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The mechanism of gentisic acid-induced relaxation of the guinea pig isolated trachea: the role of potassium channels and vasoactive intestinal peptide receptors

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Abstract

We examined some of the mechanisms by which the aspirin metabolite and the naturally occurring metabolite gentisic acid induced relaxation of the guinea pig trachea *in vitro*. In preparations with or without epithelium and contracted by histamine, gentisic acid caused concentration-dependent and reproducible relaxation, with mean EC_{50} values of 18 µM and E_{max} of 100% (N = 10) or 20 µM and E_{max} of 92% (N = 10), respectively. The relaxation caused by gentisic acid was of slow onset in comparison to that caused by norepinephrine, theophylline or vasoactive intestinal peptide (VIP). The relative rank order of potency was: salbutamol 7.9 > VIP 7.0 > gentisic acid 4.7 > theophylline 3.7. Gentisic acid-induced relaxation was markedly reduced (24 ± 7.0, 43 ± 3.9 and 78 ± 5.6%) in preparations with elevated potassium concentration in the medium (20, 40 or 80 mM, respectively). Tetraethylammonium (100 µM), a nonselective blocker of the potassium channels, partially inhibited the relaxation response to gentisic acid, while 4-AP (10 µM), a blocker of the voltage potassium channel, inhibited gentisic acid-induced relaxation by 41 ± 12%. Glibenclamide (1 or 3 µM), at a concentration which markedly inhibited the relaxation induced by the opener of ATP-sensitive K^+ channels, levcromakalim, had no effect on the relaxation induced by gentisic acid. Charybdotoxin (0.1 or 0.3 µM), a selective blocker of the large-conductance Ca^{2+}-activated K^+ channels, caused rightward shifts (6- and 7-fold) of the gentisic acid concentration-relaxation curve. L-N^G-nitroarginine (100 µM), a NO synthase inhibitor, had no effect on the relaxant effect of gentisic acid, and caused a slight displacement to the right in the relaxant effect of the gentisic acid curve at 300 µM, while methylene blue (10 or 30 µM) or ODQ (1 µM), the inhibitors of soluble guanylate cyclase, all failed to affect gentisic acid-induced relaxation. D-$_P$-Cl-Phe^6,Leu^{17}[VIP] (0.1 µM), a VIP receptor antagonist, significantly inhibited (37 ± 7%) relaxation induced by gentisic acid, whereas CGRP (8-37) (0.1 µM), a CGRP antagonist, only slightly enhanced the action of gentisic acid. Taken together, these results provide functional evidence for the direct activation of voltage and large-conductance Ca^{2+}-activated K^+ channels, or indirect modulation of potassium channels induced by VIP receptors and accounts for the predominant relaxation response caused by gentisic acid in the guinea pig trachea.

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- Vasoactive intestinal peptide
- VIP
- Trachea
- Smooth muscle

Introduction

2,5-Dihydroxybenzoic acid or gentisic acid is an active metabolite of salicylic acid degradation (1). There is an increasing amount of evidence indicating that gentisic acid has a broad spectrum of biological activity such as anti-inflammatory, antirheumatic and antioxidant properties, independent of the action of salicylic acid (2,3). Thus, gentisic acid has been demonstrated to inhibit both the cyclooxygenase and 12-lipoxygenase enzymes (4) and has also been found to decrease the production of the leukotrienes C4 on the stomach wall during the gastric lesion induced by ethanol (5).

Several potentially active compounds including some naturally occurring substances capable of directly activating potassium channels have been recently reported (6,7). The new classes of potassium channel openers might be of potential clinical interest because they are able to induce vascular (8) and nonvascular (9) smooth muscle relaxation, including in the respiratory tract (10,11).

Despite the considerable number of reported studies for gentisic acid, so far its effect on nonvascular smooth muscle has not been investigated. The aims of the present study were to examine whether gentisic acid causes relaxation in the guinea pig trachea *in vitro* and if so, to characterize some of the pharmacological mechanisms responsible for such effects using selective receptor antagonists or ion channel blockers. We present evidence that the relaxation caused by gentisic acid in the guinea pig trachea is largely mediated by activation of potassium channels, especially the high-conductance calcium-activated potassium channels, and by vasoactive intestinal peptide (VIP)-receptor interaction.

Material and Methods

Tissue preparations

Guinea pigs (250-400 g) of both sexes were killed by cervical dislocation. The trachea was rapidly removed, freed from connective tissue, and cut into six transverse rings (3-4 mm wide), each containing 3 cartilages as described previously (12,13). The rings were opened (usually 6 strips of 8-10 mm in length were obtained from the same animal) and suspended in individual 10-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 was of the following composition: 118.0 mM NaCl, 4.4 mM KCl, 1.1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 11.0 mM glucose. Tissues were allowed to equilibrate for at least 60 min before drug additions under a resting tension of 1 g, during which the buffer solution was renewed every 15 min. Isometric responses were recorded on a polygraph (Letica Scientific Instruments, Barcelona, Spain) by means of TRI-201 force displacement transducers. In some 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 (12).

Experimental procedure

After an equilibration period of at least 60 min, the preparations with or without epithelium were precontracted with histamine (1 to 3 μM), and once the tonic contraction became stable, gentisic acid (0.3 μM to 1 mM) was added to the bath by the cumulative method (14). Two to five complete cumulative concentration-response curves were obtained for gentisic acid in each preparation, at 60-min intervals between curves.

To investigate the possible mechanisms responsible for the relaxation response induced by gentisic acid in the guinea pig trachea, the preparations without epithelium were contracted by the addition of histamine (1 or 3 μM) 20 min before being incubated

with one of the following drugs: 100 µM tetraethylammonium (TEA, a nonselective blocker of K^+ channels), 10 µM 4-aminopyridine (4-AP, a selective blocker of voltage-sensitive K^+ channels), 1 or 3 µM glibenclamide (a selective blocker of ATP-sensitive K^+ channels), 0.1 or 0.3 µM charybdotoxin (a selective blocker of large-conductance Ca^{2+}-sensitive K^+ channels), 100 or 300 µM L-N^G-nitroarginine (L-NOARG, a NO synthase competitive antagonist), 10 or 30 µM methylene blue (an inhibitor of soluble guanylate cyclase), 1 µM ODQ (a selective inhibitor of soluble guanylate cyclase), 0.1 µM porcine D-$_P$-Cl-Phe^6,Leu^{17}[VIP] (a VIP receptor antagonist), and 0.1 µM human calcitonin gene-related peptide (8-37) (CGRP (8-37), a CGRP receptor antagonist). In addition, we also investigated the ability of gentisic acid to elicit relaxation in preparations containing 20, 40 or 80 mM of KCl instead of 4.7 mM.

Drugs

The following drugs were supplied by Sigma Chemical Co., St. Louis, MO, USA: gentisic acid (standard), histamine, TEA, 4-AP, glibenclamide, charybdotoxin, L-NOARG, methylene blue, ODQ, D-$_P$-Cl-Phe^6,Leu^{17}[VIP], porcine VIP, CGRP (8-37). The other chemical products and salts were supplied by Merck (Darmstadt, Germany) and were of high grade analytic purity. The stock solutions of these drugs were prepared and stored at -20ºC. The bath concentration of ethanol did not exceed 0.03%, which was shown to have no effect *per se* on the basal tonus of the preparations or on the agonist-mediated contraction or relaxation.

Statistical analysis

Responses are reported as percentage of the maximal effect of control relaxation caused by gentisic acid. Data were analyzed statistically by the unpaired Student *t*-test, with the level of significance set at $P<0.05$. The EC_{50} (i.e., the molar concentration of gentisic acid and of the reference drugs causing half-maximum responses) values were determined from individual experiments for the complete agonist concentration-response curves by graphic methods. In some experiments, the pD_2 values (i.e., log of the EC_{50}) were calculated. The EC_{50} and pD_2 values are reported as geometric means accompanied by their respective 95% confidence limits. All other results are reported as means ± SEM.

Results

Cumulative addition of gentisic acid (0.3 µM-1 mM) to the guinea pig isolated trachea with or without epithelium caused a concentration-dependent relaxation in preparations precontracted with histamine (1 to 3 µM) (a concentration inducing about 50% of the maximal contractile response). The calculated mean EC_{50} (and 95% confidence limits) were 18.72 µM (11.13-31.49) and 20.58 µM (12.01-35.28) and the maximum relaxation (E_{max}) was 100% (N = 10) and 92% (N = 10), respectively (Figure 1).

The relaxation caused by gentisic acid was of slow onset when compared to that caused by VIP, norepinephrine or theophylline. In epithelium-denuded and histamine-precontracted preparations the relative rank order of relaxant potency (pD_2) was: salbu-

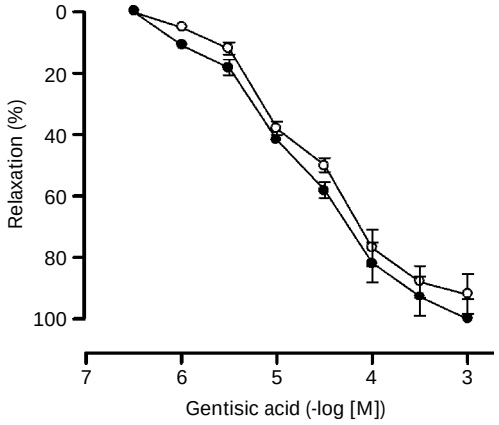

Figure 1 - Mean relaxant concentration-response curves for gentisic acid in the guinea pig isolated trachea, without (filled circles) or with (open circles) epithelium. Data are reported as means ± SEM of 10 experiments. P<0.05 for the difference between points (Student *t*-test).

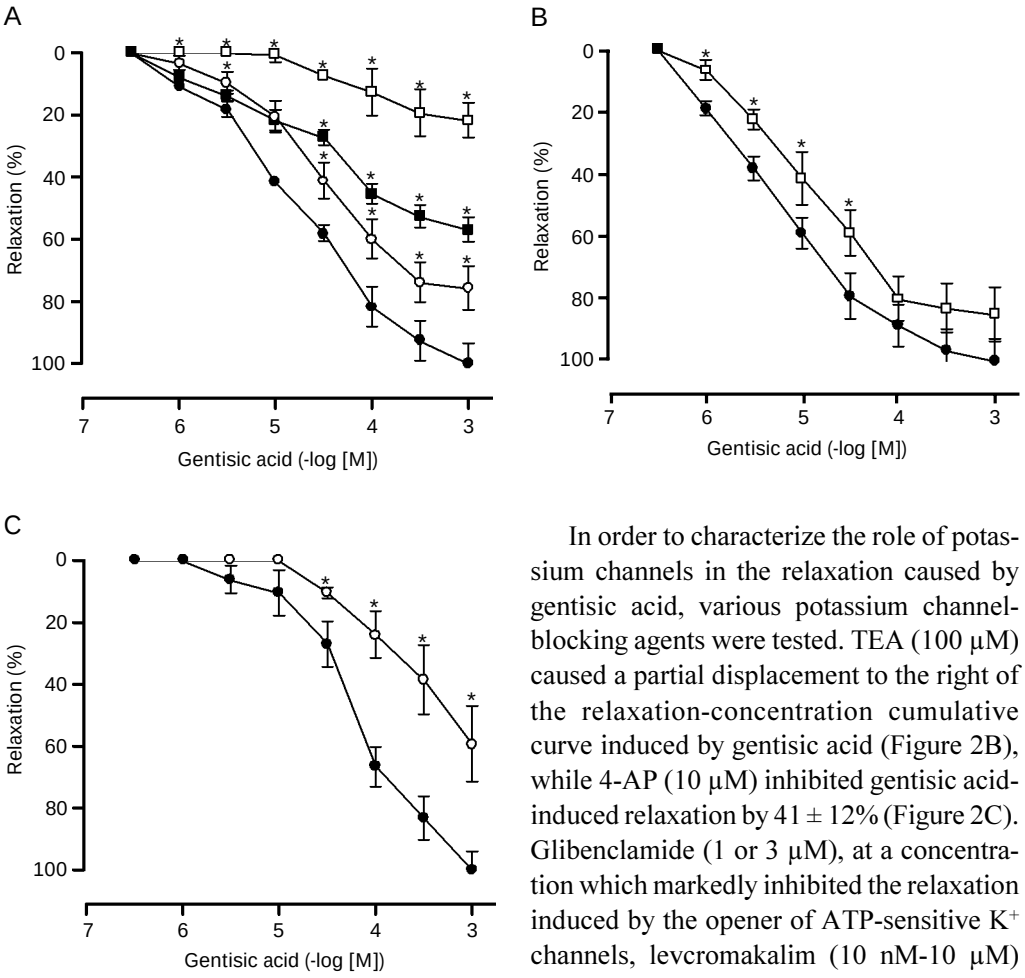

Figure 2 - Mean relaxant concentration-response curves for gentisic acid in the guinea pig trachea without epithelium in the absence (filled circles) or presence of: *A*, KCl (open circles, 20 mM; filled squares, 40 mM, or open squares, 80 mM); *B*, tetraethylammonium (open squares, 100 µM); *C*, 4-aminopyridine (open circles, 10 µM). Data are reported as means ± SEM of 6 experiments. *P<0.05 for the difference between points (Student *t*-test).

tamol 7.9 (7.5-8.5) > VIP 7.0 (6.4-7.5) > gentisic acid 4.7 (4.5-5.0) > theophylline 3.7 (2.9-4.3) (data not shown).

When the experiments were performed in Krebs-Henseleit solution containing 20 mM of KCl, the relaxation caused by gentisic acid was slightly reduced (24 ± 7%). In preparations contracted with 40 mM KCl, the relaxation caused by gentisic acid was reduced by 43 ± 4%, while in preparations contracted with 80 mM KCl, a concentration that is presumed to induce maximal depolarization of the cell membrane, the response to gentisic acid was markedly attenuated (78 ± 5.6%), although a weak relaxation response was still evident between 30 µM and 1 mM (Figure 2A).

In order to characterize the role of potassium channels in the relaxation caused by gentisic acid, various potassium channel-blocking agents were tested. TEA (100 µM) caused a partial displacement to the right of the relaxation-concentration cumulative curve induced by gentisic acid (Figure 2B), while 4-AP (10 µM) inhibited gentisic acid-induced relaxation by 41 ± 12% (Figure 2C). Glibenclamide (1 or 3 µM), at a concentration which markedly inhibited the relaxation induced by the opener of ATP-sensitive K^+ channels, levcromakalim (10 nM-10 µM) (data not shown), did not modify the relaxation induced by gentisic acid (Figure 3A), but preincubation of the preparations with charybdotoxin (0.1 or 0.3 µM) caused a 6- and 7-fold shift to the right of the concentration-relaxation curve for gentisic acid, respectively (Figure 3B).

The nitric oxide and cGMP pathways were also investigated. L-NOARG (100 µM), methylene blue (10 or 30 µM) and ODQ (1 µM) all failed to affect gentisic acid-induced relaxation, but an increase in L-NOARG concentration to 300 µM produced a slight rightward displacement of the relaxation curve for gentisic acid (Figure 4A, B and C, respectively). D-$_P$-Cl-Phe^6,Leu^{17}[VIP] (0.1 µM), a VIP receptor antagonist, also significantly inhibited (37 ± 7%) gentisic acid-induced relaxation (Figure 5A), while CGRP

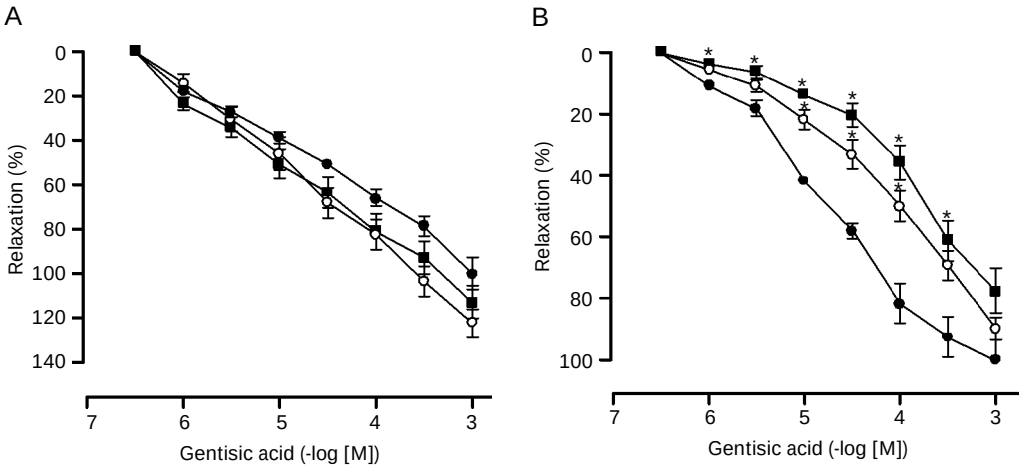

Figure 3 - Mean relaxant concentration-response curves for gentisic acid in the guinea pig trachea without epithelium in the absence (filled circles) or presence of: *A*, glibenclamide (open circles, 1 µM or filled squares, 3 µM); *B*, charybdotoxin (open circles, 0.1 µM or filled squares, 0.3 µM). Data are reported as means ± SEM of 6 experiments. *P<0.05 for the difference between points (Student *t*-test).

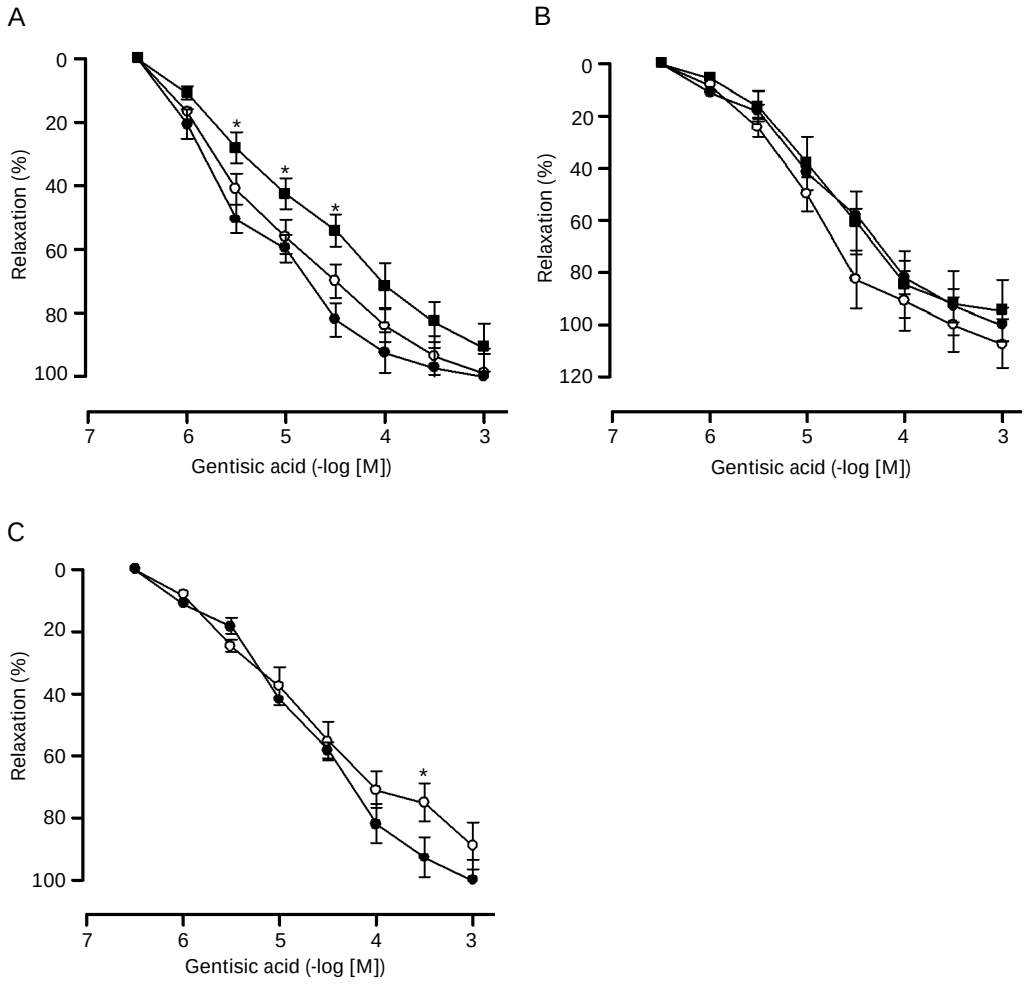

Figure 4 - Mean relaxant concentration-response curves for gentisic acid in the guinea pig trachea without epithelium in the absence (filled circles) or presence of: *A*, L-NOARG (open circles, 100 µM or filled squares, 300 µM); *B*, methylene blue (open circles, 10 µM or filled squares, 30 µM); *C*, ODQ (open circles, 1 µM). Data are reported as means ± SEM of 6 experiments. *P<0.05 for the difference between points (Student *t*-test).

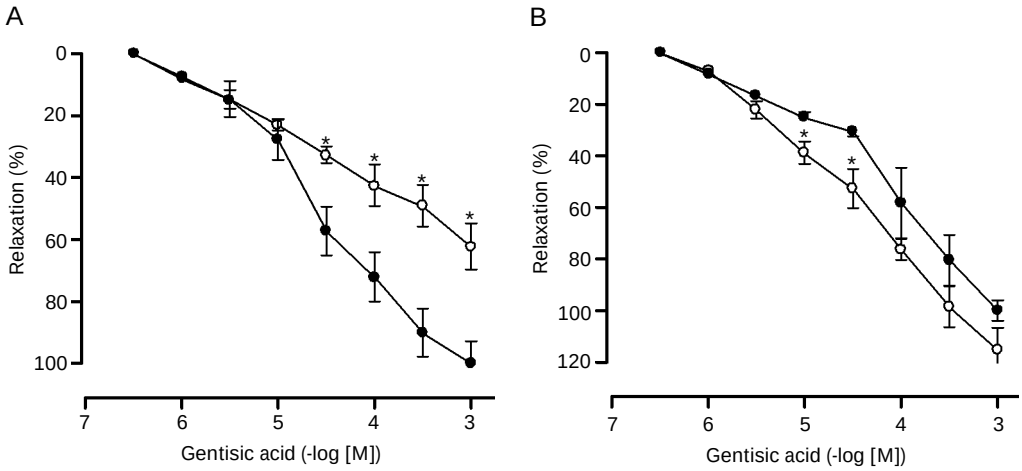

Figure 5 - Mean relaxant concentration-response curves for gentisic acid in the guinea pig trachea without epithelium in the absence (filled circles) or presence of: *A*, porcine D-$_P$-Cl-Phe^6,Leu^{17}[VIP] (open circles, 0.1 µM); *B*, CGRP (8-37) (open circles, 0.1 µM). Data are reported as means ± SEM of 6 experiments. *P<0.05 for the difference between points (Student *t*-test).

(8-37) (0.1 µM), a CGRP antagonist, only slightly enhanced the gentisic acid-induced relaxation (Figure 5B).

Discussion

Potassium channels are important target proteins which are involved in the relaxation of numerous smooth muscle cells, including airway smooth muscle, where the large-conductance calcium-activated potassium channel (BK_{Ca}) has an important regulatory role in tissue responsiveness (15,16). Several types of potassium channels have been reported to produce smooth muscle relaxation. Among them are the delayed rectifier channels (17), the large- and low-conductance calcium-activated channels (15) and also the ATP-sensitive potassium channels (16). Potassium channels also are an important effector element for several relaxant agents in the guinea pig isolated trachea, like nitric oxide (18) and VIP (19). The results of the present study provide functional evidence indicating that gentisic acid, an active metabolite of the salicylic route of degradation (1), causes concentration-dependent relaxation of the guinea pig trachea in both the presence and absence of epithelium.

The gentisic acid relaxation response was largely mediated by the opening of K^+ channels, sensitive to both TEA and 4-AP. In addition, gentisic acid-induced relaxation was markedly antagonized when a higher K^+ concentration was present in the medium, further confirming the requirement of the integrity of the cell membrane potential for its action. Also relevant are the results showing that the relaxation caused by gentisic acid in the guinea pig trachea was largely antagonized by charybdotoxin, a neurotoxin known to antagonize selectively high-conductance calcium-activated potassium channels (18). However, glibenclamide, a selective blocker of ATP-sensitive K^+ channels, at concentrations at which it completely antagonized the relaxation caused by the opener of ATP-sensitive potassium channels levcromakalim in the guinea pig trachea, failed to affect gentisic acid-mediated relaxation in this preparation, suggesting that ATP-sensitive potassium channels have no major effect on gentisic acid relaxation.

There is an increasing amount of experimental evidence suggesting that nitric oxide or nitric oxide-related substances present in the airways, including the guinea pig trachea (20,21), are able to modulate the relaxation response induced by several drugs and neurotransmitters. Our results, however, cannot fully rule out an action of nitric oxide on the relaxation produced by gentisic acid in the

guinea pig trachea, since in the presence of high L-NOARG concentrations a slight displacement of the relaxant concentration response to gentisic acid was observed. The observation that the relaxation caused by gentisic acid was not affected by the absence of the epithelium in the preparations or by the guanylate cyclase inhibitors further confirms our hypothesis that nitric oxide seems to play only a minor role in the gentisic acid relaxation of the guinea pig trachea. A possible antioxidant property of gentisic acid cannot be fully excluded, since it has been reported that gentisic acid inhibits the reaction of nitric oxide with superoxide anions generated in the medium, thus enhancing the half-time of nitric oxide (22). Our results also show that the cGMP pathway seems unlikely to be involved in the relaxant effect of gentisic acid on the guinea pig trachea, since the inhibitors of soluble guanylate cyclase, methylene blue and ODQ (23), at concentrations known to inhibit the responses mediated by the cGMP-dependent mechanism, fully failed to affect gentisic acid-mediated relaxation.

The possible role of VIP and CGRP receptors in the gentisic acid-induced relaxation of the guinea pig trachea was also assessed by the use of selective antagonists of these receptors. Preincubation of the preparations with porcine D-$_P$-Cl-Phe^6,Leu^{17}[VIP] consistently antagonized gentisic acid-mediated relaxation, suggesting some modulatory role of VIP in the gentisic acid response. There is increasing evidence suggesting that the relaxation induced by VIP in the airway smooth muscles is largely mediated by activation of potassium channels including BK_{Ca}, while ATP-sensitive potassium channels apparently play a minor role in the VIP response (19). On the other hand, human CGRP (8-37), another neuropeptide involved in the modulation of the responsiveness of airway smooth muscles (24), only slightly enhanced the action of gentisic acid, indicating that CGRP has apparently no major effect on gentisic acid-mediated relaxation. The precise mechanisms by which VIP mediated the relaxant response of gentisic acid in the guinea pig trachea are still unknown and additional studies are required to clarify this issue.

In summary, the present results have provided direct pharmacological evidence indicating that gentisic acid, an active metabolite of the salicylic acid route of degradation, produced concentration-dependent and reproducible relaxation in the guinea pig isolated trachea precontracted with histamine both in the presence and in the absence of epithelium. Its precise mechanism of action still remains partially undefined, but several mechanisms seem to account for its relaxant effect, such as its ability to activate large-conductance Ca^{2+}-activated K^+ channels sensitive to charybdotoxin and also voltage-sensitive K^+ channels highly sensitive to 4-AP. Finally, it has also been demonstrated that the VIP receptors strongly contribute to the relaxation response of gentisic acid in the guinea pig trachea.

References

1. Ohsako M, Matsumoto Y & Goto S (1993). Transport of aspirin and its metabolites through human erythrocyte membrane. *Biological and Pharmaceutical Bulletin*, 16: 154-157.
2. Liu ZC, McClelland RA & Uetrecht JP (1995). Oxidation of 5-aminosalicylic acid by hypochlorous acid to a reactive iminoquinone. Possible role in the treatment of inflammatory bowel diseases. *Drug Metabolism and Disposition*, 23: 246-250.
3. Glinkowska G, Baan B, Sommer E, Demkow U, Sokolnicka I, Strzelecka H & Skopinska E (1997). The effect of phenolic compounds of poplar leaves extract on cutaneous angiogenesis reaction induced in mice by human mononuclear leukocytes. *Acta Poloniae Pharmaceutica*, 54: 151-154.
4. Radomski M, Michalska Z, Marcinkiewicz E & Gryglewski RJ (1986). Salicylates and 12-lipoxygenase activity in human washed platelets. *Pharmacological Research Communications*, 18: 1015-1030.
5. Trautmann M, Peskar BM & Peskar BA (1991). Aspirin-like drugs, ethanol-induced rat gastric injury and mucosal eicosanoid

release. *European Journal of Pharmacology*, 201: 53-58.
6. Soleas GJ, Diamandis EP & Goldberg DM (1997). Wine as a biological fluid: history, production, and role in disease prevention. *Journal of Clinical Laboratory Analysis*, 11: 287-313.
7. McManus OB, Harris GH, Giangiacomo KM, Feigenbaum P, Reuben JP, Addy ME, Burka JF, Kaczorowski GJ & Garcia ML (1993). An activator of calcium dependent potassium channels isolated from a medicinal herb. *Biochemistry*, 32: 6128-6133.
8. Price JM, Backer CH & Bond RF (1997). Calcium-activated potassium channel-mediated arteriolar relaxation during endotoxic shock. *Shock*, 7: 294-299.
9. Hong SJ, Roan YF & Chang CC (1997). Spontaneous activity of guinea pig ileum longitudinal muscle regulated by Ca^{2+}-activated K^+ channel. *American Journal of Physiology,* 272: G962-G971.
10. Buchheit KH, Hofmann A & Pfannkuche HJ (1997). *In vitro* and *in vivo* effects of SCA40 on guinea pig airways. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 355: 217-223.
11. Müller-Schweinitzer E & Fozard JR (1997). SCA40: studies of the relaxant effects on cryopreserved human airway and vascular smooth muscle. *British Journal of Pharmacology*, 120: 1241-1248.
12. Schlemper V & Calixto JB (1994). Nitric oxide pathway-mediated relaxant effect of bradykinin in the guinea pig isolated trachea. *British Journal of Pharmacology*, 111: 83-88.
13. Schlemper V & Calixto JB (1995). Mechanisms involved in the relaxant response of bradykinin in epithelium intact strips of the guinea-pig trachea. *European Journal of Pharmacology*, 282: 177-184.
14. Van Rosum JM (1963). Cumulative dose-response curves. II. Technique for making of dose-response curves in isolated organs and the evaluation of drug parameters. *Archives Internationales de Pharmacodynamie et de Therapie*, 143: 299-330.
15. Kume H, Takai A, Tokuno H & Tomita T (1989). Regulation of Ca^{2+}-dependent K^+-channel activity in tracheal myocytes by phosphorylation. *Nature*, 341: 152-154.
16. Kotlikoff MI (1993). Potassium channels in airway smooth muscle: a tale of two channels. *Pharmacology and Therapeutics*, 58: 1-12.
17. Archer SL, Souil E, Dinhxuan AT, Schremmer B, Mercier JC, Elyaagoubi A, Nguyenhuu L, Reeve HL & Hampl V (1998). Molecular identification of the role of voltage-gated K^+ channels, kv1.5 and kv2.1, in hypoxic pulmonary vasoconstriction and control of resting membrane potential in rat pulmonary artery myocytes. *Journal of Clinical Investigation*, 101: 2319-2330.
18. Corompt E, Bessard G, Lantuejoul S, Naline E, Advenier C & Devillier P (1998). Inhibitory effects of large Ca^{2+}-activated K^+ channel blockers on beta-adrenergic- and NO-donor-mediated relaxations of human and guinea-pig airway smooth muscles. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 357: 77-86.
19. Thirstrup S, Nielsen Kudsk JE & Mellemkjaer S (1997). Involvement of K^+ channels in the relaxant effect of vasoactive intestinal peptide and atrial natriuretic peptide in isolated guinea-pig trachea. *European Journal of Pharmacology*, 319: 253-259.
20. Vaali K, Li L, Paakkari I & Vapaatalo H (1998). Relaxing effects of NO donors on guinea-pig trachea *in vitro* are mediated by calcium-sensitive potassium channels. *Journal of Pharmacology and Experimental Therapeutics*, 286: 110-114.
21. Schnackenberg CG, Welch WJ & Wilcox CS (1998). Normalization of blood pressure and renal vascular resistance in SHR with a membrane-permeable superoxide dismutase. *Hypertension*, 32: 59-64.
22. Chabot F, Mitchell JA, Gutteridge JMC & Evans TW (1998). Reactive oxygen species in acute lung injury. *European Respiratory Journal*, 11: 745-757.
23. Paulino N, Pizollatti MG, Yunes RA, Filho VC, Creczynski-Pasa TB & Calixto JB (1999). The mechanisms underlying the relaxant effect of methyl and ethyl gallates in the guinea pig trachea *in vitro*: contribution of potassium channels. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 360: 331-336.
24. Ninomiya H, Uchida Y, Endo T, Ohtsuka M, Nomura A, Saotome M & Hasegawa S (1996). The effects of calcitonin gene-related peptide on tracheal smooth muscle of guinea-pigs *in vitro*. *British Journal of Pharmacology*, 119: 1341-1346.