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Relaxation of isolated guinea-pig trachea by apigenin, a constituent of celery, *via* inhibition of phosphodiesterase

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Apigenin (PubChem CID: 5280443)
Aminophylline (PubChem CID: 9433)
Adenosine 3′,5′ cyclic monophosphate (PubChem CID: 7059571)
Guanosine 3′,5′ cyclic monophosphate (PubChem CID: 24316)
Histamine (PubChem CID: 774)
3-isobutyl-1-methylxanthine (PubChem CID: 3758)
Nifedipine (PubChem CID: 4485)
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ABSTRACT

Apigenin, was reported to have vasodilatory effects by inhibiting Ca^{2+} influx through both voltage- and receptor-operated calcium channels, but not by inhibiting cAMP- or cGMP-phosphodiesterases (PDEs) in rat thoracic aorta. However, apigenin was reported to inhibit PDE1, 2 and 3 in guinea-pig lung and heart. The aim of this study was to clarify that guinea-pig tracheal relaxation by apigenin whether *via* PDE inhibition.

We isometrically recorded the tension of isolated guinea-pig tracheal segments on a polygraph. Antagonistic effects of apigenin against cumulative contractile agents or Ca^{2+} induced contractions of the trachealis in normal or isotonic high-K^+, Ca^{2+}-free Krebs solution, respectively. Effects of apigenin (15 and 30 μM) on the cumulative forskolin- and nitroprusside-induced relaxations to histamine (30 μM)-induced precontraction were performed. The inhibitory effects of 30–300 μM apigenin and 3-isobutyl-1-methylxanthine (IBMX, positive control) on the cAMP- and cGMP-PDEs were determined.

Apigenin concentration-dependently but non-competitively inhibited cumulative histamine-, carbachol- or Ca^{2+}-induced contractions in normal or in the depolarized (K^+, 60 mM) trachealis, suggesting that Ca^{2+} influx through voltage-dependent calcium channels is inhibited. However, apigenin (15–30 μM) parallel leftward shifted the concentration-response curves of forskolin and nitroprusside, and significantly increased the pD_2 values of these two cyclase activators. Both apigenin and IBMX, a reference drug, concentration (10–300 μM)-dependently and significantly, but non-selectively inhibited the activities of cAMP- and cGMP-PDEs in the trachealis. In conclusion, the relaxant effect of apigenin may be due to inhibition of both enzyme activities and reduction of intracellular Ca^{2+} by inhibiting Ca^{2+} influx in the trachealis.

1. Introduction

Phosphodiesterases (PDEs) are classified according to their primary protein and complementary DNA sequences, co-factors, substrate specificities, and pharmacological roles. It is now known that PDEs comprise at least 11 distinct enzyme families that hydrolyze adenosine 3′,5′ cyclic monophosphate (cAMP) and/or guanosine 3′,5′ cyclic monophosphate (cGMP) (Lee et al., 2002). Thus PDEs are roughly classified to cAMP- and cGMP-PDEs. cAMP and cGMP are synthesized from ATP and GTP, when adenylate cyclase and guanylate cyclase are activated, respectively. If cAMP- or cGMP-PDEs are inhibited, the intracellular content of cAMP or cGMP is enhanced and subsequently activates cAMP- or cGMP-dependent protein kinase which may phosphorylate and inhibit myosin light-chain kinase, thus inhibiting contractions (Westfall et al., 1998).

Flavonoids at least divide into five classes (flavones, flavonols, flavanones, isoflavones, and chalcones). We previously reported that flavones, similar to isoflavones, are the most potent among these classes in guinea-pig tracheal relaxation (Ko et al., 2003). Apigenin, a member of flavones and also a constituent of *Apium graveolens* L. (Apiaceae), was reported to have vasodilatory effects by inhibiting Ca^{2+} influx through both voltage- and receptor-operated calcium channels, but not by enhancing cAMP or cGMP in rat thoracic aorta (Ko et al., 1991; Ajay et al., 2003). Their results suggest that the vasodilating effects of apigenin were unrelated to inhibition of PDEs in rat thoracic aorta. However, we reported that apigenin inhibited PDE1 (calcium/calmodulin-dependent), PDE2 (cGMP-stimulated) and PDE3 (cGMP-inhibited) of guinea-pig lung and heart with the IC_{50} values of 25.4, 16.7 and 10.5 μM, respectively (Ko et al., 2004). The inconsistency between our result and theirs may be due to tissue difference. The aim of this study was to clarify guinea-pig tracheal relaxant effects of apigenin whether *via* PDE inhibition.

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2. Materials and methods

2.1. Reagents and drugs

Apigenin (4′,5,7-trihydroxyflavone, molecular weight 270.24), aminophylline, calmodulin, cAMP, carbachol, α-chymotrypsin, cGMP, *Crotalus atrox* snake venom, 2′,5′-dideoxyadenosine, dl-dithiothreitol, Dowex resin, forskolin, glibenclamide, histamine, indomethacin, 3-isobutyl-1-methylxanthine (IBMX), methylene blue, nifedipine, N^{ω}-nitro-L-arginine (L-NNA), nitroprusside, propranolol, sodium ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and Tris-HCl were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). [^3H]cAMP and [^3H]cGMP were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents, including KCl, were of analytical grade. Glibenclamide was dissolved in dimethyl sulfoxide (DMSO). Apigenin, 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.

2.2. Guinea-pig trachea

Male guinea-pigs (Hartley) weighing 250–450 g were purchased from the Animal Center of the Ministry of Science and Technology, Taipei, Taiwan, and housed in ordinary cages at 22 ± 1 °C with a humidity of 50–60% under a constant 12/12-h light/dark cycle and provided with food and water *ad libitum*. Under a protocol approved by the Animal Care and Use Committee of Taipei Medical University, these guinea-pigs were anesthetized by an intraperitoneal (i.p.) injection of pentobarbital (50 mg/kg) 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) 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 calcium, but with 60 mM KCl instead of 60 mM NaCl. 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.

2.3. Antagonistic effects against contractile agents

Histamine, carbachol or KCl was cumulatively added to the organ bath containing normal Krebs solution and repeated until the contraction reached constancy after washout. The maximal contraction was set as 100%. After the tissues were preincubated with apigenin or its vehicle for 15 min, these three contractile agents were also cumulatively added to the organ bath. Then, log concentration-response curves were constructed. The antagonistic potencies of apigenin were expressed as pD_2' values, when the antagonistic effects were in a non-competitive manner.

2.4. Isotonic high-K^+-depolarized trachealis

After equilibration, the normal Krebs solution was replaced by the isotonic high-K^+, Ca^{2+}-free Krebs solution with 2 mM EGTA until no contraction was elicited, then it was replaced by the solution without EGTA at the last. Subsequently, Ca^{2+} (0.01–30 mM) were cumulatively added into the isotonic high-K^+, -Ca^{2+}-free Krebs solution and contractions were elicited in the depolarized trachealis. The maximal contractile response elicited by Ca^{2+} was taken as 100%, and the log concentration-response curve was constructed. The inhibitory effect of apigenin preincubated for 15 min on cumulative Ca^{2+}-induced contractions in isotonic high-K^+ (60 mM)-depolarized tracheas were expressed as a pD_2' value, when the inhibitory effect was in a non-competitive manner. Nifedipine (1 μM) was used as a positive control.

2.5. Effects of antagonists on the apigenin-induced relaxation

All antagonists, including propranolol, glibenclamide, 2′,5′-dideoxyadenosine, methylene blue, L-NNA, α-chymotrypsin, and their respective vehicles were individually incubated after the histamine (30 μM)-precontraction reached a steady state for 15 min prior to the first addition of apigenin. Then apigenin (1–300 μM) was cumulatively added to the organ bath, and log concentration-response curves were constructed.

2.6. Effects of apigenin on the forskolin- and nitroprusside-induced relaxations

Apigenin (15 and 30 μM) was incubated for 15 min prior to the addition of histamine (30 μM) in the trachealis. The influence of apigenin on the relaxant response of forskolin or nitroprusside to the histamine-induced precontraction was determined. Forskolin or nitroprusside was cumulatively added to the organ bath after the sustained contraction by histamine had reached constancy. At the end of the experiment without washout, aminophylline (1 mM) was added to standardize the maximal tissue relaxation (100%).

2.7. Effect of epithelium removal on the apigenin-induced relaxation

Some tracheal segments were denuded by rubbing with a moistened cotton-tipped applicator, while the intact epithelium was retained in other segments, to investigate the influences of epithelium on the relaxant response of cumulative apigenin preincubated for 15 min prior to the first addition to the histamine (30 μM)-induced precontraction. 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 (Holroyde, 1986).

2.8. Phosphodiesterase activity

According to the previously reported method (Ko et al., 2002), cAMP- and cGMP-PDE activities in homogenate of trachealis were measured. Shortly, 25 μl upper layer of homogenate after centrifugation was 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. The reaction mixture contained various concentrations of apigenin (10–300 μM) or IBMX (10–300 μM) as the positive control to test enzyme inhibition.

2.9. Statistical analysis

According to the previous method (Ariens and van Rosssum, 1957), antagonistic potencies of apigenin on these log concentration-response curves in a non-competitive manner are expressed as pD_2' values, where $pD_2' = pD_x' + \log(x-1)$, pD_x' is negative logarithm of molar concentration of apigenin and x is the ratio between maximal effect of contractile agent in the absence and presence of apigenin. Relaxing potencies of forskolin and nitroprusside against histamine (30 μM)-induced precontractions are expressed as pD_2 values. The pD_2 values