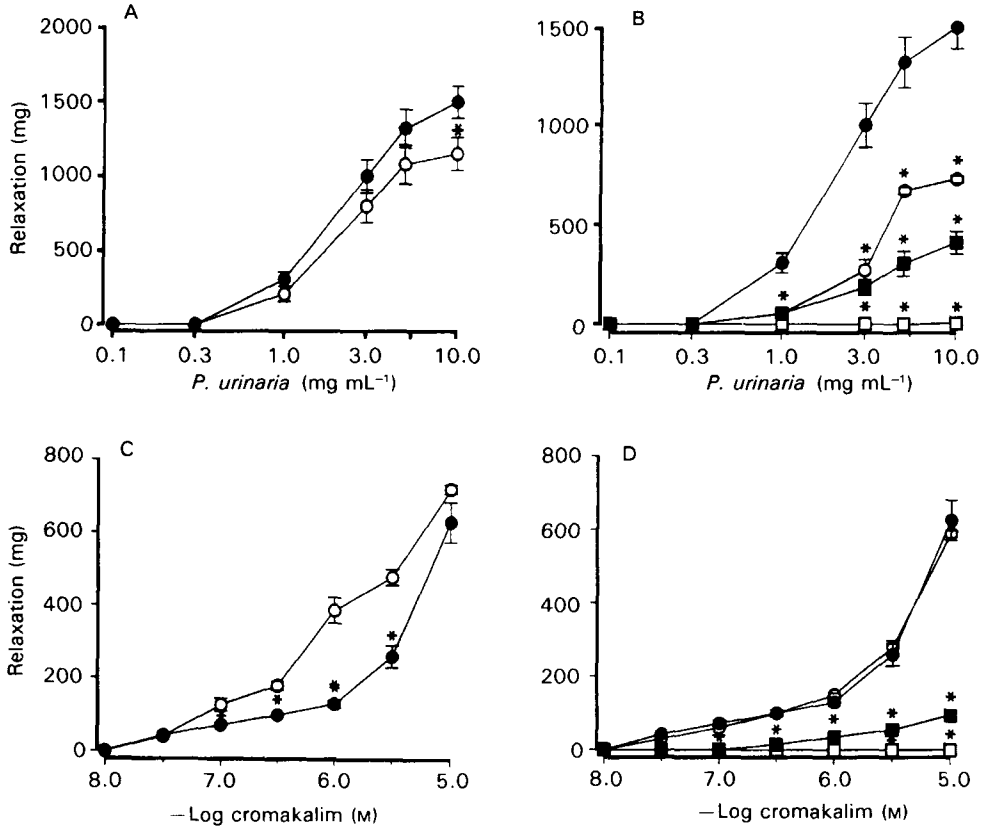

FIG. 1. Mean relaxant concentration–response curve for the HE of *Phyllanthus urinaria* (A and B) or cromakalim (C and D) in the guinea-pig isolated trachea with (○) or without(●) epithelium (A and C), or in normal medium (●) or in presence of increasing concentrations of KCl in the medium (mM): 20 (○), 40 (■), 80 (□) (B and D). Values are mean ± s.e.m. of 4 or 5 experiments.

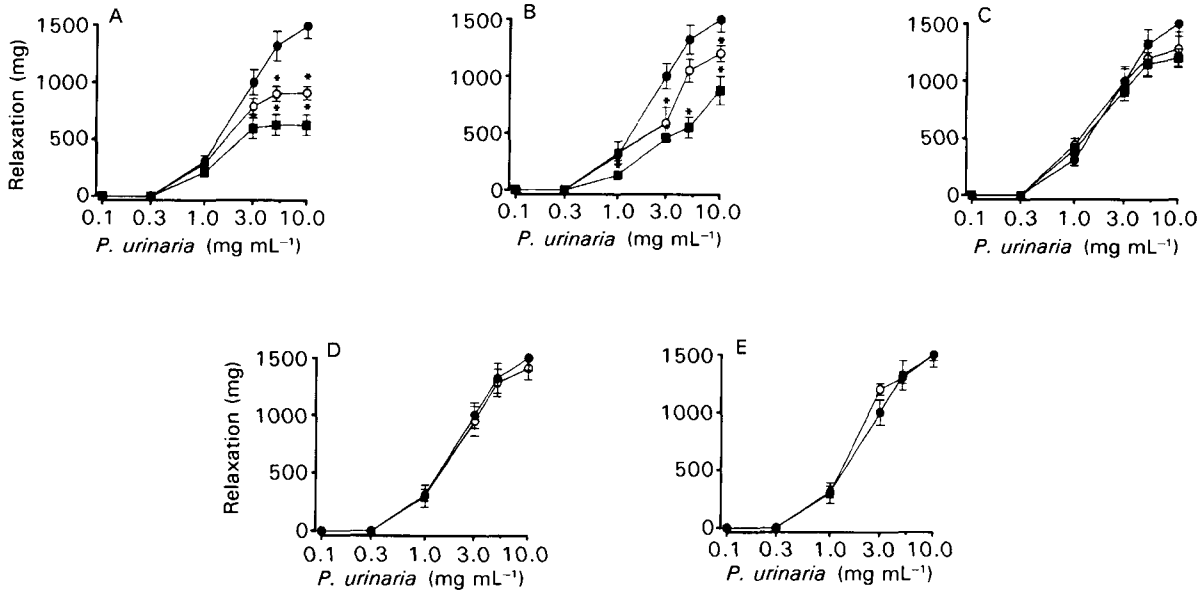

FIG. 2. Mean relaxant concentration–response curves for the HE of *Phyllanthus urinaria* in the guinea-pig isolated trachea without epithelium, in the absence (●) or presence of: (A) tetraethylammonium (○ 10 mM and ■ 30 mM), (B) glibenclamide (○ 0.1 μM and ■ 3 μM), (C) apamin (○ 0.1 μM and ■ 0.3 μM), (D) L-NOARG (○ 100 mM) or (E) methylene blue (○ 10 μM). Values are means ± s.e.m. of 4 or 5 experiments.

phate-buffered saline, concentration: NaCl 137 mM, KCl 2.7 mM and phosphate buffered 10 mM) (all from Sigma Chemical Co., St Louis, MO, USA). FK 888 ({(4*R*)-4-hydroxy-1-([1-methyl-1H-indol-3-yl] carbonyl)-L-*N*-benzyl-*N*-methyl-3-2(2-naphthyl)-L-alaninamide}) (Fujisawa Pharmaceutical Co., Osaka, Japan), SR 48968 ({(*S*)-*N*-methyl-*N*-[4-acetyl amino-4-phenylpiperidino-2-(3,4-dichlorophenyl) butyl] benzamide}) (Sanofi Recherche, Montpellier, France) and dimethylsulphoxide (DMSO) (VETEQ BR, RJ, Brazil). The stock solutions of these drugs (1–100 mM) were prepared and stored at −20°C. The bath concentration of ethanol or dimethylsulphoxide did not exceed 0.03%, which was shown to have no effect on the basal tonus of the preparation or on the agonist-mediated relaxation.

Statistical analysis

Responses were expressed as absolute changes in mg of tension. Statistical analysis of the results was carried out by means of the unpaired Student's *t*-test. $P < 0.05$ or less was considered as indicative of significance. The EC50 (i.e. the concentration of HE or drug causing half maximum response) values were determined from individual experiments for the complete agonist concentration–response curves by graphical interpolation. The EC50 values are reported as geometric means accompanied by their respective 95% confidence limits. All other reported results are means ± s.e.m.

Results

Cumulative addition of the HE of *Phyllanthus urinaria* (0.1–10 mg mL^{-1}) to the guinea-pig trachea, with or without epithelium, caused a concentration–dependent relaxation. The calculated mean EC50 (and 95% confidence limits) and maximum relaxation for these effects were: 1.94 (1.41–2.67) and 2.00 (1.47–2.78) mg mL^{-1} and 717 (± 16) mg and 627 (± 12) mg, respectively (Fig. 1A). However, the HE of *P. urinaria* caused significantly higher relaxation (23.3 ± 5.0%) in preparations where the epithelium was denuded (Fig. 1A). The relaxation induced by the HE was well reproducible and completely reversed when experiments were carried out at 60- to 90-min intervals between curves. Fig. 1B shows that the relaxant effect of HE was significantly antagonized by increasing concentrations of potassium in the bath (20 and 40 mM), being completely abolished in preparations contracted by 80 mM of KCl. Similarly, cumulative addition of cromakalim (10 nM–10 μM) to preparations pre-contracted by carbachol (0.1 μM) caused a concentration-dependent relaxation with mean EC50 and maximum relaxation of 3.57 (2.75–4.64) μM and 627 (± 17) mg, respectively (Fig. 1C). Similar to that reported for the HE of *P. urinaria*, the relaxation caused by cromakalim was fully abolished in the presence of higher KCl concentration (80 mM) (Fig. 1D).

The relaxations induced by both HE of *P. urinaria* and cromakalim in GPT without epithelium were antagonized in a concentration–dependent and reversible manner by both TEA (10 or 30 mM, a non-selective K^+ channel blocker) and glibenclamide (0.1 or 3 μM, an ATP-sensitive K^+ channel blocker) (Figs. 2 A, B). In contrast, apamin (0.1 or 0.3 μM, a calcium-sensitive K^+ channel blocker), did not significantly affect the relaxation induced by either the hydroalcoholic extract of *P. urinaria* (Fig. 2C) or cromakalim (data not shown). Interestingly, neither L-NOARG (100 μM) nor methylene blue (10 μM) interfered with the relaxation produced by the hydroalcoholic extract of *P. urinaria* (Figs. 2 D, E).

Pre-incubation of guinea-pig trachea (GPT) with propranolol (1 μM), or with selective VIP receptor antagonist (D-p-Cl-Phe^6,Leu^{17})VIP (0.1 μM), produced parallel displacement to the right of approximately two-fold for the relaxant response

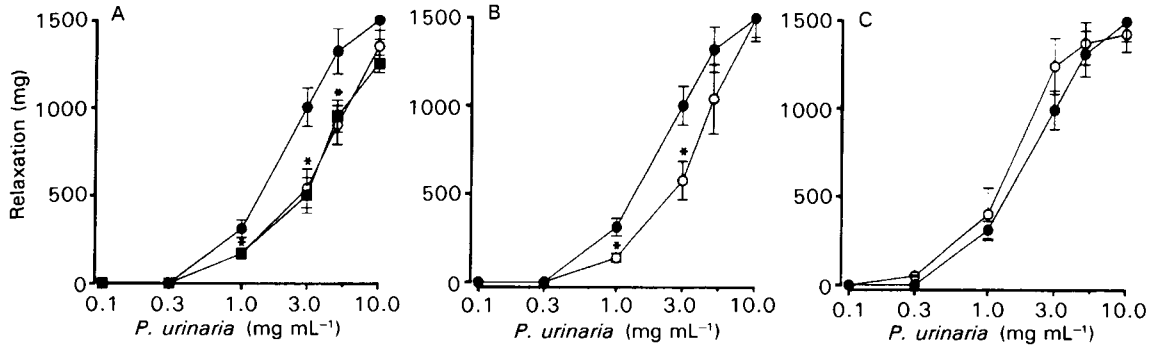

FIG. 3. Mean relaxant concentration–response curves for the HE of *Phyllanthus urinaria* in the guinea-pig isolated trachea, without epithelium, in the absence (●) or presence of: (A) propranolol (○ 1 μM and ■ 3 μM), (B) [D-p-Cl-Phe^6,Leu^{17}] VIP (○ 0.1 μM) or (C) CGRP (8–37) (○ 0.1 μM). Values are means ± s.e.m. of 4 or 5 experiments.

induced by HE of *P. urinaria* (Figs 3 A, B). The increase in the concentration of propranolol to 3 μM did not result in any further displacement to the right of the relaxant concentration–response to HE (Fig. 3A). In the incubation of preparations with VIP and β-blocker antagonists plus glibenclamide, the degree of shift to the right on the relaxant response induced by the hydroalcoholic extract of *P. urinaria* did not differ significantly from that obtained from preparations pre-incubated with glibenclamide alone (results not shown). However, at the same concentrations, propranolol and VIP receptor antagonists almost completely abolished the relaxant response induced by noradrenaline or VIP in the GPT (results not shown). On the other hand, the CGRP selective receptor antagonist, CGRP (8–37) (0.1 μM), did not significantly affect the relaxation induced by the hydroalcoholic extract of *P. urinaria* (Fig. 3C) in conditions where it almost completely abolished the relaxation elicited by CGRP (results not shown).

Discussion

The results of the present study have demonstrated that the hydroalcoholic extract obtained from leaves, stems and roots of *P. urinaria*, besides inducing concentration-dependent contraction in GPT (Paulino et al 1996) and guinea-pig urinary bladder (Dias et al 1995), at higher concentrations produces concentration-dependent and reversible relaxation in the GPT with or without epithelium pre-contracted by carbachol or under spontaneous tonus.

The relaxation induced by the hydroalcoholic extract of *P. urinaria* seems to involve multiple mechanisms of action. This is revealed by the finding that its relaxant effects, like those caused by cromakalim – a potassium channel activator (Arch et al 1988; Lijnen et al 1989; Ichinose & Barnes 1990; Spinelli et al 1990), were consistently and concentration-dependently prevented by the non-selective potassium channel blocker TEA (De Man et al 1993, 1994), as well as by glibenclamide, a selective ATP-sensitive potassium-channel blocker (Schmid-Antomarchi et al 1987; Murray et al 1989). In contrast, the neurotoxin apamin isolated from bee venom, which was found to selectively antagonize calcium-activated potassium channels of low conductance (Romey et al 1984; Cooke & Hayllet 1985), did not affect the relaxation induced by either the hydroalcoholic extract of *P. urinaria* or by cromakalim. Such results indicate that the ATP-sensitive potassium channel, but not the small-conductance calcium-activated potassium channel, seems to play an important role in the relaxation induced by the HE of *P. urinaria* in GPT. Another piece of evidence, supporting the involvement of the ATP-sensitive potassium channel, was the fact that the hydroalcoholic extract caused partial relaxation in GPT contracted by low concentrations of KCl (20 mM), being virtually ineffective in preparations pre-contracted with higher KCl concentration (80 mM). Similar results have been reported for other activators of the ATP-sensitive potassium channel (Hamilton et al 1986; Findlay, 1994; Morley, 1994; and the results of the present study). Furthermore, the direct stimulation of β_2, VIP receptors and/or release of noradrenaline and VIP from GPT seem, at least in part, to be involved in the relaxation induced by the HE. This observation is substantiated by the fact that propranolol and [D-p-Cl-Phe^6,Leu^{17}]VIP, a VIP antagonist (Pandol et al 1986), at concentrations where they almost completely abolished the relaxation induced by noradrenaline and VIP in GPT, caused a partial, though significant shift to the right of the relaxant concentration-response curves elicited by the hydroalcoholic extract of *P. urinaria* in GPT. On the other hand, the hydroalcoholic extract-mediated relaxation in GPT seems not to involve the release or activation of CGRP receptors, as indicated by the finding that the selective CGRP receptor antagonist, CGRP (8–37) (Chiba et al 1989; Maggi et al 1991), in conditions where it consistently antagonized CGRP-mediated relaxation in guinea-pig ureter (Maggi et al 1994) and in GPT (present study), had no significant effect on the relaxant response caused by hydroalcoholic extract of *P. Urinaria*.

Pharmacological and immunohistochemical studies have revealed that nitric oxide (NO) or an NO-derived substance are present in the airways of several animal species, including GPT (Tucker et al 1990; Belvise et al 1991; Li Schlemper & Calixto 1994). However, both NO biosynthesis inhibitors L-NOARG and methylene blue, an agent which inhibits the activation of soluble guanylate cyclase by NO, at concentrations where they consistently antagonize NO-mediated responses (Gruetter et al 1981; Palmer et al 1987; Moore et al 1990; Schlemper & Calixto 1994), failed to prevent the relaxation caused by HE of *P. urinaria* in the GPT. Taken together, these results support the view that the L-arginine-NO

pathway is unlikely to play a role in the relaxation caused by HE of *P. urinaria* in the GPT.

The constituents responsible for the relaxant effect of the HE of *P. urinaria* in the GPT are currently unknown. However, this effect is unlikely to be related to the presence of steroids isolated from *P. corcovadensis* (Santos et al 1995b), which are also present in *P. urinaria*. Recently, we have isolated and characterized two tannins, geraniin and furosin, from leaves, stems and roots of *P. sellowianus*, which caused antinociception when assessed in mice (Miguel et al 1995a, b). Whether or not these compounds can account for the relaxant effect of the HE of *P. urinaria* in GPT still requires further investigation. Chemical and pharmacological studies are now in progress to isolate and to characterize the constituents responsible for such effects, and also to investigate in more detail the mechanisms underlying the relaxant action of the active principle(s) of *P. urinaria* in GPT.

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References

Arch, J. R., Buckle, D. R., Bumstead, J., Clarke, G. D., Taylor, J. F., Taylor, S. G. (1988) Evaluation of the potassium channel activator cromakalim (BRL 34915) as a bronchodilator in the guinea pig: comparison with nifedipine. Br. J. Pharmacol. 95: 763–770

Belvise, M. G., Stretton, D., Barnes, J. P. (1991) Nitric oxide as an endogenous modulator of cholinergic neurotransmission in guinea pig airways. Eur. J. Pharmacol. 198: 219–221

Chiba, T., Yamaguchi, A., Yamatani, T., Nakamura, A., Morishita, T., Inui, T., Fukase, M., Noda, T., Fujita, T. (1989) CGRP receptor antagonist hCGRP (8-37). Am. J. Physiol. 256: E331–335

Cooke, N. S., Hayllet, D. G. (1985) Effects of apamin, quinidine and neuromuscular blockers on calcitonin-activated potassium channels in guinea pig hepatocytes. J. Physiol. Lond. 358: 373–375

De Man, J. G., Boeckxstaens, G. E., Pelckmans, P. R. P., De Winter, B. Y., Herman, A. G., Van Maercke, Y. M. (1993) Prejunctional modulation of the nitrergic innervation of the canine ileocolonic junction via potassium channels. Br. J. Pharmacol. 110: 559–564

De Man, J. G., Boeckxstaens, G. E., Herman, A. G., Pelckmans, P. R. P. (1994) Effect of potassium channel blockade and α_2-adrenoceptor activation on the release of nitric oxide from non-adrenergic non-cholinergic nerves. Br. J. Pharmacol. 112: 341–345

Dias, M. A., Campos, A. H., Cechinel-Filho, V., Yunes, R. A., Calixto, J. B. (1995) Analysis of the mechanisms underlying the contractile response induced by the hydroalcoholic extract of *Phyllanthus urinaria* in the guinea pig urinary bladder in vitro. J. Pharm. Pharmacol. 47: 847–855

Findlay, I. (1994) Interactive regulation of the ATP-sensitive potassium channel of cardiac muscle. J. Cardiovasc. Pharmacol. 24 suppl. 4: S6–S11

Gorski, F., Corrêa, C. R., Cechinel-Filho, V., Yunes, R. A., Calixto, J. B. (1993) Potent antinociceptive activity of the hydroalcoholic extract from *Phyllanthus corcovadensis*. J. Pharm. Pharmacol. 45: 1046–1049

Gruetter, C. A., Gruetter, D. Y., Lyon, J. E., Kadowitz, P. J., Ignarro, L. J. (1981) Relationship between cyclic guanosine 3′,5′-monophosphate formation and relaxation of coronary arterial smooth muscle by glyceryl trinitrate, nitroprusside, nitrite and nitric oxide: effects of methylene blue and methemoglobin. J. Pharmacol. Exp. Ther. 249: 181–186

Hamilton, T. C., Weir, S. W., Weston, A. H. (1986) Comparation (Query?) of the effects of BRL 34915 and verapamil on electrical and mechanical activity in rat portal vein. Br. J. Pharmacol. 88: 103–111

Ichinose, M., Barnes, P. J. (1990) A potassium channel activator modulates both excitatory noncholinergic and cholinergic neurotransmission in guinea pig airways. J. Pharmacol Exp. Ther. 252: 1207–1212

Li, C. G., Rand, M. J. (1991) Evidence that participation of the NANC relaxant responses of guinea-pig trachea to electrical field stimulation is mediated by nitric oxide. Br. J. Pharmacol. 102: 91–94

Lijnen, P., Fagard, R., Staessen, J., Weiping, T., Moerman, E., Amery, A. (1989) Humoral and cellular effects of the K^+-channel activator cromakalim in man. Eur. J. Clin. Pharmacol. 37: 609–611

Maggi, C. A., Chiba, T., Giulinai, S. (1991) Human a-calcitonin gene-related peptide (8–37) as an antagonist of exogenous and endogenous calcitonin gene-related peptide. Eur. J. Pharmacol. 192: 85–88

Maggi, C. A., Giuliani, S., Santicioli, P. (1994) Multiple mechanisms in the smooth muscle relaxant action of calcitonin gene-related peptide (CGRP) in the guinea pig ureter. Naunyn-Schmiedebergs Arch. Pharmacol. 350: 537–547

Miguel, O. G., Cechinel-Filho, V., Niero, R., Silva, G. O., Pizzolatti, M. G., Yunes, R. A., Calixto, J. B., Santos, A. R. (1995a) Constituents of *Phyllanthus sellowianus*. Fitoterapia 66: 275

Miguel, O. G., Cechinel-Filho, V., Pizzolatti, M. G., Santos, A. R., Calixto, J. B., Ferrari, F., Messana, I., Yunes, R. A. (1995b) A triterpene and phenolic compounds from leaves and stems of *Phyllanthus sellowianus*. Planta Med. 61: 391

Moore, P. K., Al-Swayeh, D. A., Chong, N. W. S., Evans, R. S., Gibson, A. (1990) L-N^G-Nitroarginine (L-NOARG) a novel L-arginine-reversible inhibitor of endothelium-dependent vasodilation in vitro. Br. J. Pharmacol. 99: 408-412

Morley, J. (1994) K^+ channel openers and suppression of airway hyperreactivity. Trends Pharmacol. Sci. 15: 463–468

Murray, M. A., Boyle, J. P., Small, R. C. (1989) Cromakalim-induced relaxation of guinea-pig isolated trachealis: antagonism by glibenclamide and by phentolamine. Br. J. Pharmacol. 98: 865–874

Palmer, R. M. J., Ferrige, A. G., Mocada, S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327: 524–526

Pandol, S. J., Dharmsathaphorn, K., Schoeffield, M. S., Vale, W., Rivier, J. (1986) Vasoactive intestinal peptide receptor antagonist [4Cl-D-Phe^6,leu^{17}]VIP. Am. J. Physiol. 250: G-553–G555

Paulino, N., Cechinel-Filho, V., Pizzolatti, M. G., Yunes, R. A., Calixto, J. B. (1996) Mechanisms involved in the contractile response induced by the hydroalcoholic extract of *Phyllanthus urinaria* on the guinea pig isolated trachea. Evidence for participation of tachykinin and influx of extracellular Ca^{2+} sensitive to ruthenium red. Gen. Pharmacol. 27: 795–802

Romey, G., Hughes, M., Schimid-Antomarchi, H., Lazdunski, M. (1984) Apamin: a specific toxin to study a class of Ca^{2+}-dependent K^+ channels. J. Physiol. Lond. 79: 259–264

Santos, A. R. S., Cechinel-Filho, V., Niero, R., Viana, A. M., Moreno, F. N., Campos, M. M., Yunes, R. A., Calixto, J. B. (1994) Analgesic effect of callus culture extracts from selected species of Phyllanthus in mice. J. Pharm. Pharmacol. 46: 755–759

Santos, A. R. S., Cechinel-Filho, V., Yunes, R. A., Calixto, J. B. (1995a) Further studies on the antinociceptive action of the hydroalcoholic extracts from the plants of the genus Phyllanthus. J. Pharm. Pharmaol. 47: 66–71

Santos, A. R. S., Niero, R., Cechinel-Filho, V., Pizzolatti, M. G., Yunes, R. A., Calixto, J. B. (1995b) Antinociceptive properties of steroids isolated from *Phyllanthus corcovadensis*. Planta Med. 61: 297–392

Schlemper, V., Calixto, J. B. (1994) Nitric oxide pathway-mediated relaxant effect of bradykinin in the guinea pig isolated trachea. Br. J. Pharmacol. 111: 83–88

Schlemper, V., Calixto, J. B. (1995) Mechanisms involved in the relaxant response of bradykinin in epithelium intact strips of the

guinea-pig trachea. Eur. J. Pharmacol. 282: 177–184

Schmid-Antomarchi, H., De Weille, J., Fosset, M., Ladzdunski, M. (1987) The receptor for antidiabetic sulfonylureas controls the activity of the ATP-modulated K^+ channel in insulin-secreting cells. J. Biol. Chem. 262: 15840–15845

Spinelli, W. Follmer, C., Parsons, R., Colatsky, T. (1990) Effects of cromakalim, pinacidil and nicorandil on cardiac refractoriness and arterial pressure in open-chest dogs. Eur. J, Pharmacol. 179: 243–252

Tucker, J. F., Brane, S. R., Charalambons, L., Hobbs, A. J., Gibson, A. (1990) L-N^G-Nitro arginine inhibits non-adrenergic, non-cholinergic relaxations of the guinea pig isolated tracheal smooth muscle. Br. J. Pharmacol. 100: 663–664

Van Rossum, J. M. (1963) Cumulative dose-response curves. II. Technique for making of dose-response curves in isolated organs and the evaluation of drug parameters. Arch. Int. Pharmacodyn. Ther. 143: 299–330