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Relaxation of Isolated Guinea Pig Trachea by Genistein via Inhibition of Phosphodiesterase

Abstract

We investigated the mechanisms of the relaxant action of genistein, an isoflavone, phytoestrogen and non-specific protein tyrosine kinase inhibitor. Changes in tension of guinea pig tracheal segments were isometrically recorded on a polygraph. Genistein concentration-dependently relaxed histamine (30 μM)-, carbachol (0.2 μM)-, KCl (30 mM)- and leukotriene D_4 (10 nM)-induced precontractions and inhibited cumulative histamine- and carbachol-induced contractions in a non-competitive manner. Genistein also concentration-dependently and non-competitively inhibited the cumulative, Ca^{2+}-induced contractions in the depolarized (K^+, 60 mM) trachealis. The remaining nifedipine (10 μM)-induced tension of the histamine (30 μM)-induced precontraction was further relaxed by genistein, suggesting that regardless of whether voltage-dependent calcium channels are blocked genistein may have other mechanisms of relaxant action. These other mechanisms of the relaxant effect of genistein appeared to be epithelium-independent and were not affected 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), suggesting that the mechanisms are unrelated to activation of the β-adrenoceptor, of adenylate cyclase, of guanylate cyclase, of adenosine triphosphate-sensitive potassium channel opening, of nitric oxide formation or of neuropeptide release, respectively. However, genistein (17.5 – 35 μM) produced parallel, leftward shifts in the concentration-response curves of forskolin and nitroprusside and significantly increased the pD_2 values of these two agonists. Both genistein and 3-isobutyl-1-methylxanthine at various concentrations (10 – 300 μM) concentration-dependently and significantly inhibited cAMP- and cGMP-phosphodiesterase (PDE) activities of the trachealis. The $-\log IC_{50}$ values of genistein were estimated to be 4.28 and 4.17, respectively. The above results reveal that the mechanisms of the relaxant action of genistein may be due to its non-selective inhibition of both PDE activities.

Key words
Genistein · isoflavone · phosphodiesterase inhibitor · guinea pig tracheal relaxation · cyclic AMP-phosphodiesterase · cyclic GMP-phosphodiesterase

Abbreviations

IBMX:	3-ixobutyl-1-methylxanthine
VDCCs:	voltage-dependent calcium channels
cAMP:	adenosine 3′,5′-cyclic monophosphate
cGMP:	guanosine 3′,5′-cyclic monophosphate
ATP:	adenosine triphosphate
PDE:	phosphodiesterase
LTD_4:	leukotriene D_4
L-NNA:	N^{ω}-nitro-L-arginine
DMSO:	dimethyl sulfoxide
EGTA:	*N,N,N′,N′*-tetraacetic acid
ANOVA:	analysis of variance

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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], [2], [3]. Genistein, an isoflavone and phytoestrogen found in high concentrations in soybean milk and tofu, has been reported to non-specifically inhibit protein tyrosine kinases [4]. Genistein, therefore, facilitates canine bronchial smooth muscle relaxation [5], [6] and attenuates antigen-induced guinea pig airway contractions [7]. In 1997, Stringfield and Morimoto reported that genistein can modulate adenosine 3′,5′-cyclic monophosphate (cAMP) levels in the HT4.7 neural cell line [8]. In the absence of phosphodiesterase (PDE) inhibitors, genistein causes increased intracellular cAMP levels. However, when PDE inhibitors are included, cAMP levels decrease as a function of the concentration of genistein. This suggests that genistein inhibits both cAMP synthesis and degradation. Cyclic AMP is mainly synthesized from adenosine triphosphate (ATP) via activation of adenylate cyclase and is degraded by PDE. Therefore, some investigators have focused on regulation of PDE by tyrosine phosphorylation either indirectly [9] or through direct interaction with a protein tyrosine kinase [10]. In 1999, Nichols and Morimoto reported that HT4.7 PDE activity can be regulated by genistein through a tyrosine kinase-independent mechanism [11]. It seems contradictory that genistein inhibits both cAMP synthesis and degradation. Therefore, we were interested in the mechanism of tracheal relaxation by genistein.

Materials and Methods

Reagents and drugs

Genistein (Fig. **1**, with a purity of > 98 %), 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, guanosine 3′,5′-cyclic monophosphate (cGMP), calmodulin, leukotriene D_4 (LTD_4), Dowex resin and *Crotalus atrox* snake venom were purchased from Sigma Chemical (St. Louis, MO, USA). [^3H]cAMP and [^3H]cGMP were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). 3-Isobutyl-1-methylxanthine (IBMX) was purchased from Aldrich Chemical (Milwaukee, WI, USA). All other reagents, including KCl, were of analytical grade. Glibenclamide was dissolved in dimethyl sulfoxide (DMSO). Genistein, IBMX, forskolin, indomethacin, and 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.

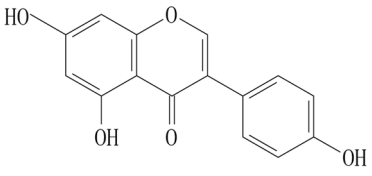

Fig. **1** Chemical structure of genistein (mol wt 270.23).

Guinea pig trachea

Using a protocol approved by the Animal Care and Use Committee of the Taipei Medical University, male Hartley guinea pigs (National Laboratory Animal Center, Taipei, Taiwan) weighing 250 – 450 g were sacrificed by cervical dislocation, and their 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, a segment was tied at one end to a holder via silk sutures, 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 its other end to a force displacement transducer (Grass FT03, Grass; Quincy, MA, USA) for the isometric recording of tension changes on a polygraph (Gould RS3200, Gould; Valley View, OH, USA). 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 $CaCl_2$, with the 60 mM NaCl being 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), KCl (30 mM) or LTD_4 (10 nM), genistein (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 maximal tissue relaxation. The relaxant potencies of genistein were expressed as $-\log IC_{50}$ values. To determine the antagonistic effects of genistein 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 contraction of a trachea without incubation of drugs or their vehicles was set to 100 %. After the tissues were preincubated with genistein or its vehicle for 15 min, these two contractile agonists were also cumulatively added to the normal Krebs solution. The antagonistic potencies of genistein were expressed as pD_2' values when the antagonistic effect on these cumulative concentration-response curves occurred in a non-competitive manner. In the case of isotonic high-K^+ (60 mM)-depolarized tracheal preparations, the normal Krebs solution was replaced after equilibration by a Ca^{2+}-free Krebs solution without EGTA, and the segments were washed with the Ca^{2+}-free solution with 2 mM EGTA after the tracheal contraction reached constancy, followed by incubation for 5 min. After repeating the above procedure until no contraction was observed, Ca^{2+} (0.01 – 10 mM) was cumulatively 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 genistein on cumulative Ca^{2+}-induced contractions in isotonic high-K^+ (60 mM)-depolarized tracheas were expressed as $-\log IC_{50}$ values. The tracheal relaxant effects of cumulative genistein (10 – 100 μM) on the histamine (30 μM)-induced precontraction were allowed to reach a steady state at each concentration. All antagonists, including propranolol, glibenclamide, 2′,5′-dideoxadenosine, methylene blue, L-NNA, α-chymotrypsin and their respective vehi-

cles were individually incubated after the precontraction reached a steady state for 15 min prior to the first addition of genistein. Similarly, nifedipine (10 μM) was added after the histamine (30 μM)-induced precontraction reached a steady state, at 15 min prior to the addition of genistein (100 μM) or its vehicle. At the end of the experiment without washout, 1 mM aminophylline was added to standardize the maximal tissue relaxation (100%). To observe the effect of genistein on the relaxant response of forskolin or nitroprusside to the histamine (30 μM)-induced precontraction, genistein (17.5 – 35 μM) was incubated for 15 min prior to the addition of histamine. Forskolin or nitroprusside was cumulatively added to the organ bath after the sustained contraction had reached constancy. At the end of the experiment, aminophylline (1 mM) was also added to maximally relax the tissue. To investigate the effects of the epithelium on the relaxant response of genistein to the histamine (30 μM)-induced precontraction, some tracheal segments were denuded by rubbing with a moistened cotton-tipped applicator while the intact epithelium was retained in other segments. 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 (H&E) to determine the effectiveness of the epithelium removal procedure [12].

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. [13]. 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 genistein (10 – 300 μM) or IBMX (10 – 300 μM) as the 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 was 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 genistein on these cumulative concentration-response curves are expressed as pD_2' values, and the relaxing effects of forskolin and nitroprusside against histamine (30 μM)-induced precontractions are expressed as pD_2 values, according to the method described by Ariëns and van Rossum [14]. The pD_2 values are the negative logarithm of the molar concentrations of forskolin and nitroprusside at which the 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 genistein and x is the ratio between the maximal effect of the agonist in the absence and presence of genistein. The $-\log IC_{50}$ value was considered to be equal to the negative logarithm of the molar concentrations of genistein at which a half-inhibitory effect on agonist-induced precontractions, the 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 the mean ± SEM. Differences among these values were statistically calculated by one-way analysis of variance (ANOVA), then determined by Dunnett's test. The difference between the two values, however, was determined using Student's unpaired *t*-test. Differences were considered statistically significant if the *p* value was < 0.05.

Results

Genistein concentration-dependently and almost completely relaxed the histamine (30 μM), carbachol (0.2 μM), KCl (30 mM) and LTD_4 (10 nM)-induced precontractions (Fig. **2**). Their $-\log IC_{50}$ values were 4.55 ± 0.06 (n = 7), 4.41 ± 0.04 (n = 7), 4.40 ± 0.05 (n = 6) and 4.57 ± 0.07 (n = 6), respectively. They are not statistically different. Genistein (35 – 100 μM) concentration-dependently inhibited the log concentration-response curves of cumulative histamine in a non-competitive manner (Fig. **3A**). The same was true for genistein (45 – 200 μM) in the curves for carbachol (Fig. **3B**). The pD_2' values were 4.04 ± 0.15 (n = 5) and 3.61 ± 0.01 (n = 6), respectively, which significantly differ from each other. This suggests that the antispasmodic effects of genistein against histamine are more potent than those against carbachol. In addition, these pD_2' values were significantly less than the $-\log IC_{50}$ values of genistein against histamine- and carbachol-in-

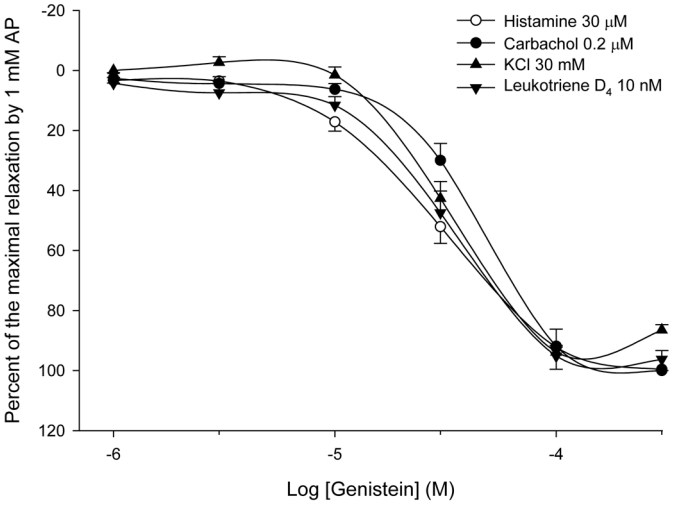

Fig. **2** The relaxant effects of genistein on histamine-, carbachol-, KCl-, and leukotriene D_4-induced precontractions in guinea pig trachealis. The relaxant effects do not include those of the respective vehicle. Each point represents the mean ± SEM of 6 or 7 experiments. AP = aminophylline.

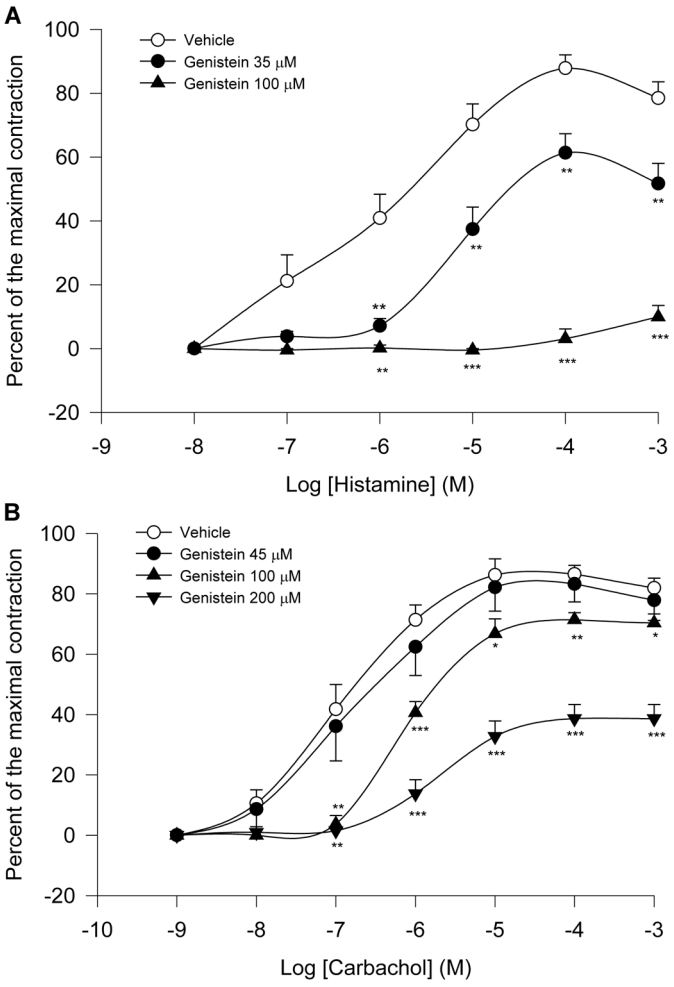

Fig. **3** Inhibitory effects of genistein on cumulative histamine (**A**)- and carbachol (**B**)-induced contractions in guinea pig trachealis in normal Krebs solution. Each point represents the mean ± SEM of 5 or 6 experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared with the corresponding value of the vehicle.

duced precontractions, respectively. In isotonic Ca^{2+}-free high-K^+ (60 mM)-depolarized tracheas, genistein (25 – 100 μM) also concentration-dependently inhibited the log concentration-response curves of cumulative Ca^{2+} (0.01 – 10 mM) in a non-competitive manner (Fig. **4**). The -logIC_{50} value was 4.47 ± 0.06 (n = 4), which did not significantly differ from that against the KCl-induced precontraction. Nifedipine (1 μM), a selective voltage-dependent calcium channel (VDCC) blocker [15], has been reported to completely inhibit calcium-induced contractions in the deporalized trachealis [16]. In this present experiment, nifedipine (10 μM), however, only relaxed 10.4% of the histamine (30 μM)-induced precontraction in the trachealis. The remaining nifedipine (10 μM)-induced tension of the trachealis was further relaxed by genistein (100 μM) to approximately 90% and then completely relaxed by the addition of 1 mM aminophylline (Fig. **5**). This suggests that regardless of whether genistein blocks the VDCCs, it may have other mechanism(s) of relaxant action. However, neither removal of the epithelium nor the presence of an 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), affected the log concentration-relaxing response curves of cumulative genistein on the histamine (30 μM)-induced precontraction in normal Krebs solution (data not shown).

In contrast, genistein (17.5 – 35 μM) shifted the log concentration-response curves of forskolin (Fig. **6A**) and nitroprusside (Fig. **6B**) to histamine (30 μM)-induced precontractions of the trachealis to the left in a parallel manner and significantly increased the pD_2 values of forskolin and nitroprusside (Table **1**). This reveals that the relaxant effect of genistein may occur via the inhibition of cAMP- and cGMP-PDE and subsequent increases in these two cyclic nucleotides. Indeed, in the present study, genistein at various concentrations (10 – 300 μM), concentration-dependently and significantly inhibited cAMP- and cGMP-PDE activities. The -logIC_{50} values of genistein were estimated

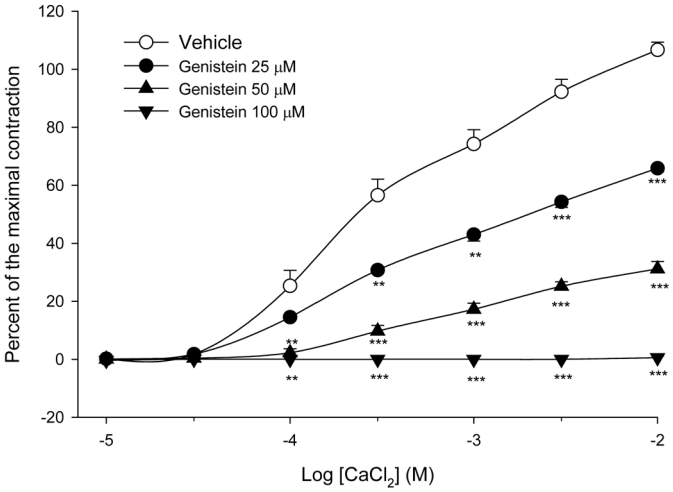

Fig. **4** Inhibitory effects of genistein on cumulative calcium-induced contractions in guinea pig trachealis depolarized by 60 mM KCl in Ca^{2+}-free medium. Each point represents the mean ± SEM of 4 experiments. ** $P < 0.01$, *** $P < 0.001$ when compared with the corresponding value of the vehicle.

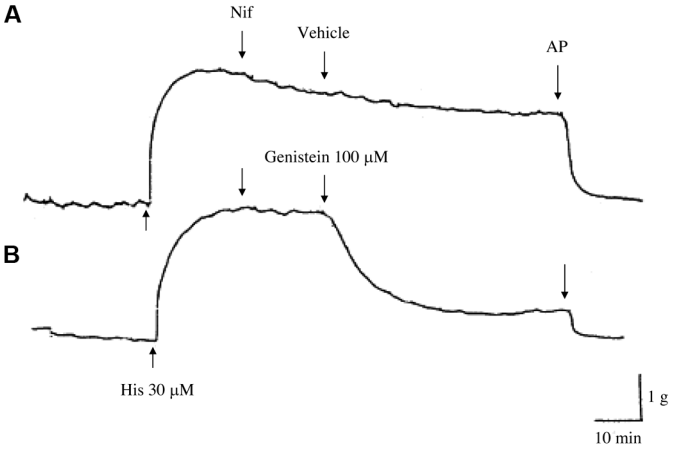

Fig. **5** Tracing of the relaxant effect of genistein on the histamine (30 μM)-induced precontraction in guinea pig trachealis in normal Krebs solution. Genistein (100 μM), compared to its vehicle (**A**), further relaxed the remaining nifedipine (Nif, 10 μM)-induced tension (**B**). At the end of the experiment, aminophylline (AP, 1 mM) was added to completely relax the trachealis.

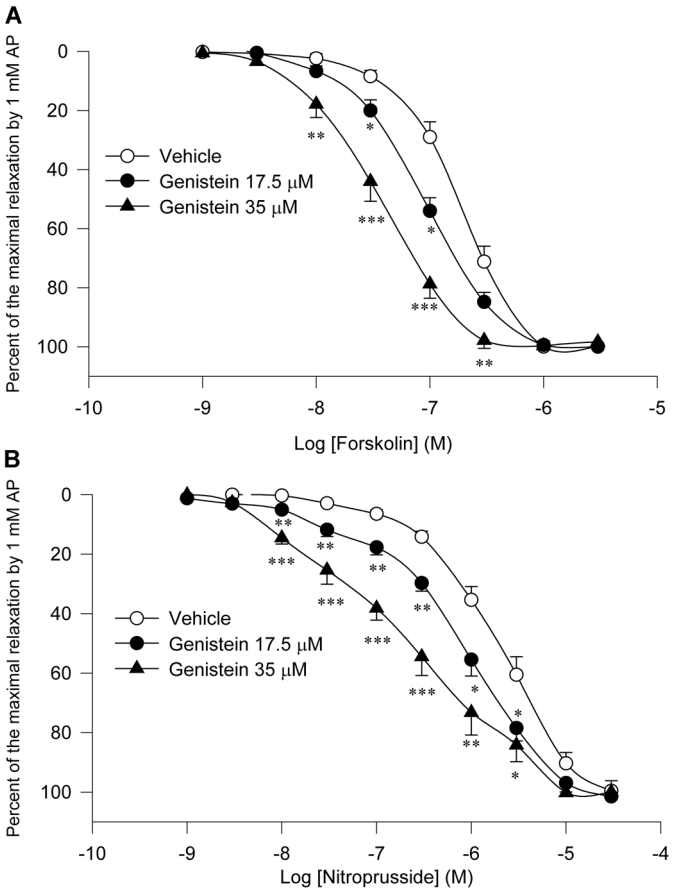

Fig. **6** Potentiating effects of genistein on the relaxant responses of cumulative forskolin (**A**) and nitroprusside (**B**) to the histamine (30 μM)-induced precontraction in guinea pig trachealis. Each point represents the mean ± SEM of 5 or 6 experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when the compared with corresponding value of the vehicle. AP = aminophylline.

to be 4.28 ± 0.06 (n = 8) and 4.17 ± 0.07 (n = 4), respectively, which do not significantly differ from each other. Therefore, genistein appeared to have non-selective inhibitory effects on both PDE activities, although the inhibitory effect of genistein at 100 μM on cAMP-PDE activity was statistically more potent ($p <$ 0.01) than that on cGMP-PDE activity (Fig. **7**). The -log IC_{50} values of IBMX, the positive control, were estimated to be 5.61 ± 0.36 (n = 4) and 4.84 ± 0.34 (n = 4), respectively, which also did not significantly differ from each other. There was also no selectivity for either PDE activity observed when IBMX was used (Fig. **7**).

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

	Forskolin	*Nitroprusside*
Genistein		
Vehicle	6.81 ± 0.06 (6)	5.67 ± 0.10 (6)
17.5 μM	7.06 ± 0.06 (5)*	6.16 ± 0.07 (5)*
35 μM	7.45 ± 0.09 (5)*	6.65 ± 0.14 (5)*

Values are presented as the mean ± SEM (n); n is the number of experiments.
* $P < 0.05$, when compared to the corresponding value of the vehicle.

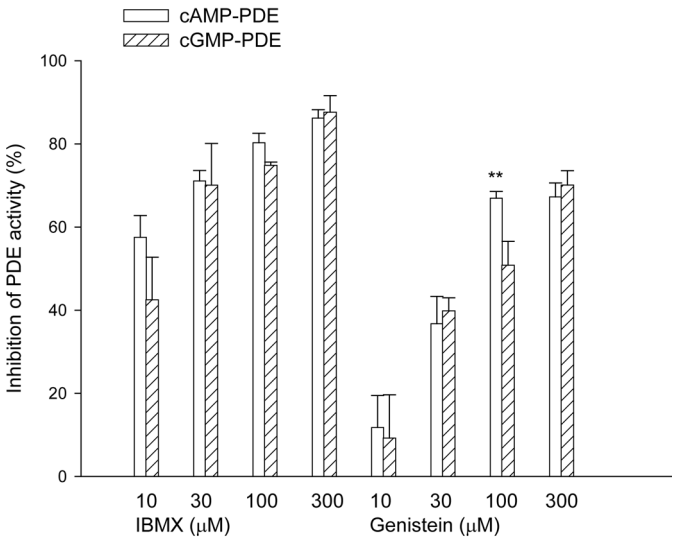

Fig. **7** Inhibitory effects of genistein and IBMX, a positive control, on cAMP- and cGMP-PDE activities. The inhibitory effects do not include those of the respective vehicle. Each column represents the mean ± SEM of 4–8 experiments. ** $P < 0.01$ when compared with the corresponding column of cGMP-PDE activity.

Discussion

Removal of the epithelium did not affect the log concentration-relaxing response curve of cumulative genistein for histamine (30 μM)-induced precontraction suggesting that the relaxant effect of genistein is epithelium-independent. The log concentration-relaxing response curve of cumulative genistein to the 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 activation of the β-adrenoceptor. Neither 2′,5′-dideoxyadenosine, an adenylate cyclase inhibitor [17], nor methylene blue, a soluble guanylate cyclase inhibitor [18], affected the log concentration-response curve of genistein, although baseline tensions were mildly relaxed during incubation with these two inhibitors (data not shown). However, relaxation of the baseline tension was unrelated to inhibition of adenylate cyclase or soluble guanylate cyclase by these two inhibitors. Therefore, this reveals that the relaxant effect of genistein occurs via activation of neither adenylate cyclase nor guanylate cyclase. Glibenclamide, an ATP-sensitive potassium channel blocker [19], also did not affect the log concentration-response curve of genistein, 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 [20], did not affect the log concentration-response curve of genistein, 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 genistein, suggesting that its relaxant effect is unrelated to the neuropeptides.

Genistein (25–100 μM) concentration-dependently and non-competitively inhibited cumulative Ca^{2+}-induced contractions in the depolarized (K^+, 60 mM) trachealis. Therefore, it may inhibit Ca^{2+} influx via VDCCs opened by 60 mM KCl. For example, nifedipine, a selective VDCC blocker, at concentrations below 1 μM, also inhibited those contractions in a non-competitive

manner. Nifedipine at 1 μM can completely inhibit such contractions [16]. In the present study, nifedipine (10 μM) only partially (10.4%) relaxed the histamine-induced precontraction in normal Krebs solution. The remaining nifedipine-induced tension was further (90%) relaxed by 100 μM genistein, suggesting that regardless of whether it blocks the VDCCs or not, it may have other mechanisms of relaxant action. Genistein concentration-dependently relaxed histamine (30 μM), carbachol (0.2 μM), KCl (30 mM) and LTD_4 (10 nM)-induced precontractions. The $-\log IC_{50}$ values against these four agonists did not significantly differ from each other. However, the $-\log IC_{50}$ values of genistein against histamine- and carbachol-induced precontractions were significantly greater than the pD_2' values of genistein against cumulative histamine- and carbachol-induced contractions, respectively. It has been reported that the phasic response to agonists involves the release of stored Ca^{2+}, and the tonic response is due to an increased influx of Ca^{2+} across the membrane [21], [22]. This suggests that genistein more selectively inhibits calcium influx than calcium release from calcium stores. In addition, the pD_2' value of genistein against cumulative histamine-induced contractions was significantly greater than that against carbachol. This suggests that the antispasmodic effects of genistein 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, which inhibits adenylate cyclase activity [23] and causes an indirect contraction thus attenuating the relaxant effects of genistein. Although the highest concentrations (100 and 200 μM) of genistein used with histamine and carbachol, respectively, are impossible to reach in the blood due to their cytotoxicity in *in vivo* studies, the above results of the *in vitro* study clearly suggest that genistein is a non-specific antispasmodic [24]. Genistein (17.5 – 35 μM) shifted both the log concentration-response curves of forskolin, an activator of adenylate cyclase [25], and that of nitroprusside, an activator of guanylate cyclase [26], to histamine (30 μM)-induced precontractions of the trachealis to the left in a parallel manner and significantly increased the pD_2 values of forskolin and nitroprusside (Table **1**). This reveals that the relaxant effect of genistein may occur via inhibition of cAMP- and cGMP-PDE, and the subsequent increase in 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 contractions [27]. 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 in $[Ca^{2+}]_i$ may be due to a reduced influx of Ca^{2+}, enhanced Ca^{2+} uptake into the sarcoplasmic reticula or enhanced Ca^{2+} extrusion through the cell membrane [27]. In the present study, genistein or IBMX, a positive control, at various concentrations (10 ~ 300 μM), significantly inhibited cAMP- and cGMP-PDE activities. Therefore, we can not exclude the possibility that the relaxant effects of genistein 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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