Mechanisms of Relaxant Action of Luteolin in Isolated Guinea Pig Trachea

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Abstract

We have investigated the mechanisms of action of luteolin, a flavone found in *Perilla frutescens*, a Chinese herbal medicine for treating asthma. In fact, luteolin occurs mostly as a glycoside in many plant species. The tension changes of tracheal segments were isometrically recorded on a polygraph. Luteolin concentration-dependently relaxed histamine (30 μM)-, carbachol (0.2 μM)- and KCl (30 mM)-induced precontractions, and inhibited cumulative histamine- and carbachol-induced contractions in a non-competitive manner. Luteolin also concentration-dependently and non-competitively inhibited cumulative Ca^{2+}-induced contractions in depolarized (K^+, 60 mM) guinea-pig trachealis. The nifedipine (10 μM)-remaining tension of histamine (30 μM)-induced precontractions was further relaxed by luteolin, suggesting that no matter whether VDCCs were blocked or not, luteolin may have other mechanisms of relaxant action. The relaxant effect of luteolin was unaffected by the removal of epithelium or by the presence of propranolol (1 μM), 2′,5′-dideoxyadenosine (10 μM), methylene blue (25 μM), glibenclamide (10 μM), N^{ω}-nitro-l-arginine (20 μM), or α-chymotrypsin (1 U/mL). However, luteolin (10 – 20 μM) produced parallel and leftward shifts of the concentration-response curve of forskolin or nitroprusside. Luteolin or IBMX at various concentrations (10 – 300 μM) concentration-dependently and significantly inhibited cAMP- and cGMP-PDE activities of the trachealis. The IC_{50} values of luteolin were estimated to be 32.4 and 34.6 μM, respectively. IBMX at various concentrations (10 – 300 μM) selectively inhibited neither cAMP-, nor cGMP-PDE activity. In contrast to IBMX, luteolin at 100 and 300 μM more potently ($P < 0.05$) inhibited cGMP-, than cAMP-PDE activity. The above results indicate that the mechanisms of relaxant action of luteolin may be due to its inhibitory effects on both PDE activities and its reduction on $[Ca^{2+}]_i$ of the trachealis.

Key words
Luteolin · flavonoid · guinea-pig trachea · intracellular calcium · phosphodiesterase

Abbreviations

IBMX: 3-isobutyl-1-methylxanthine
VDCCs: voltage dependent calcium channels
cAMP: adenosine 3′,5′-cyclic monophosphate
cGMP: guanosine 3′,5′-cyclic monophosphate
PDE: phosphodiesterase

Introduction

Flavonoids are naturally occurring polyphenolic compounds with a wide distribution in the plant kingdom. They possess antioxidant, antitumor, antiangiogenic, anti-inflammatory, antiallergic, and antiviral properties [1]. Luteolin (Fig. **1**), a flavone found in high concentrations in celery, green pepper, perilla leaf and seed, and chamomile, has been reported to inhibit lipopoly-

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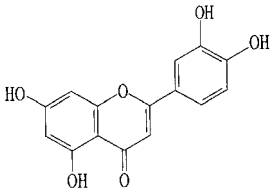

Fig. **1** Chemical structure of luteolin (mol wt: 286.23).

saccharide (LPS)-induced tumor necrosis factor-α (TNF-α) and interleukin-6 production as well as inducible nitric oxide expression [2]. Luteolin has been reported to interfere with LPS signalling by reducing the activation of several mitogen-activated protein kinase family members, and to suppress TNF-α release by inhibiting extracellular signal-regulated kinase, p38, casein kinase 2 activation [3]. *In vivo*, luteolin was found to be an active anti-inflammatory and anti-allergic constituent after orally administration of the extract of *Perilla frutescens* leaf, a component of the Chinese medicine "Tsai-Pu-Tang" for treating cough and bronchial asthma, in mice [4]. Luteolin also attenuates TNF-α production and intracellular adhesion molecule-1 expression, and abolishes infiltration of leukocytes in the lung and liver of LPS-treated mice [5]. Recently, luteolin has been reported to alleviate bronchoconstriction and airway hyperreactivity in ovalbumin sensitized mice. Therefore, Das et al. [6] concluded that luteolin could be used either as a lead molecule to identify an effective anti-asthma therapy or as a means to identify novel anti-asthma targets. We have reported that luteolin has a high potency in relaxing tracheal smooth muscle [7]. However, little is known about the influence of luteolin on tracheal smooth muscle. Therefore we were interested to investigate its mechanisms of relaxant action.

Materials and Methods

Reagents and drugs

Luteolin (Fig. **1**), with a purity of 99%, was purchased from Indofine Chemical Co., Hillsborough, NJ, USA. Aminophylline, carbachol, histamine, propranolol, 2′,5′-dideoxyadenosine, methylene blue, glibenclamide, N^{ω}-nitro-L-arginine (L-NNA), α-chymotrypsin, nifedipine, indomethacin, forskolin, sodium nitroprusside, ethylene glycol bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), Trizma base, *dl*-dithiothreitol, β-mercaptoethanol, cyclic AMP, cyclic GMP, calmodulin, Dowex resin, and *Crotalus atrox* snake venom, etc., were purchased from Sigma Chemical, St. Louis, MO, USA. [^3H]cAMP and [^3H]cGMP were purchased from Amersham Pharmacia Biotech AB, Uppsala, Sweden. 3-Isobutyl-1-methylxanthine (IBMX) was purchased from Aldrich Chem., Milwaukee, WI, USA. All reagents, including KCl, were of analytical grade. Glibenclamide was dissolved in dimethyl sulfoxide (DMSO). Luteolin, IBMX, forskolin, indomethacin, or nifedipine were dissolved in ethyl alcohol. Other drugs were dissolved in distilled water. The final concentration of ethyl alcohol or DMSO was less than 0.1% and did not significantly affect the contraction of the trachea.

Guinea-pig trachea

Under a protocol approved by the Animal Care and Use Committee of Taipei Medical University, male Hartley guinea pigs weighing 250 to 450 g were killed by cervical dislocation and the tracheas were removed. Each trachea was cut into six segments. Each segment consisted of three cartilage rings. All segments were cut open opposite the trachealis. After the segments were randomized to minimize regional variability, they were tied at one end to holders via silk suture, placed in 5 mL of normal or Ca^{2+}-free Krebs solution containing indomethacin (3 μM), gassed with a 95% O_2 – 5% CO_2 mixture at 37 °C, and attached by the other end of each segment to force displacement transducers (Grass FT03) for the isometric recording of tension changes on a polygraph (Gould RS3200). The composition of the normal Krebs solution was (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and dextrose 10.1. The isotonic high K^+, Ca^{2+}-free Krebs solution consisted of the above composition without $CaCl_2$, but 60 mM NaCl was replaced by 60 mM KCl. The tissues were suspended in normal Krebs solution under an initial tension of 1.5 g and allowed to equilibrate for at least 1 h with washing at 15-min intervals. After the tissues were precontracted with histamine (30 μM), carbachol (0.2 μM) or KCl (30 mM), Luteolin (1 – 300 μM) was cumulatively added to the organ bath, and its tracheal relaxant effects were allowed to reach a steady state at each concentration. At the end of the experiment without washout, 1 mM of aminophylline was added to standardize the tissue relaxing maximally. The relaxant potencies of luteolin were expressed as -log IC_{50} values. To determine the antagonistic effects of luteolin against contractile agonists, either histamine or carbachol was then cumulatively added to the normal Krebs solution, and the procedure was repeated until the contraction reached constancy after washout. Then, cumulative concentration-response curves were constructed. The maximal contractions of the tracheas without incubation of drugs or their vehicles were set as 100%. After the tissues were preincubated with luteolin or its vehicle for 15 min, these two contractile agonists were also cumulatively added into the normal Krebs solution. The antagonistic potencies of luteolin were expressed as pD_2' values, when the antagonistic effect on these cumulative concentration-response curves was in a non-competitive manner. In the case of isotonic high K^+ (60 mM)-depolarized tracheal preparations, normal Krebs solution was replaced after equilibration by Ca^{2+}-free Krebs solution without EGTA, and washed with the Ca^{2+}-free solution with 2 mM EGTA after tracheal contraction reached constancy and then incubated for 5 min. After repeating the above procedure until no contraction was observed, cumulative Ca^{2+} (0.01 – 10 mM) was added and contractions were elicited in the depolarized trachealis. The maximal contractile response elicited by Ca^{2+} (10 mM) was taken as 100%, and the cumulative concentration-response curve was constructed. The inhibitory effects of luteolin on cumulative Ca^{2+}-induced contractions in isotonic high K^+ (60 mM)-depolarized tracheas were expressed by -log IC_{50} values. The tracheal relaxant effects of cumulative luteolin (10 – 100 μM) on histamine (30 μM)-induced precontraction were allowed to reach a steady state at each concentration. After the precontraction reached a steady state, all antagonists, including propranolol, glibenclamide, 2′,5′-dideoxadenosine, methylene blue, L-NNA, and α-chymotrypsin or their vehicles were incubated for 15 min prior to the first addition of luteolin. Similarly, nifedipine (10 μM) was added at 15 min prior to the addition of luteolin (100 μM) or its vehicle, after histamine (30 μM)-induced precontraction reached a steady state. At the end of the experiment without washout, 1 mM of aminophylline was added to standardize the maximal tissue relaxation (100%).

To observe the effect of luteolin on the relaxant response of forskolin or nitroprusside to histamine (30 μM)-induced precontraction, luteolin (10 – 20 μM) was incubated for 15 min prior to the addition of histamine. Forskolin or nitroprusside was cumulatively added into the organ bath after the sustained contraction reached a constancy. At the end of the experiment, aminophylline (1 mM) was also added to maximally relax the tissue. To investigate the effects of epithelium on the relaxant response of luteolin to histamine (30 μM)-induced precontraction, some tracheal segments were denuded by rubbing with a moistened cotton-tipped applicator, while some were kept with the epithelium intact. At the end of the experiment, aminophylline (1 mM) was also added to maximally relax the tissue. The denuded and intact tissues were examined using light microscopy after staining with hematoxylin and eosin to determine the effectiveness of the epithelium removal procedure.

Phosphodiesterase activity

The isolated trachealis was homogenized with a glass/teflon homogenizer (Glas-Col, Terre Haute, IN, USA) in 20 volumes of cold medium (pH 7.4) containing 100 mM Tris-HCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol. cAMP- and cGMP-PDE activities in the homogenate were measured by a modification of the method of Cook et al. [8]. The homogenate was centrifuged at 9500 rpm for 15 min, and the upper layer was decanted. Twenty-five microliters of the upper layer were taken for determination of enzyme activity in a final volume of 100 μL containing 40 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 3.75 mM mercaptoethanol, 0.1 unit calmodulin (PDE activator), 10 μM $CaCl_2$, and either 1 μM cAMP with 0.2 μCi [^3H]-cAMP or 1 μM cGMP with 0.2 μCi [^3H]-cGMP. In tests of enzyme inhibition, the reaction mixture contained various concentrations of luteolin (10 – 300 μM) or IBMX (10 – 300 μM), a positive control. The reagents and homogenate were mixed on ice, and the reaction was initiated by transferring the mixture to a water bath at 37 °C. Following a 30-min incubation, the reaction was stopped by transferring the reaction vessel to a bath of boiling water for 3 min. After cooling on ice, 20 μL of a 1 mg/mL solution of *Crotalus atrox* venom were added to the reaction mixture, and the mixture was incubated at 37 °C for 10 min. Unreacted [^3H]-cAMP or [^3H]-cGMP was removed by the addition of 500 μL of a 1-in-1 Tris-HCl (40 mM) buffer suspension of Dowex resin (1 × 8 – 200) with incubation on ice for 30 min. Each tube was then centrifuged for 2 min at 6000 rpm, and 150 μL of the supernatant were removed for liquid scintillation counting. Less than 10% of the tritiated cyclic nucleotide was hydrolyzed in this assay.

Statistical analysis

The antagonistic effects of luteolin on these cumulative concentration-response curves were expressed as pD_2' values, and the relaxing effects of forskolin and nitroprusside against histamine (30 μM)-induced precontractions were expressed as pD_2 values, according to the method described by Ariëns and van Rossum [9]. The pD_2 values are the negative logarithm of the molar concentrations of forskolin and nitroprusside at which half-relaxing effects on histamine (30 μM)-induced precontractions were observed. $pD_2' = pD_x' + \log(x - 1)$, where pD_x' is the negative logarithm of the molar concentration of luteolin and x is the ratio between the maximal effect of the agonist in the absence of luteolin and that in the presence of luteolin. The $-\log IC_{50}$ value was considered to be equal to the negative logarithm of the molar concentrations of luteolin at which a half-inhibitory effect on agonist-induced precontractions, Ca^{2+} (10 mM)-induced contraction, or cyclic nucleotide PDE activity was observed. The IC_{50} value was calculated by linear regression. All values are shown as means ± SEM. The differences among these values were statistically calculated by one-way analysis of variance (ANOVA), then determined by least significant difference (LSD). The difference between two values, however, was determined by use of Student's unpaired *t*-test. The differences were considered statistically significant if the *P* value was less than 0.05.

Results

Luteolin concentration-dependently relaxed the histamine (30 μM)-, carbachol (0.2 μM)-, and KCl (30 mM)-induced precontractions (Fig. **2**). The $-\log IC_{50}$ values were 4.65 ± 0.11 (n = 6), 4.64 ± 0.06 (n = 7) and 4.58 ± 0.13 (n = 7), respectively. The $-\log IC_{50}$ values did not significantly differ from each other. Luteolin (3 – 100 μM) concentration-dependently inhibited the concentration-response curves of cumulative histamine and carbachol in a non-competitive manner (Figs. **3A,B**). The pD_2' values were 4.41 ± 0.13 (n = 6), and 4.03 ± 0.08 (n = 6), respectively, which significantly differ from each other. This suggests that the antispasmodic effects of luteolin against histamine are more potent than those against carbachol. In isotonic Ca^{2+}-free high K^+ (60 mM)-depolarized tracheas, luteolin (10 – 100 μM) concentration-dependently inhibited the concentration-response curves of cumulative Ca^{2+} (0.01 – 10 mM) in a non-competitive manner (Fig. **4**). The $-\log IC_{50}$ value was 4.56 ± 0.13 (n = 6), which is not significantly different from that against KCl (30 mM)-induced precontractions. Nifedipine, a voltage-dependent calcium channels (VDCCs) blocker, at 10 μM, however, only relaxed by 14.4 ± 2.9% (n = 6) the histamine (30 μM)-induced precontraction in the tracheas. The nifedipine (10 μM)-remaining tension of the trachea was further relaxed by luteolin (100 μM) to 98.8 ± 3.8% (n = 6).

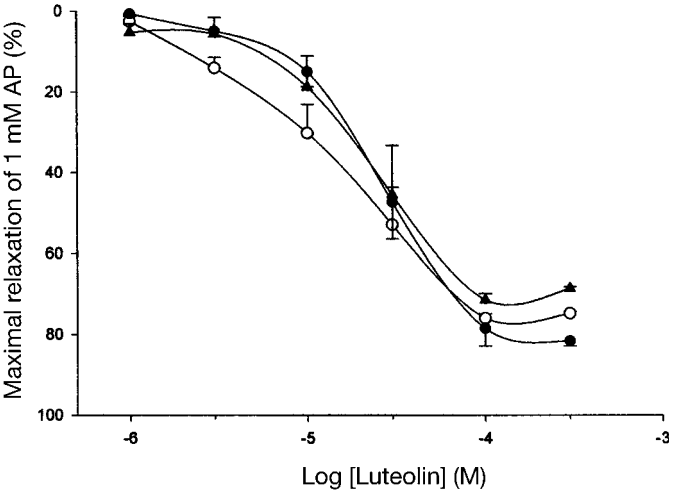

Fig. **2** The relaxant effects of luteolin on, histamine (○, 30 μM)-, carbachol (●, 0.2 μM)-, and KCl (▲, 30 mM)-induced precontractions in guinea pig trachealis. The relaxant effects do not include those of the vehicle. Each point represents the mean ± SEM of 6 – 7 experiments. AP: aminophylline.

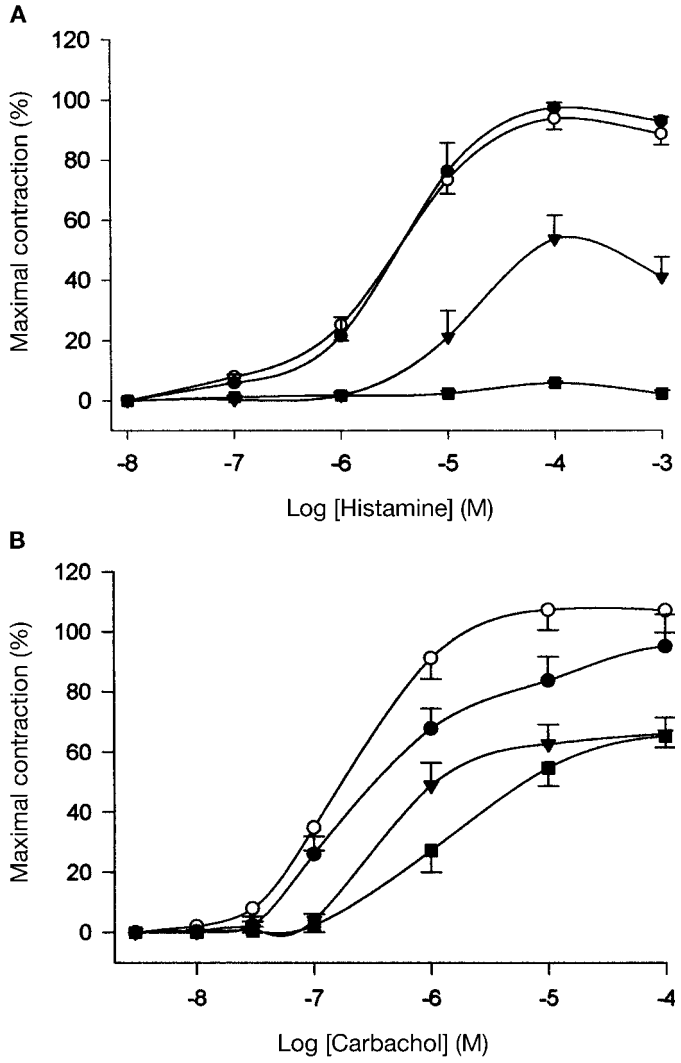

Fig. **3** The inhibitory effects of luteolin (○, vehicle; ●, 3 μM; ▼, 30 μM; ■, 100 μM) on cumulative histamine (**A**)- and carbachol (**B**)-induced contractions in guinea pig trachealis in normal Krebs solution. Each point represents the mean ± SEM of 6 experiments.

This suggests that no matter whether luteolin blocks the VDCCs or not, luteolin may have other relaxant action mechanism(s).

However, the removal of epithelium, and the presence of antagonist, such as propranolol (1 μM), 2′,5′-dideoxyadenosine (10 μM), methylene blue (25 μM), glibenclamide (10 μM), l-NNA (20 μM), or α-chymotrypsin (1 U/mL), did not affect the log concentration-relaxing response curves of cumulative luteolin to histamine (30 μM)-induced precontraction in normal Krebs solution (data not shown).

In contrast, luteolin (10 – 20 μM) shifted in parallel leftwards the log concentration-response curves of forskolin (Fig. **5A**) and nitroprusside (Fig. **5B**) to histamine (30 μM)-induced precontractions of the trachealis. Furthermore, at 20 μM it significantly increased the pD_2 values of forskolin, and nitroprusside (Table **1**). Luteolin at various concentrations (10 – 300 μM), concentration-dependently and significantly inhibited cAMP- and cGMP-PDE activities. The IC_{50} values of luteolin were 32.4 ± 7.0 (n = 4) and 34.6 ± 6.3 (n = 4) μM, respectively, which did not significantly differ from each other, though the inhibitory effects of luteolin at 100 and 300 μM on cGMP-PDE were more potent ($P < 0.05$) than on cAMP-PDE activity (Fig. **6**). The IC_{50} values of IBMX, a positive control, were estimated to be 5.5 ± 2.5 (n = 4) and 16.3 ± 7.3 (n = 4) μM, respectively, which also did not significantly differ from each other. In contrast to luteolin, IBMX at various concentrations (10 – 300 μM) selectively inhibited neither cAMP-, nor cGMP-PDE activity (Fig. **6**).

Discussion

The removal of epithelium did not affect the log concentration-relaxing response curve of cumulative luteolin to histamine (30 μM)-induced precontraction suggesting that the relaxant effect of luteolin is epithelium-independent. The log concentration-relaxing response curve of cumulative luteolin to histamine (30 μM)-induced precontraction was not affected by propranolol (1 μM), a non-selective β-adrenoceptor blocker, suggesting that its relaxant effect is not via the activation of β-adrenoceptor. 2′,5′-Dideoxyadenosine, an adenylate cyclase inhibitor [10], and methylene blue, a soluble guanylate cyclase inhibitor [11], also did not affect the log concentration-response curve of luteolin. This reveals that its relaxant effect is neither via the activation of adenylate cyclase nor via that of guanylate cyclase. Glibenclamide, an ATP-sensitive potassium channel blocker [12], also did not affect the log concentration-response curve of luteolin, suggesting that its relaxant effect is not via the opening of ATP-sensitive potassium channels. L-NNA (20 μM), a nitric oxide (NO) synthase inhibitor [13], did not affect the log concentration-response curve of luteolin, suggesting that its relaxant effect is unrelated to NO formation. α-Chymotrypsin (1 U/mL), a peptidase, also did not affect the log concentration-response curve of luteolin, suggesting that its relaxant effect is unrelated to the neuropeptides.

Luteolin (10 – 100 μM) concentration-dependently and non-competitively inhibited cumulative Ca^{2+}-induced contractions in the

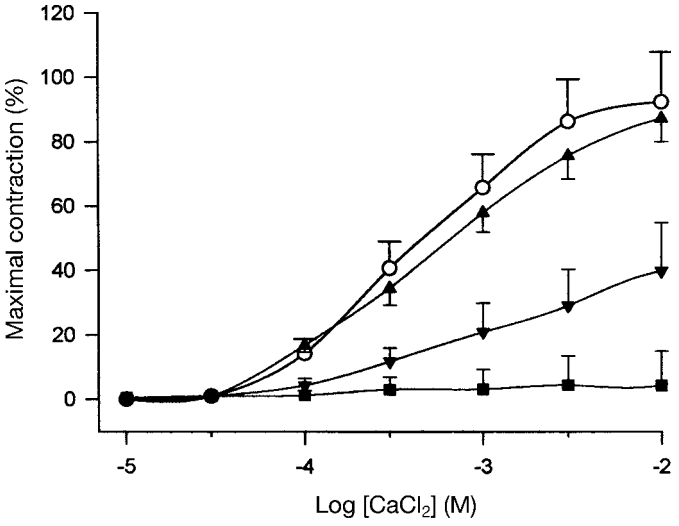

Fig. **4** The inhibitory effects of luteolin (○, vehicle; ▲, 10 μM; ▼, 30 μM, ■, 100 μM) on cumulative calcium-induced contractions in guinea pig trachealis depolarized by KCl 60 mM in Ca^{2+}-free medium. Each point represents the mean ± SEM of 6 experiments.

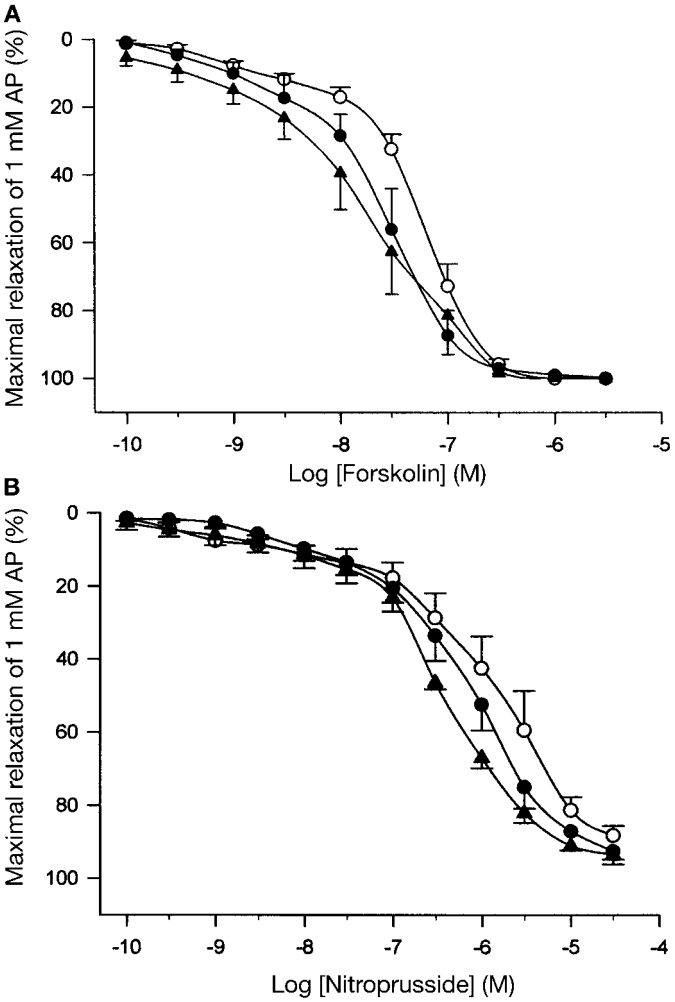

Fig. **5** The potentiating effects of luteolin (○, vehicle; ●, 10 μM; ▲, 20 μM) on the relaxant responses of cumulative forskolin (**A**) and nitroprusside (**B**) to the histamine (30 μM)-induced precontractions in the guinea pig trachealis. Each point represents the mean ± SEM of 6 experiments. AP: aminophylline.

depolarized (K^+, 60 mM) trachealis. Therefore, it may inhibit Ca^{2+} influx via VDCCs opened by 60 mM KCl. For example, nifedipine, a selective VDCCs blocker [14], at concentrations below 1 μM, also inhibits those contractions in a non-competitive manner. Nifedipine at 1 μM can completely inhibit those contractions. In the present study, nifedipine (10 μM) relaxed the histamine-induced precontraction in normal Krebs solution by only 14.4%.

Table **1** The pD_2 values of forskolin and nitroprusside against histamine (30 μM)-induced precontractions in the absence and presence of luteolin

	Forskolin	*Nitroprusside*
Luteolin		
Vehicle	7.33 ± 0.08 (6)	5.85 ± 0.18 (6)
10 μM	7.57 ± 0.19 (6)	6.20 ± 0.18 (6)
20 μM	7.81 ± 0.23 (6)*	6.39 ± 0.07 (6)*

Values are presented as means ± SEM (n); n is the number of experiments.
* $P < 0.05$ when compared with their corresponding values of vehicle.

The nifedipine-remaining tension was further (98.8%) relaxed by luteolin at 100 μM suggesting that no matter whether it blocked the VDCCs or not, it may have other mechanisms of relaxant action. Luteolin concentration-dependently relaxed the histamine (30 μM)-, carbachol (0.2 μM)-, and KCl (30 mM)-induced precontractions. The -logIC_{50} values against these three contractile agents did not significantly differ from each other, suggesting that the ability of luteolin to inhibit calcium influx from the extracellular space may be similar. It has been reported that tonic, but not phasic, contraction is maintained by calcium influx [15]. However, The pD_2' value of luteolin against cumulative histamine-induced contractions was significantly greater than that against carbachol. This suggests that the antispasmodic effects of luteolin against histamine are more potent than those against carbachol. Although the exact reason is not clear, it has been established that carbachol may activate muscarinic M_2 receptors, a major (80%) receptor population, via a pertussis-toxin-sensitive G protein, G_i, to inhibit adenylate cyclase activity [16] and cause an indirect contraction which attenuates the relaxant effects of luteolin. Luteolin (10–20 μM) shifted in parallel leftward both the log concentration-response curves of forskolin, an activator of adenylate cyclase [17], and those of nitroprusside, an activator of guanylate cyclase [18], to histamine (30 μM)-induced precontractions of the trachealis, and significantly increased the pD_2 values of forskolin and nitroprusside (Table **1**). This reveals that the relaxant effect of luteolin may be via the inhibitions of cAMP- and cGMP-PDE, and the subsequent increase of these two cyclic nucleotides. The increased cAMP or cGMP level subsequently activates cAMP- or cGMP-dependent protein kinase which may phosphorylate and inhibit myosin light-chain kinase, thus inhibiting contraction [19]. The precise mechanism by which relaxation is produced by this second-messenger pathway is not known, but it may result from decreased intracellular Ca^{2+} ($[Ca^{2+}]_i$). The decrease of $[Ca^{2+}]_i$ may be due to reduced influx of Ca^{2+}, enhanced Ca^{2+} uptake into the sarcoplasmic reticula, or enhanced Ca^{2+} extrusion through the cell membrane [19]. In this present study, indeed, luteolin or IBMX, a posi-

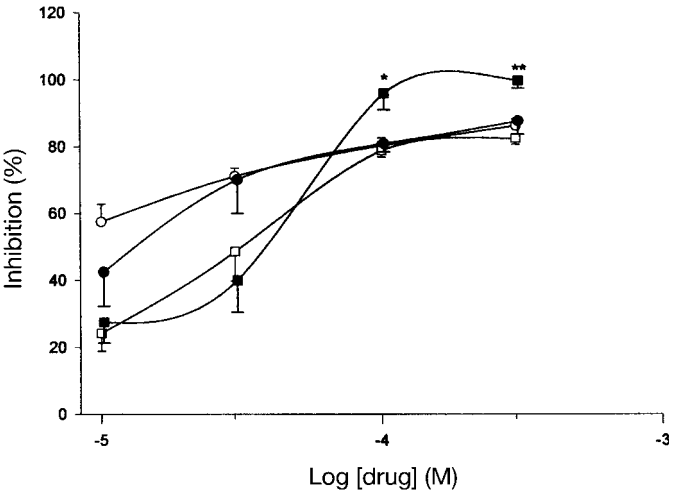

Fig. **6** The inhibitory effects of luteolin (□, ■) and IBMX (○, ●), a positive control, on cAMP- (○, □) and cGMP-PDE (● , ■) activities. The inhibitory effects do not include those of their vehicle. Each point represents the mean ± SEM of at least 4 experiments. * $P < 0.05$, ** $P < 0.01$ when compared with corresponding value on cAMP-PDE activity.

tive control, at various concentrations (10 – 300 μM), significantly inhibited cAMP- and cGMP-PDE activities. The -log IC_{50} values of luteolin were 4.49 and 4.46, respectively. These -log IC_{50} values were similar to those of luteolin on relaxant effects in the trachealis, precontracted by histamine, carbachol or KCl (see Results). It has been reported that there is a strong positive correlation between the IC_{50} values of IBMX either on cAMP- [20] or on cGMP-PDE activity [21] and its EC_{50} values for the tracheal muscle relaxation. Therefore, we cannot exclude the possibility that the relaxant effects of luteolin may be due to its inhibitory effect on both enzyme activities and its subsequent reducing effect on $[Ca^{2+}]_i$ of the trachealis.

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