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Mechanisms involved in the relaxant action of the ethanolic extract of propolis in the guinea-pig trachea in-vitro

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Abstract

This study examines the mechanisms by which the standardised ethanolic extract of propolis induces relaxation of the guinea-pig trachea in-vitro. In guinea-pig trachea with or without epithelium and contracted by histamine, the propolis extract caused reproducible and graded relaxation, with a mean EC50 value of 3.8 or 10.5 μg mL^{-1} and E_{max} of 100%, respectively. The propolis extract-induced relaxation was markedly reduced (26±9 and 96±3%) when guinea-pig tracheas were exposed to Krebs solution containing elevated K^+ in the medium (40 or 80 mM). Pre-incubation of guinea-pig tracheas with tetraethylamonium (100 mM) or with 4-aminopyridine (10 mM) reduced the propolis extract-induced relaxation by 31±10% and 28±2%. Likewise, apamin (0.1 μM), charybdotoxin (0.1 μM) or iberiotoxin (0.1 μM) caused marked inhibition of propolis extract-mediated relaxation in guinea-pig trachea (percentage of inhibition: 65±3%, 60±5% and 65±9%, respectively). Also, glibenclamide (1 μM) inhibited the relaxant response caused by the propolis extract by 57±4%. ω-Conotoxin GIVA (0.1 μM) or capsaicin (1 μM) produced small but significant inhibition (30±5% or 47±7%, respectively) of the propolis extract-induced relaxation. The vasoactive intestinal peptide (VIP) antagonist D-p-Cl-Phe^6,Leu^{17}[VIP] porcine (0.1 μM) inhibited relaxation by 55±5%, while propranolol (1 μM) induced a parallel rightward displacement (about 20 fold) of the propolis extract concentration–response curve. Finally, the propolis extract-induced relaxation was inhibited by the nitric oxide synthase inhibitor L-N^G-nitroarginine (L-NOArg, 100 μM) (48±6%), and by the soluble guanylate cyclase inhibitor methylene blue (10 μM) (37±6%), while the more selective soluble guanylate cyclase inhibitor 1*H*-[1,2,4]oxadiazolol[4,3-a]quinoxalin-1-one (ODQ, 1 μM) produced only a parallel (about 3 fold) rightward displacement of the propolis extract concentration–response curve. Collectively, these results support the notion that the propolis extract-mediated relaxation in the guinea-pig trachea involves the release of nitric oxide, probably from sensory neurons, besides the activation of soluble guanylate cyclase and activation of Ca^{2+}- and ATP-sensitive K^+ channels. Furthermore, the stimulation of β_2-adrenergic and VIP receptors also seems to account for its relaxant action.

Introduction

Propolis, a naturally occurring substance, is a red or yellow-brown to dark-brown lipophilic mixture that is produced by mixing the exudates collected from various plants by honeybees (Miyataka et al 1997). Propolis, also known as bee glue, is a traditional remedy that is widely used in many countries for the management

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of numerous diseases, including airway affections, cutaneo-mucosal infections and viral infections (Amoros et al 1994).

There are numerous reports in the literature indicating that propolis and its active constituents exert a great range of biological actions such as free radical scavenger and antioxidant properties (Matsushige et al 1996), anti-carcinogenic properties (Jaiswal et al 1997), antiviral effects (Amoros et al 1994), antibacterial activity (Bankova et al 1995), anti-protozoal action against *Trypanosoma cruzi* (De Castro & Higashi 1995) and immunomodulatory and anti-inflammatory properties (Ivanovska et al 1995), as well as a liver-protective effect (Mahran et al 1996).

We have demonstrated that the ethanolic extract of propolis induces a pronounced antinociceptive effect according to evaluation in chemical models of nociception in mice (de Campos et al 1998).

Several naturally occurring constituents, such as phenolic acids, terpenes, lignans, cinnamic acid, caffeic acid, several esters and flavonoids, have been described in different propolis samples (Bankova et al 1995, Banskota et al 1998). The majority of such compounds are known to exhibit important pharmacological properties. Thus, the prenylated derivative present in propolis exhibits in-vitro cytotoxic actions against carcinoma and leukaemic cells (Banskota et al 1998; Kimoto et al 2001a, b). The clerodane diterpenoid inhibits chemical substance-induced skin tumours in mice (Mitamura et al 1996) and exhibits antimicrobial actions (Aga et al 1994). It has also been reported that some phenolic components present in propolis extract are able to inhibit the neutrophils' chemiluminescence (Krol et al 1996) apart from exerting an anticomplementary activity (Georgieva et al 1997).

In this study we therefore investigated, by use of receptor- and ion-channel-selective antagonists, some of the mechanisms underlying the relaxant effect caused by the Brazilian ethanolic extract of propolis on the guinea-pig isolated trachea in-vitro.

Materials and Methods

Drugs

The drugs used were obtained from the following sources: standardised ethanolic extract of propolis (P1) (from Apis Nativa Produtos Naturais Ltda, Araranguá, Brazil), histamine, tetraethylammonium, 4-aminopyridine, apamin, charybdotoxin, iberiotoxin, glibenclamide, ω-conotoxin GIVA, D-$_p$-Cl-Phe^6,Leu^{17}[VIP] porcine, capsaicin, propranolol, L-N^G-nitroarginine (L-NOArg), methylene blue and 1*H*-[1,2,4]oxadiazolol[4,3-a]quinoxalin-1-one (ODQ) (all from Sigma Chemical Co., St Louis, MO). The other chemical products and salts were obtained from Merck (Germany), and had a high degree of analytic purity. The stock solutions of these drugs were prepared and stored at −20°C. The bath concentration of ethanol did not exceed 0.03%, which was shown to have no effect on the basal tonus of the preparations (see below) or on the agonist-mediated relaxation. The concentration of antagonists or ionic-channel blockers used in this experiment did not change the basal tonus of preparations.

Propolis extract preparations

Standardised ethanolic extract of propolis, P1, obtained from commercial preparations available in southern Brazil, supplied by Apis Nativa Produtos Naturais Ltda (http://www.prodapys.com.br), lot P1 n.10/2000, was used. The alcohol of this preparation was evaporated and the dry resin was diluted in stock solution at a concentration of 10% (w/v). The propolis was collected from the beehive in March (1999) near Araranguá city, in Santa Catarina state, Brazil (following a sample stocked in our labaratory). Propolis was triturated and mixed with an extractive solution containing 96GL alcohol. The mixture was left for 10 days, with a single mixing of 10 min once a day. After this period, the mixture was concentrated in Soxhlet, an extractor of compounds from plant and natural products, and the alcohol was removed from the solution to make a dry residue. The product of this extraction was diluted at a concentration of 10% (w/v) in 96GL alcohol.

High-performance liquid chromatographic assay

The ethanolic extracts of propolis were analysed using HPLC apparatus (Merck-Hitachi, Germany) equipped with a pump (model L-6200, Merck-Hitachi, Germany) and a diode array detector (L-3000, Merck-Hitachi, Germany). Separation was achieved on a Lichrochart 125-4 column (Merck, Darmstadt, Germany) (RP-18, 12.5×0.4 cm, 5 μm particle size) using water–formic acid (95:5, v/v) (solvent A) and methanol (solvent B). The elution was carried out with a linear gradient and a flow rate of 1 mL min^{-1}. The detection was monitored at 280 nm and the compounds were identified using standards as references (according to Marcucci 2000). For data analysis, the Merck-Hitachi D-6000 (Chromatography Data Station – DAD Manager) was used.

The sample used in this experiment presented the following phenolic compound composition: 3-prenyl-4-hydroxycinnamic acid (1.72 mgmL^{-1}), 2,2-dimethyl-6-carboxyethenyl-2*H*-1-benzopyran (38.04 mg mL^{-1}) and 3,5-diprenyl-4-hydroxycinnamic acid (26.21 mg mL^{-1}).

Tissue preparations

Guinea-pigs (250–400 g) of both sexes were anaesthetised and killed by cervical dislocation (Paulino et al 1996) (protocol approved by ethical committee at Universidade do Sul de Santa Catarina). The trachea was rapidly removed and, after being freed from connective tissue, was cut into six transverse rings (3–4 mm wide), each containing 3 cartilages as described previously (Paulino et al 1999). The rings were opened (usually 6 strips of 8–10 mm in length, obtained from the same guinea-pig) and were suspended in individual 10-mL jacketed organ baths containing Krebs-Henseleit solution maintained at 37°C, pH 7.2, gassed with 95% O_2–5% 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 addition, 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 to induce relaxation (Paulino et al 1996). The guinea-pigs were used in accordance with current ethical guidelines for the care of laboratory animals.

Experimental procedure

After the equilibration period of at least 60 min, the preparations with or without epithelium were pre-contracted with histamine (1–3 μM, approximately the EC50) and, when tonic contraction became stable (usually after 5 min), were exposed to the propolis extract (10^{-7}–10^{-3} g mL^{-1}), which was added to the bath by cumulative method (Van Rossum 1963). Usually, 2–5 complete cumulative concentration–response curves were obtained for the propolis extract in each preparation, at 60-min intervals between curves.

To investigate the possible mechanisms responsible for the relaxation response induced by propolis extract in the guinea-pig trachea, the preparations without epithelium were pre-contracted by addition of histamine (1 or 3 μM), 20 min before being incubated with one of the following drugs: tetraethylammonium (a non-selective blocker of K^+ channels, 100 mM), 4-aminopyridine (a selective blocker of voltage-sensitive K^+ channels (Kv), 10 mM), apamin (a selective blocker of Ca^{2+}-sensitive K^+ channels, 0.1 μM), charybdotoxin (a selective blocker of large Ca^{2+}-sensitive K^+ channels, 0.1 μM), iberiotoxin (a selective blocker of large Ca^{2+}-sensitive K^+ channels, 0.1 μM), glibenclamide (a selective blocker of ATP-sensitive K^+ channels, 1 μM), ω-conotoxin GIVA (a selective blocker of voltage-activated N-type Ca^{2+} channels and inhibitor of neurotransmitter release at neuronal synapses, 0.1 μM), D-$_p$-Cl-Phe^6,Leu^{17}[VIP] porcine (a vasoactive intestinal peptide (VIP) receptor antagonist, 0.1 μM), capsaicin (an excitatory and desensitising agent on a subset of primary afferent sensory neurons, 1 μM), propranolol (a β-adrenergic blocker, 1 μM), L-NOArg (a nitric oxide synthase competitive antagonist, 100 μM), methylene blue (a non-selective inhibitor of soluble guanylate cyclase, 10 μM) and ODQ (a selective inhibitor of soluble guanylate cyclase, 1 μM). In addition, we also investigated the ability of the propolis extract to elicit relaxation in preparations maintained in Krebs solution containing 40 or 80 mM of KCl instead of 4.7 mM.

Statistical analysis

Responses were expressed as absolute changes in % of tension and reported as means$\pm$s.d. Statistical analysis of the results was carried out by means of the unpaired Student's *t*-test (Graph Pad Instat program), by comparison of individual points of the treated groups with the control groups, during the relaxant concentrated cumulative curve. $P < 0.05$ was considered as indicative of significance. The EC50 values were determined from individual experiments for the complete concentration–response curves by graphical interpretation test (Graph Pad Instat program). The EC50 values are reported as geometric means accompanied by their respective 95% confidence limits.

Results

The chemical constitution of propolis was evaluated by HPLC analysis, and the chromatogram showed the characteristic profile of samples from the south of Brazil

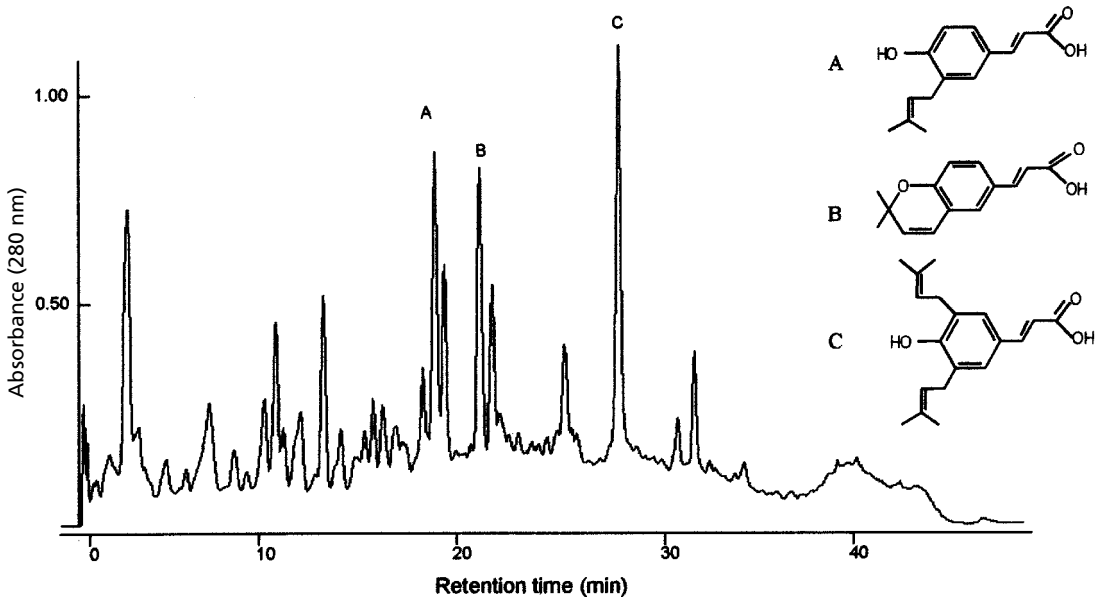

Figure 1 Chromatographic analysis by HPLC of the propolis extract sample from South Brazil. The HPLC was run on a Merck-Hitachi D-6000 (model L-3000, Merck-Hitachi, Germany), Lichrochart 125-4 column (Merck, Darmstadt, Germany) (RP-18, 12.5 × 0.4 cm, 5 μm particle size) using water–formic acid (95:5, v/v) (solvent A) and methanol (solvent B). The elution was carried out with a linear gradient and flow rate of 1 mL min^{-1}. The detection was monitored at 280 nm. A, 3-prenyl-4-hydroxycinnamic acid; B, 2,2-dimethyl-6-carboxyethenyl-2*H*-1-benzopyran; C, 3,5-diprenyl-4-hydroxycinnamic acid.

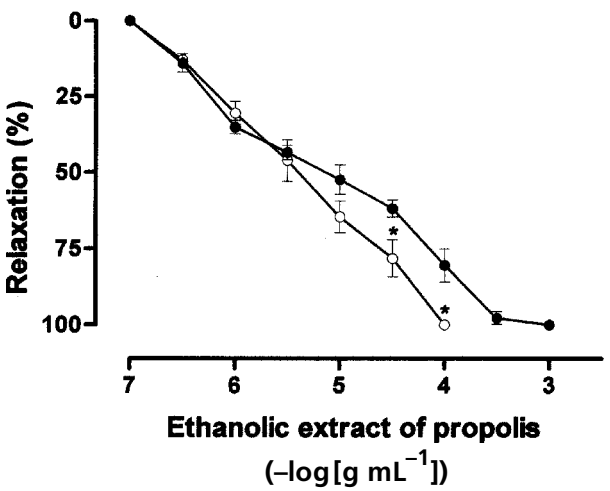

Figure 2 Mean relaxant concentration–response curves for the ethanolic extract of propolis in guinea-pig trachea without epithelium (●) or with intact epithelium (○). Values are mean ± s.d. of 6 experiments. *$P < 0.05$ for the difference between points.

P1 (Figure 1). The presence of phenolic compounds was observed and determined in all the samples of propolis. Chemical analysis revealed the presence of the 3-prenyl-4-hydroxycinnamic acid, in 1.72 mg mL^{-1}, peak A; 2,2-dimethyl-6-carboxyethenyl-2*H*-1-benzopyrane, in 38.04 mg mL^{-1}, peak B; 3,5- diprenyl-4-hydroxy-cinnamic acid, in 26.21 mg mL^{-1}, peak C, in the samples of propolis studied (Figure 1).

Cumulative addition of the standardised propolis extract (P1) (10^{-7}–10^{-3} g mL^{-1}) to the guinea-pig trachea, with or without epithelium and pre-contracted by histamine, resulted in a concentration-dependent complete relaxation of the preparations. The calculated mean EC50 values (and 95% confidence limits) for these effects were 10.5 (2.5–43.6) μg mL^{-1} and 3.8 (1.2–12.0) μg mL^{-1}, respectively (Figure 2). The relaxation induced by the propolis extract was well reproducible with no evidence of tachyphylaxis when experiments were carried out at 60-min intervals between curves (results not shown).

The results in Figure 3A show that the maximal relaxant effect of propolis extract (P1) in preparations without epithelium was significantly antagonised (26 ± 9%) when the preparations were transferred to Krebs solution containing 40 mM KCl, and these responses were practically abolished (96 ± 3% of inhibition) by increasing concentrations of potassium in the bath to 80 mM.

The maximal relaxation induced by propolis extract (P1) in guinea-pig trachea without epithelium was anta-

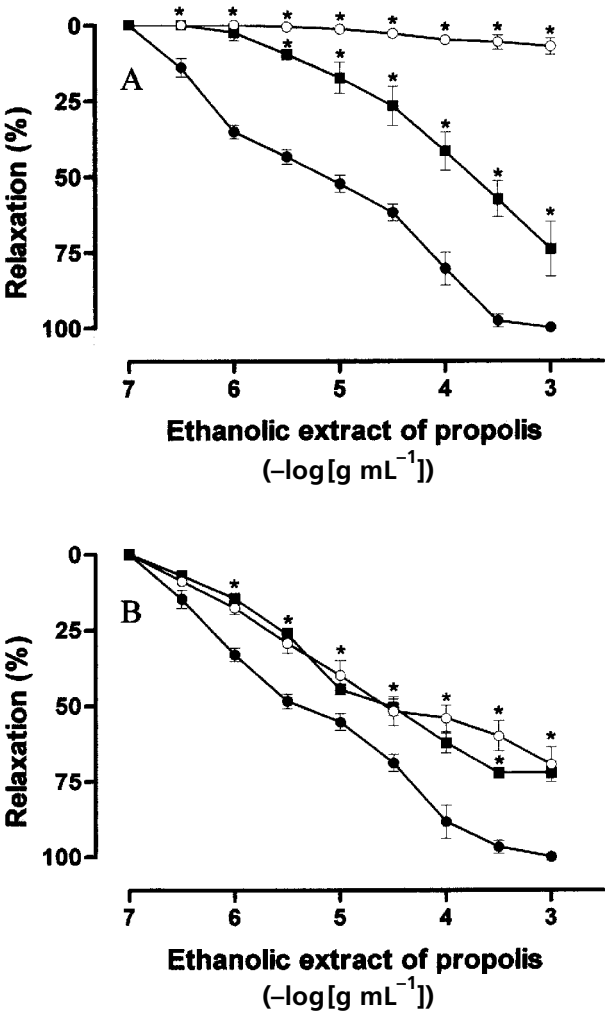

Figure 3 Mean relaxant concentration–response curves for the ethanolic extract of propolis in guinea-pig trachea without epithelium in the absence (●) or presence of (A) KCl, 80 mM (○) or 40 mM (■), (B) tetraethylammonium 100 mM (○) or 4-aminopyridine 10 mM (■). Values are means±s.d. of 6 experiments. *$P < 0.05$ vs values obtained in the absence of KCl.

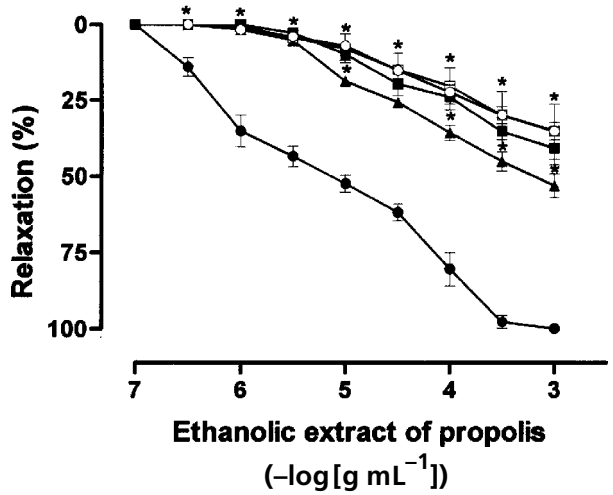

Figure 4 Mean relaxant concentration–response curves for the ethanolic extract of propolis in guinea-pig trachea without epithelium in the absence (●) or presence of apamin 0.1 μM (○), charybdotoxin 0.1 μM (■), iberiotoxin 0.1 μM (□) or glibenclamide 1 μM (▲). Values are means±s.d. of 6 experiments. *$P < 0.05$ vs values obtained in the absence of compounds.

gonised in a reversible manner by both tetraethylammonium (100 mM, a non-selective blocker of K^+ channels) (31±10%) and 4-aminopyridine (10 mM, at this concentration, a selective blocker of voltage-sensitive K^+ channels (Kv) (28±2%) (Figure 3B). The pre-incubation of preparations with apamin (0.1 μM, a selective blocker of small Ca^{2+}-sensitive K^+ channels) inhibited the propolis-mediated relaxation in a concentration-dependent manner (65±3%). Similarly, charybdotoxin (0.1 μM, a selective blocker of large Ca^{2+}-sensitive K^+ channels) and iberiotoxin (0.1 μM, a highly selective blocker of large Ca^{2+}-sensitive K^+ channels) each consistently inhibited the propolis extract-mediated relaxation in guinea-pig trachea (% of inhibition, 60±5 and 65±9, respectively). Likewise, pre-incubation of preparations with glibenclamide (1 μM, a selective blocker of ATP-sensitive K^+ channels) also consistently inhibited, in a concentration-dependent fashion (57±5%), the relaxation caused by propolis extract (Figure 4).

Pre-incubation of the preparations with ω-conotoxin GIVA (0.1 μM, a selective blocker of voltage-activated N-type Ca^{2+} channels and neurotransmitter release inhibitor at neuronal synapses) or with capsaicin (1 μM, an excitatory and desensitizing agent on a subset of primary afferent sensory neurons) significantly inhibited the propolis extract-mediated relaxation in guinea-pig trachea (30±5 and 47±7%, respectively) (Figure 5). Pre-incubation of preparations with D-$_p$-Cl-Phe^6,Leu^{17}[VIP] porcine (0.1 μM, a VIP receptor antagonist) produced a 55±6% inhibition of propolis extract-mediated relaxation in the isolated guinea-pig trachea, while propranolol (1 μM, a β-adrenergic antagonist) induced a marked parallel rightward displacement (about 20 fold) of the propolis extract concentration–response curve (Figure 5). At the same concentrations, propranolol and the VIP receptor antagonists almost completely abolished the relaxant response induced, respectively, by noradrenaline (norepinephrine) and VIP in the preparations (results not shown).

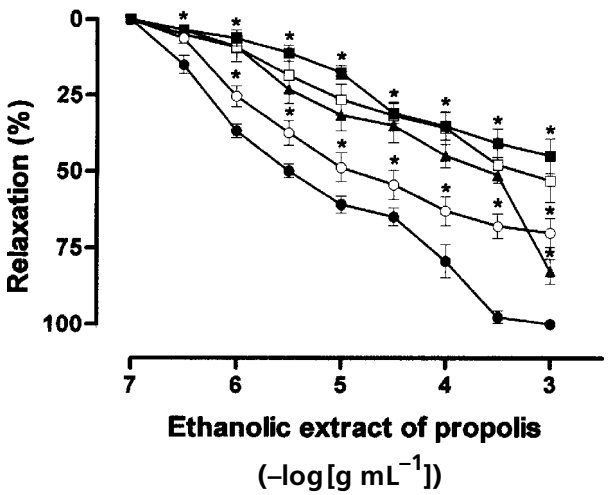

Figure 5 Mean relaxant concentration–response curves for the ethanolic extract of propolis in guinea-pig trachea without epithelium in the absence (●) or presence of ω-conotoxin GIVA 0.1 μM (○), D-$_P$-Cl-Phe^6,Leu^{17}[VIP] (porcine) 0.1 μM (■), capsaicin 1 μM (□) or propranolol 1 μM (▲). Values are means±s.d. of 6 experiments. *$P < 0.05$ vs values obtained in the absence of compounds.

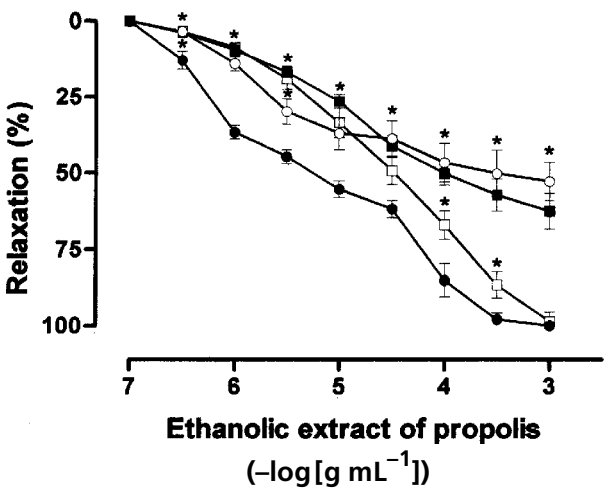

Figure 6 Mean relaxant concentration–response curves for the ethanolic extract of propolis in guinea-pig trachea without epithelium in the absence (●) or presence of L-NOArg 100 μM (○); methylene blue 10 μM (■) or ODQ 1 μM (□). Values are means±s.d. of 6 experiments. *$P < 0.05$ vs values obtained in the absence of compounds.

Finally, the nitric oxide pathway was also investigated by means of incubation of the guinea-pig trachea without epithelium with L-NOArg (100 μM, a nitric oxide synthase competitive antagonist), and it was found that the propolis extract-induced relaxation (Figure 6) was inhibited in a graded manner (48±6%). Furthermore, methylene blue (10 μM, a non-selective inhibitor of soluble guanylate cyclase) inhibited by 37±6%, while ODQ (1 μM, a selective inhibitor of soluble guanylate cyclase) induced a small but significant rightward displacement (about 3 fold) of the propolis extract concentration–response curve (Figure 6). None of the test-drugs significantly affected the tonus of the guinea-pig isolated trachea without epithelium.

Discussion

Despite the existence of several in-vivo and in-vitro studies with propolis extract, to the best of our knowledge this represents the first study which demonstrates that the standardised propolis extract causes, at low concentrations, a concentration-dependent, complete and well-reproducible relaxation response in guinea-pig trachea in-vitro pre-contracted by histamine, both in the presence and in the absence of epithelium.

Since K^+ channels are important modulators of the smooth muscle tonus, including in the airways smooth muscle (Kotlikoff 1993; Viana-Jorge et al 2000), we also investigated the possible contribution of various K^+ channel types to the relaxant action of propolis extract in the guinea-pig trachea. Our results clearly show that both the small and large conductance Ca^{2+}-activated K^+ channels are molecular targets of the propolis extract in the guinea-pig trachea. This notion derives from the results indicating that the selective antagonists of small and large conductance Ca^{2+}-activated K^+ channels such as apamin, charybdotoxin and iberiotoxin (Gimenez-Gallego et al 1988; Suarez-Kurtz et al 1991) each consistently affected the guinea-pig trachea relaxation caused by propolis extract. Furthermore, the relaxation caused by propolis extract in the guinea-pig trachea involved indirect or direct activation with ATP-sensitive K^+ channels, because glibenclamide, at a concentration known to inhibit these channels (Winquist et al 1989; Paulino et al 1999), greatly antagonised the action of the propolis extract. However, the non-selective K^+-channel antagonist tetraethylammonium and the non-selective voltage dependent K^+-channel antagonist 4-aminopyridine also partially but significantly antagonised the propolis extract-mediated relaxation in guinea-pig trachea. An additional piece of evidence showing the contribution of K^+ channels to the relaxant action of propolis extract in the guinea-pig trachea was that the increase of K^+ in the medium by 40 and 80 mM largely antagonised its relaxant effect, indicating the require-

ment of the integrity of the cell membrane for the propolis extract-mediated relaxation.

A growing amount of evidence now suggests that nitric oxide can be released in airway tissues including the trachea, either at the epithelium or in sensory neurons (Vaali et al 2000). It has been reported that nitric oxide is capable of stimulating Ca^{2+}-activated K^+ channels (Bolotina et al 1994), including those in airway smooth muscles (Abderrahmane et al 1998), causing membrane hyperpolarization, a decrease in calcium influx and muscle relaxation (Lincoln & Cornwell 1991). Results of this study show that despite the fact that propolis extract-induced relaxation was unaffected by removal of the trachea epithelium, the incubation of nitric oxide synthase antagonist significantly prevented its relaxant response, suggesting that the nitric oxide, probably released from sensory neurons, participates in guinea-pig trachea relaxation in response to propolis extract. We next explored whether or not the relaxant action of propolis extract on guinea-pig trachea was mediated by activation of soluble guanylate cyclase. Our results indicate that the less-selective soluble guanylate cyclase inhibitor methylene blue (Ellis 1997) greatly inhibited the propolis extract-mediated relaxation. In addition, the more selective soluble guanylate cyclase inhibitor ODQ, at a concentration known to inhibit this enzyme (Schrammel et al 1996), produced a rightward displacement (about 3 fold) of the relaxant response caused by propolis extract. Taken together, these findings indicate that activation of soluble guanylate cyclase and increase of cGMP take part in the relaxant action of propolis extract in guinea-pig trachea.

One important result presented here was the finding that pre-incubation of preparations with the selective antagonist of VIP receptors prevented, by more than 50%, the propolis extract-induced relaxation, suggesting a role exerted by VIP in its relaxant effect. Also, the involvement of β-adrenoceptors in the relaxant action caused by propolis extract in the guinea-pig trachea is evident from the fact that the non-selective β_1- and β_2-receptor antagonist propranolol largely prevented the propolis extract-mediated relaxation. The release of neuropetide from sensory neurons was confirmed by pre-treatment of preparations with the neurotoxin capsaicin which selectively depletes neuropetides from sensory neurons. Furthermore, pre-incubation of guinea-pig trachea with ω-conotoxin GIVA, a selective blocker of voltage-activated N-type voltage-sensitive Ca^{2+} channels and potent neurotransmitter release inhibitor at the neuronal synapse (Nielsen et al 2000), significantly reduced the relaxant response caused by the propolis extract. These findings further indicate the relevant role played by the indirect release of relaxant substances from neuronal synapses in the relaxant action of propolis extract in guinea-pig trachea.

The constituents responsible for the relaxant effect of propolis extract in guinea-pig trachea are currently not completely known. However, this effect is likely to be related to the presence of three phenolic compounds (Marcucci et al 2001) already reported in the sample of propolis (P1) used in this study. Chemical and pharmacological studies are now in progress to isolate and chemically characterise the constituents responsible for such effects, and also to investigate in more detail the precise mechanisms by which these compounds exert their relaxant action in the guinea-pig isolated trachea in-vitro.

In conclusion, our results show that propolis induces a relaxant effect on the guinea-pig isolated trachea by means of several mechanisms that include direct or indirect activation of potassium channels or modulation of VIP, adrenergic or nitric oxide pathways.

References

Abderrahmane, A., Salvail, D., Dumoulin, M., Garon, J., Cadieux, A., Rousseau, E. (1998) Direct activation of K(Ca) channel in airway smooth muscle by nitric oxide: involvement of a nitrothiosylation mechanism? *Am. J. Respir. Cell Mol. Biol.* **19**: 485–497

Aga, H., Shibuya, T., Sugimoto, T., Kurimoto, M., Nakajima, S. (1994) Isolation and identification of antimicrobial compounds in Brazilian propolis. *Biosci. Biotech. Biochem.* **58**: 945–946

Amoros, M., Lurton, E., Boustie, J., Girre, L. (1994) Comparison of the anti-herpes simplex virus activities of propolis and 3-methyl-but-2-enyl caffeate. *J. Nat. Prod.* **57**: 644–647

Bankova, V., Christov, R., Kujumgiev, A., Marcucci, M. C., Popov, S. (1995) Chemical composition and antibacterial activity of Brazilian propolis. *Z. Naturforsch.* **50**: 167–172

Banskota, A. H., Tezuka, Y., Prasain, J. K., Matsushige, K., Saiki, I., Kadota, S. (1998) Chemical constituents of Brazilian propolis and their cytotoxic activities. *J. Nat. Prod.* **61**: 896–900

Bolotina, V. M., Najibi, S., Palacino, J. J., Pagano, P. J., Cohen, R. A. (1994) Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* **368**: 850–853

De Campos, R. O., Paulino, N., Da Silva, C. H., Scremin, A., Calixto, J. B. (1998) Anti-hyperalgesic effect of an ethanolic extract of propolis in mice and rats. *J. Pharm. Pharmacol.* **50**: 1187–1193

De Castro, S. L., Higashi, K. O. (1995) Effect of different formulations of propolis on mice infected with *Trypanosoma cruzi*. *J. Ethnopharmacol.* **46**: 55–58

Ellis, J. L. (1997) Role of soluble guanylyl cyclase in the relaxations to a nitric oxide donor and to nonadrenergic nerve stimulation in guinea pig trachea and human bronchus. *J. Pharmacol. Exp. Ther.* **280**: 1215–1218

Georgieva, P., Ivanovska, N., Bankova, V., Popov, S. (1997) Anti-complement activity of lysine complexes of propolis phenolic constituents and their synthetic analogs. *Z. Naturforsch.* **52C**: 6064

Gimenez-Gallego, G., Navia, M. A., Reuben, J. P., Katz, G. M., Kaczorowski, G. J., Garcia, M. L. (1988) Purification, sequence, and model structure of charybdotoxin, a potent selective inhibitor of calcium-activated potassium channels. *Proc. Natl Acad. Sci. USA* **85**: 3329–3335

Ivanovska, N., Dimov, V., Pavlova, S., Bankova, V., Popov, S. (1995) Immunomodulatory action of propolis. V. Anticomplementary activity of a water-soluble derivative. *J. Ethnopharmacol.* **47**: 135–143

Jaiswal, A. K., Venugopal, R., Mucha, J., Carothers, A. M., Grunberger, D. (1997) Caffeic acid phenethyl ester stimulates human antioxidant response element-mediated expression of the NAD(P)H: quinone oxidoreductase (NQO1) gene. *Cancer Res.* **57**: 440–446

Kimoto, T., Aga, M., Hino, K., Koya-Miyata, S., Yamamoto, Y., Micallef, M. J., Hanaya, T., Arai, S., Ikeda, M., Kurimoto, M. (2001a) Apoptosis of human leukemia cells induced by Artepillin C, an active ingredient of Brazilian propolis. *Anticancer Res.* **21**: 221–228

Kimoto, T., Koya-Miyata, S., Hino, K., Micallef, M. J., Hanaya, T., Arai, S., Ikeda, M., Kurimoto, M. (2001b) Pulmonary carcinogenesis induced by ferric nitrilotriacetate in mice and protection from it by Brazilian propolis and artepillin C. *Virchows Arch.* **438**: 259–270

Kotlikoff, M. I. (1993) Potassium channels in airway smooth muscle: a tale of two channels. *J. Exp. Pharmacol. Ther.* **58**: 1–12

Krol, W., Scheller, S., Czuba, Z., Matsuno, T., Zydowicz, G., Shani, J., Mos, M. (1996) Inhibition of neutrophils' chemiluminescence by ethanol extract of propolis (EEP) and its phenolic components. *J. Ethnopharmacol.* **55**: 19–25

Lincoln, T. M., Cornwell, T. L. (1991) Towards an understanding of the mechanism of action of cyclic AMP and cyclic GMP in smooth muscle relaxation. *Blood Vessels* **28**: 129–137

Mahran, L. G., El-Khatib, A. S., Agha, A. M., Khayyal, M. T. (1996) The protective effect of aqueous propolis extract on isolated rat hepatocytes against carbon tetrachloride toxicity. *Drugs Exp. Clin. Res.* **22**: 309–316

Marcucci, M. C. (2000) Process to tipify natural products. Patent requested PI000 6272 in INPI, Brasil.

Marcucci, M. C., Ferreres, F., García-Vigueira, C., Bankova, V., De Castro, S. L., Dantas, A. P., Paulino, N. (2001) Phenolic compounds from Brazilian propolis with pharmacological activities. *J. Ethnopharmacol.* **74**: 105–112

Matsushige, K., Basnet, P., Hase, K., Kadota, S., Tanaka, K., Namba, T. (1996) Propolis protects pancreatic beta-cells against the toxicity of streptozotocin (STZ). *Phytomedicine* **3**: 203–209

Mitamura, T., Matsuno, T., Sakamoto, S., Maemura, M., Kudo, H., Suzuki, S., Kuwa, K., Yoshimura, S., Sassa, S., Nakayama, T., Nagasawa, H. (1996) Effects of a new clerodane diterpenoid isolated from propolis on chemically induced skin tumors in mice. *Anticancer Res.* **16**: 2669–2672

Miyataka, H., Nishiki, M., Matsumoto, H., Fujimoto, T., Matsuka, M., Satoh, T. (1997) Evaluation of propolis. I. Evaluation of Brazilian and Chinese propolis by enzymatic and physico-chemical methods. *Biol. Pharm. Bull.* **20**: 496–501

Nielsen, K. J., Schroeder, T., Lewis, R. (2000) Structure–activity relationships of omega-conotoxins at N-type voltage-sensitive calcium channels. *J. Mol. Recognit.* **13**: 55–70

Paulino, N., Cechinel-Filho, V., Yunes, R. A., Calixto, J. B. (1996) The relaxant effect of extract of *Phyllanthus urinaria* in the guinea-pig isolated trachea. Evidence for involvement of ATP-sensitive potassium channels. *J. Pharm. Pharmacol.* **48**: 1158–1163

Paulino, N., Pizollatti, M. G., Yunes, R. A., Filho, V. C., Creczynski-Pasa, T. B., Calixto, J. B. (1999) The mechanisms underlying the relaxant effect of methyl and ethyl gallates in the guinea pig trachea in vitro: contribution of potassium channels. **360**: 331–336

Schrammel, A., Behrends, S., Schmidt, K., Koesling, D., Mayer, B. (1996) Characterization of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanylyl cyclase. *Mol. Pharmacol.* **50**: 1–5

Suarez-Kurtz, G., Garcia, M. L., Kaczorowski, G. J. (1991) Effects of charybdotoxin and iberiotoxin on the spontaneous motlity and tonus of different guinea pig smooth muscle tissues. *J. Pharmacol. Exp. Ther.* **259**: 439–445

Vaali, K., Li, L., Lähteenmäki, T., Vapaatalo, H. (2000) Role of BK(Ca) channels and cyclic nucleotides in synergistic relaxation of trachea. *Eur. J. Pharmacol.* **399**: 75–84

Van Rosum, 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

Viana-Jorge, R., Oliveira, C. F., Garcia, M. L., Kaczorowski, G. J., Suarez-Kurtz, G. (2000) Correolide, a nor-triterpenoid blocker of *Shaker*-type Kv1 channels elicits twitches in guinea-pig ileum by stimulating the enteric nervous system and enhancing neurotransmitter release. *Br. J. Pharmacol.* **131**: 772–778

Winquist, R. J., Heaney, L. A., Wallace, D. G., Baskin, E. P., Stein, R. G., Garcia, M. L., Kaczorowski, G. J. (1989) Glyburide blocks the relaxation response to BRL-34915 (Cromakalin), minoxidil sulfate and diazoxide in vascular smooth muscle. *J. Pharmacol. Exp. Ther.* **248**: 149–155