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Mechanisms of Relaxant Action of 3-*O*-Methylquercetin In Isolated Trachea (1/3)

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Abstract We investigated the mechanisms of action of 3-methylquercetin (3-MQ), isolated from *Rhamnus nakaharai* (Hayata) Hayata (Rhamnaceae) which is used as a folk medicine for treating constipation, inflammation, tumors and asthma in Taiwan. The tension changes of tracheal segments were isometrically recorded on a polygraph. 3-MQ 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. 3-MQ 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 precontraction was further relaxed by 3-MQ, suggesting that no matter whether VDCCs were blocked or not, 3-MQ may have other mechanisms of relaxant action. The relaxant effect of 3-MQ 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, 3-MQ (7.5-15 μM) and IBMX (3-6 μM), a positive control, produced parallel and leftward shifts of the concentration-response curve of forskoline (0.01-3 μM) or nitroprusside (0.01-30 μM). 3-MQ 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 3-MQ were estimated to be 13.8 and 14.3 μM, respectively. The inhibitory effects of 3-MQ on both enzyme activities were not significantly different from those of IBMX, a non-selective PDE inhibitor. The above results reveal that the mechanisms of relaxant action of 3-MQ may be due to its inhibitory effects on both PDE activities and its subsequent reducing effect on $[Ca^{2+}]_i$ of the trachealis.

Key words: 3-Methylquercetin, *Rhamnus nakaharai*, Rhamnaceae, guinea-pig trachea, intracellular calcium, phosphodiesterase.

Abbreviations: 3-MQ: 3-methylquercetin, 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

3-*O*-Methylquercetin (3-MQ) was identified as one of the 3-methoxyflavones responsible for the pronounced antiviral activity of the extracts from *Veronia amygdalina* Del. (Compositae) and from different *Euphorbia* species, used as a traditional medicine in Central Africa (1). The antiviral activity of 3-MQ on poliovirus RNA synthesis was reported by many authors (2), (3). However, little is known about the influence of 3-MQ on smooth muscle. In 1986, Laekeman et al. reported the pharmacological effects of 3-MQ, including an anti-aggregatory effect on rabbit platelets, inhibitory effects on cyclooxygenase and lipooxygenase of the platelets, a vasodilating effect on rabbit central ear artery, an anti-arrhythmic effect on the left atrium and a positive chronotropic effect on the right atrium of guinea-pig. 3-MQ also potentiated the positive chronotropic effect of isoproterenol. However, they concluded that 3-MQ at a lower concentration (< 60 μM) did not significantly reveal any of the above effects (4). *Rhamnus nakaharai* (Hayata) Hayata has been used as a folk medicine, similar to other *Rhamnus* species in Taiwan, for treating constipation, inflammation, tumors, and asthma (5). Therefore, we are interested in

investigating the mechanisms of the tracheal relaxant action of 3-MQ, a main constituent of the plant.

Materials and Methods

Reagents and drugs

3-MQ (Fig. **1**) was isolated from of *Rhamnus nakaharai* (Hayata) Hayata (6), and identified by spectral methods, including UV, IR, MS, and NMR spectroscopic techniques (spectral data of 3-MQ are obtainable on request from the author of correspondence). The content and purity of 3-MQ were 0.01% and 99%, respectively. Fresh stem bark of the plant was collected at Ali, Wu-Tai Shian, Ping-Tung Hsien, Taiwan in July of 1990 and identified by Professor Chung-Nan Lin, School of Pharmacy, Kaoshiung Medical University, Kaoshiung, Taiwan. Voucher specimens (9001) are deposited in the herbarium of the School of Pharmacy, Kaoshiung Medical University. Aminophylline, carbachol, histamine, propranolol, 2',5'-dideoxyadenosine, methylene blue, glibenclamide, N^{ω}-nitro-L-arginine (L-NNA), α-chymotrypsin, nifedipine, indomethacin, 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 *Ophiophagus hannah* snake venom, etc., were purchased from Sigma Chemical, St. Louis, MO, USA. [^{3}*H*]cAMP and [^{3}*H*]cGMP were purchased from DuPont, Boston, MA, USA. 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). 3-MQ, IBMX, cromakalim, forskolin, indomethacin, or nifedipine was 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

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 ^{o}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), 3-MQ (1-30 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 3-MQ were expressed as $-\log IC_{50}$ values. To determine the

antagonistic effects of 3-MQ 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 3-MQ or its vehicle for 15 min, these two contractile agonists were also cumulatively added into the normal Krebs solution. The antagonistic potencies of 3-MQ 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+} (0.1-30 mM) was added and contractions were elicited in the depolarized trachealis. The maximal contractile response elicited by Ca^{2+} (30 mM) was taken as 100%, and the cumulative concentration-response curve was constructed. The inhibitory effects of 3-MQ 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 3-MQ (1-100 μM) on 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, and α-chymotrypsin or their vehicles were incubated after the precontraction reached a steady state for 15 min prior to the first addition of 3-MQ. In a similar manner, nifedipine (10 μM) was added after histamine (30 μM)-induced precontraction reached a steady state, at 15 min prior to the addition of 3-MQ (30 μM) or its vehicle. 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 3MQ on the relaxant responses of forskolin and nitroprusside to histamine (30 μM)-induced precontractions, 3MQ and IBMX, a positive control, were 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 constant value. 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 3-MQ 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 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 (7).

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 3-MQ (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 ^oC. 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 *Ophiophagus hannah* venom were added to the reaction mixture, and the mixture was incubated at 37 ^oC 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 3-MQ 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. The $pD_2' = pD_x' + \log (x-1)$, where pD_x' is the negative logarithm of the molar concentration of 3-MQ and x is the ratio between the maximal effect of the agonist in the absence of 3-MQ and that in the presence of 3-MQ. The $-\log IC_{50}$ value was considered to be equal to the negative logarithm of the molar concentrations of 3-MQ at which a half-inhibitory effect on Ca^{2+} (30 mM)-induced contraction 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

3-MQ concentration-dependently relaxed the histamine (30 μM)-, carbachol (0.2 μM)-, and KCl (30 mM)-induced precontractions (Figs. **2A, B, C**). Their -log IC_{50} values were 4.92 ± 0.09 (n = 7), 4.67 ± 0.04 (n = 6) and 4.68 ± 0.08 (n = 9), respectively. The -log IC_{50} value against histamine was significantly different from that against carbachol. 3-MQ (1-30 μM) concentration-dependently inhibited concentration-response curves of cumulative histamine and carbachol in a non-competitive manner (Figs. **3A, B**). The pD_2' values were 5.07 ± 0.12 (n = 12), and 4.55 ± 0.21 (n = 12), respectively, which are significantly different from each other. It suggests that the antispasmodic effects of 3-MQ against histamine are more potent than those against carbachol. In isotonic Ca^{2+}-free high K^+ (60 mM)-depolarized tracheas, 3-MQ (3-30 μM) concentration-dependently inhibited concentration-response curves of cumulative Ca^{2+} (0.1-30 mM) in a non-competitive manner (Fig. **4**). The -log IC_{50} value was 5.02 ± 0.14 (n = 18), which is not significantly different from that against KCl (30 mM)-induced precontraction. Nifedipine (1 μM), a voltage-dependent calcium channel

blocker, can completely inhibit the calcium-induced contractions in the deporalized trachealis (10). In this present experiment, nifedipine (10 μM), however, only relaxed 30.2 ± 5.6% (n = 6) of the histamine (30 μM)-induced precontraction in the tracheas. The nifedipine (10 μM)-remaining tension of the trachea was further relaxed by 3-MQ (30 μM) to 92.3 ± 3.8% (n = 6). This suggests that no matter whether 3-MQ blocks the voltage-dependent calcium channels (VDCCs) or not, 3-MQ 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 3-MQ to histamine (30 μM)-induced precontraction in normal Krebs solution (data not shown).

In contrast, 3-MQ (7.5-15 μM) and IBMX (3-6 μM) parallelly shifted left-ward the log concentration-response curves of forskolin (Fig. **5A**, **B**) and nitroprusside (Fig. **5C, D**) to histamine (30 μM)-induced precontractions of the trachealis, and significantly increased the pD_2 values of forskolin, and nitroprusside (Tab **1**). This reveals the relaxant effect of 3-MQ may be via the inhibition of cAMP- and cGMP-PDE, and the subsequent increase of these two cyclic nucleotides. Indeed, in this present study, 3-MQ or IBMX, a positive control, at various concentrations (10-300 μM), concentration-dependently and significantly inhibited cAMP- and cGMP-PDE activities. The inhibitory effects of 3-MQ were not significantly different from those of IBMX. The IC_{50} values of 3-MQ or IBMX were estimated to be 13.8 or 8.3 and 14.3 or 9.9 μM, respectively. IBMX at various concentrations (10-300 μM) selectively inhibited neither cAMP-, nor cGMP-PDE activity. In contrast to IBMX, 3-MQ at 300 μM more selectively ($P < 0.01$) inhibited cGMP-, than cAMP-dependent PDE activity (Fig. **6**).

Discussion

The removal of epithelium did not affect the log concentration-relaxing response curve of cumulative 3-MQ to histamine (30 μM)-induced precontraction suggesting that the relaxant effect of 3-MQ is epithelium-independent. The log concentration-relaxing response curve of cumulative 3-MQ 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 (11), and methylene blue, a soluble guanylate cyclase inhibitor (12), also did not affect the log concentration-response curve of 3-MQ. 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 (13), also did not affect the log concentration-response curve of 3-MQ, 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 (14), did not affect the log concentration-response curve of 3-MQ, 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 3-MQ, suggesting that its relaxant effect is unrelated to the neuropeptides.

3-MQ (3-30 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 (15), at concentrations below 1 μM, also inhibits those contractions in a non-competitive manner. Nifedipine at 1 μM can further completely inhibit those contractions (10). In the present study, nifedipine (10 μM) only (25-39%) relaxed the histamine-induced precontraction in normal Krebs solution. The nifedipine-remaining tension was further (87-96%) relaxed by 3-MQ at 30 μM suggesting that no matter whether it blocked the VDCCs or not, it may have other mechanisms of relaxant action.

3-MQ concentration-dependently relaxed the histamine (30 μM)-, carbachol (0.2 μM)-, and KCl (30 mM)-induced precontractions. The -log IC_{50} value against histamine was significantly greater than that against carbachol. The pD_2' value of 3-MQ against cumulative histamine-induced contractions was also significantly greater than that against carbachol. This suggests that the antispasmodic effects of 3-MQ 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 3-MQ. 3-MQ (7.5-15 μM) and IBMX (3-6 μM) parallelly shifted left-ward 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 (Tab **1**). This reveals that the relaxant effect of 3-MQ 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, 3-MQ or IBMX, a positive control, at various concentrations (10-300 μM), significantly inhibited cAMP- and cGMP-PDE activities. The inhibitory effects of 3-MQ were not significantly different from those of IBMX. The IC_{50} values of 3-MQ were 13.8 and 14.3 μM, therefore, the -log IC_{50} values of 3-MQ were 4.86 and 4.84, respectively. These -log IC_{50} values were similar to those of 3-MQ 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 can not exclude the possibility that the relaxant effects of 3-MQ 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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Tab. **1** The pD_2 values of forskolin and nitroprusside against histamine (30 μM)-induced precontractions in the absence and presence of 3-MQ and IBMX.

	Forskolin	Nitroprusside
3-MQ		
Vehicle	5.98 ± 0.08 (6)	5.65 ± 0.04 (7)
7.5 μM	6.25 ± 0.08 (5)*	6.08 ± 0.14 (6)**
15 μM	6.41 ± 0.15 (7)*	6.23 ± 0.13 (7)***
IBMX		
Vehicle	6.05 ± 0.09 (6)	5.64 ± 0.09 (5)
3 μM	6.46 ± 0.12 (6)*	5.97 ± 0.12 (5)*
6 μM	6.66 ± 0.12 (6)***	6.22 ± 0.07 (6)***

Values are presented as means ± SEM (n); n is the number of experiments.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared with their corresponding values of vehicle.

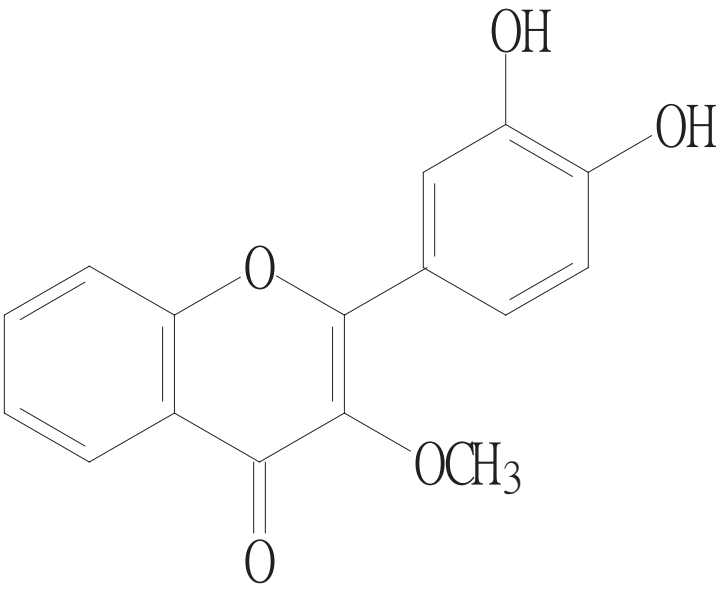

Fig. **1** Chemical structure of 3-methylquercetin (3-MQ).

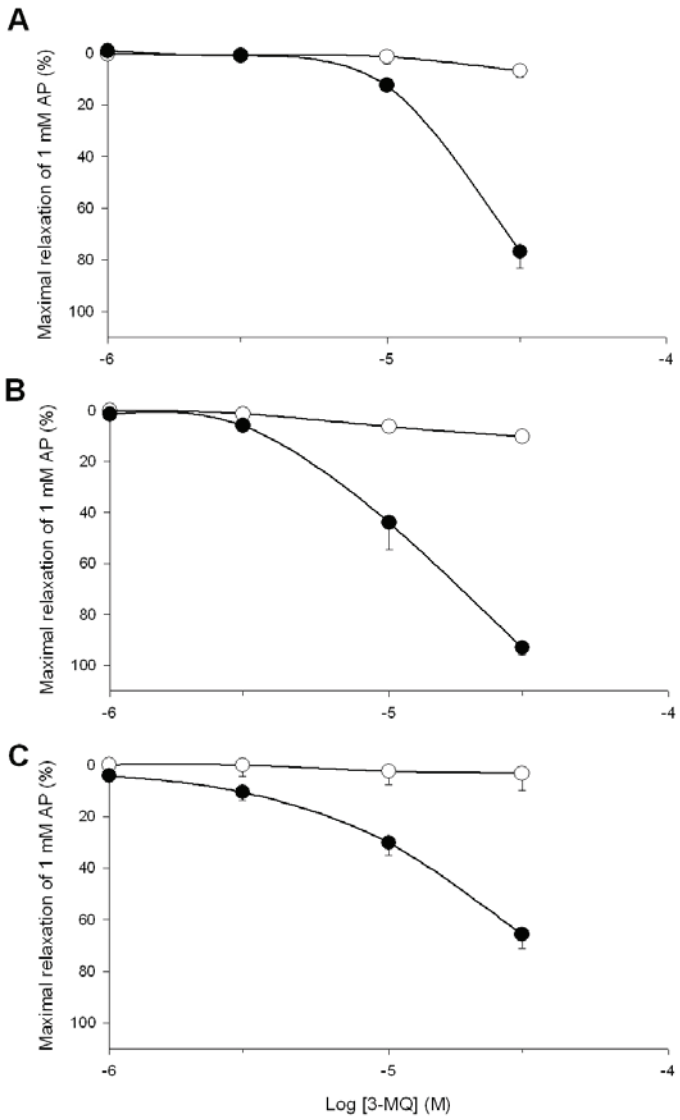

Fig. **2** The relaxant effects of 3-MQ (●) and its vehicle (○) on carbachol (**A**, 0.2 μM)-, histamine (**B**, 30 μM)- and KCl (**C**, 30 mM)-induced precontractions in guinea-pig trachealis. AP: aminophylline.

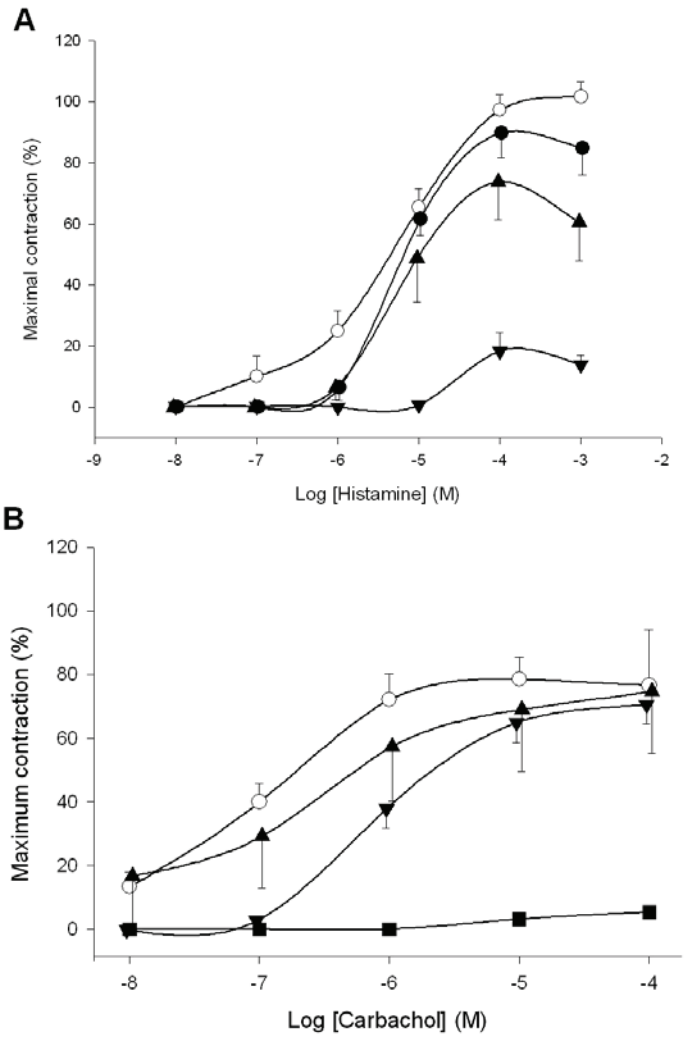

Fig. **3** The inhibitory effects of 3-MQ (○, vehicle; ●, 3 μM; ▲, 10 μM; ▼, 30 μM; ■, 100 μM) on cumulative histamine (**A**)- and carbachol (**B**)-induced contractions in guinea-pig trachealis in normal Krebs solution. Each point represents the mean ± SEM of 4-8 experiments.

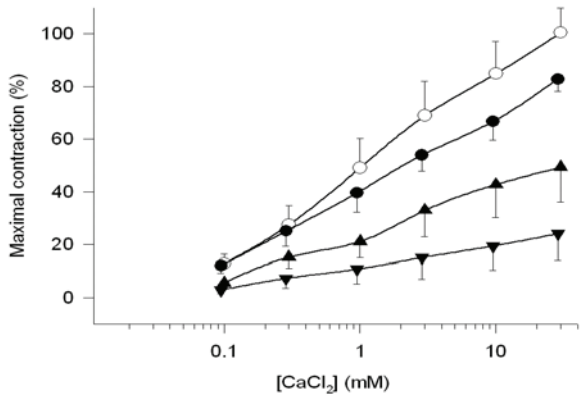

Fig. **4** The inhibitory effects of 3-MQ (○, vehicle; ●, 3 μM; ▲, 10 μM; ▼, 30 μM) on cumulative calcium-induced contractions in guinea-pig trachealis depolarized by KCl 60 mM in Ca^{2+}-free medium. Each point represents the mean ± SEM of 6 experiments.

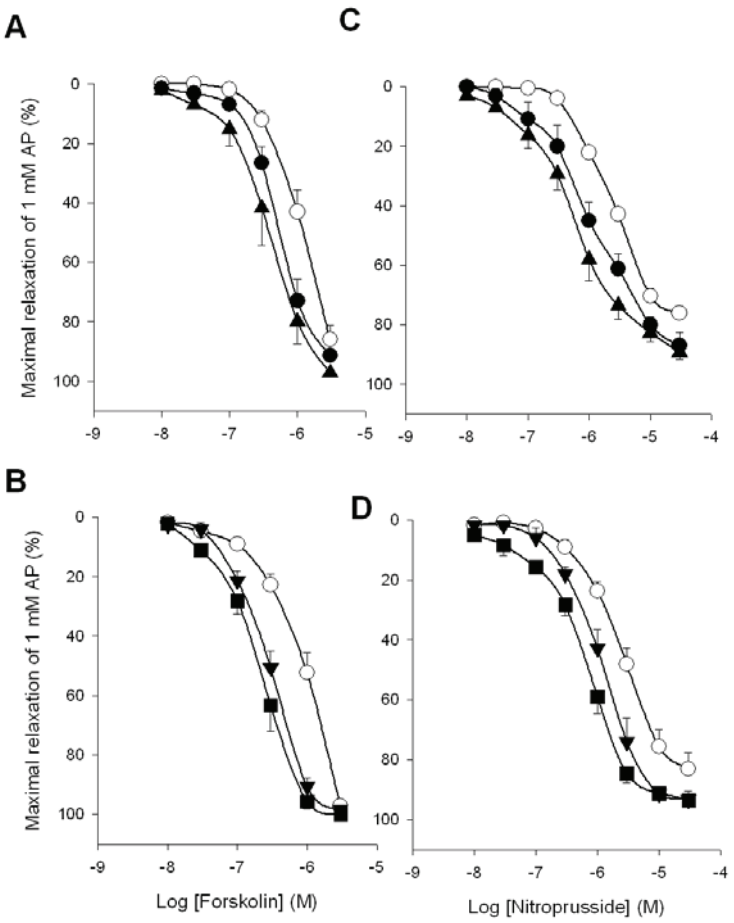

Fig. **5** The potentiating effects of 3-MQ (**A**, **C**; ○, vehicle; ●, 7.5 μM; ▲, 15 μM) and IBMX (**B**, **D**; ○, vehicle; ▼, 3 μM; ■, 6 μM), a positive control, on the relaxant responses of cumulative forskolin (**A**, **B**) and nitroprusside (**C**, **D**) to the histamine (30 μM)-induced precontractions in the guinea-pig trachealis. Each point represents the mean ± SEM of 5-7 experiments. AP: aminophylline.

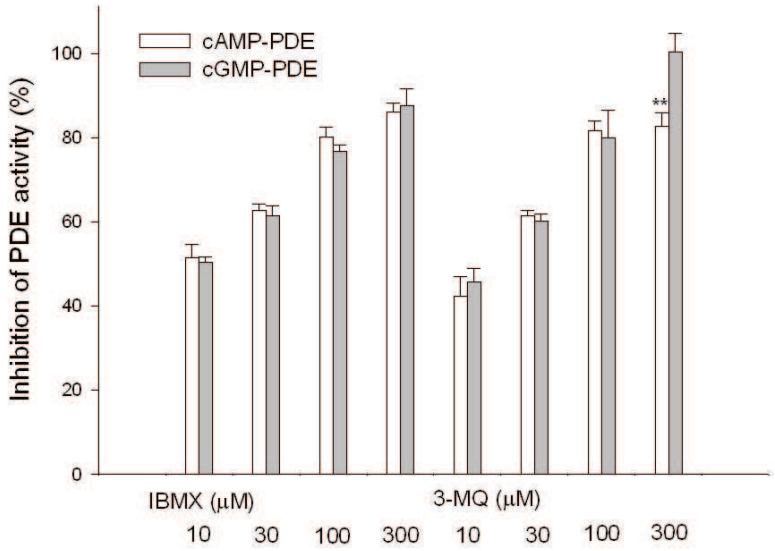

Fig. **6** The inhibitory effects of 3-MQ and IBMX, a positive control, on cAMP- and cGMP-PDE activities. The inhibitory effects do not include those of their vehicle. Each column represents the mean ± SEM of 3-9 experiments. ** $P<0.01$ when compared with corresponding value on cGMP-PDE activity.

Relaxant Action Mechanisms of 3-*O*-Methylquercetin in Isolated Trachea (3/3)

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Abstract: *Rhamnus nakaharai* Hayata (Rhamnaceae), has been used as a folk medicine in Taiwan for treating constipation, inflammation, tumors and asthma. 3-*O*-Methylquercetin (3-MQ), a main constituent of the plant, has been reported to inhibit total cAMP- and cGMP-phosphodiesterase (PDE) of guinea pig trachealis. Therefore we are interested in investigating the inhibitory effect of 3-MQ on various PDE isozymes from guinea pig lungs and hearts. Isolated guinea pig lungs and hearts were homogenized and centrifuged. The supernatant was chromatographed over a column of Q-sepharose, and eluted with various concentrations of NaCl. In the following order, PDE subtype 1, 5, 2, 4 from lungs, and 3 from hearts were separated. The IC_{50} values of 3-MQ on these isozymes were 31.9, 86.9, 18.6, 28.5 and 1.6 μM, respectively. 3-MQ (10-100 μM) non-competitively inhibited PDE2, but competitively inhibited PDE4. 3-MQ (1-10 μM) also competitively inhibited PDE3. However, 3-MQ (10-100 μM) did not competitively inhibit PDE1 and 5, though it had a tendency to competitively inhibit PDE1 at concentrations of 10-30 μM. The present results showed that Ki values of 3-MQ was similar to that of milrinone in PDE3, and was not significantly different from that of Ro 20-1724 in PDE4, respectively. In conclusion, 3-MQ revealed selective and competitive PDE3/PDE4 inhibitor, although its inhibitory effect on PDE4 was not potent. Therefore, 3-MQ may have a potential in the treatment of asthma beside its antiviral activity.

Key words: 3-*O*-Methylquercetin, *Rhamnus nakaharai*, Rhamnaceae, guinea pig, phosphodiesterase, isozymes

Abbreviations

3-MQ: 3-*O*-methylquercetin
PDE: phosphodiesterase
cAMP: adenosine 3', 5' cyclic monophosphate
cGMP: guanosine 3', 5' cyclic monophosphate
EGTA: ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*',*N*'-tetraacetic acid
EDTA: ethylenediaminetetraacetic acid
BSA: bovine serum albumin
PMSF: phenylmethanesulfonyl fluoride
EHNA: erythro-9-(2-hydroxy-3-nonyl)-adenine·HCl
Ro 20-1724: 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone.

Introduction

Phosphodiesterases (PDE) have been classified according to their primary protein and cDNA sequences, co-factor and substrate specifity, and pharmacological role. It is now known that PDE comprise at least 11 distinct enzyme families that hydrolyse cAMP and/or cGMP [1]. PDE1~5 isozymes, which are calcium/calmodulin-dependent (PDE1), cGMP-stimulated (PDE2), cGMP-inhibited (PDE3), cAMP-specific (PDE4), and cGMP-specific (PDE5), have been found to be present in canine trachea [2], guinea pig lung [3], and human bronchi [4]. In guinea pig airways, PDE3 and 4 have been identified [5], but other isozymes might also be present. PDE3, 4 or dual 3/4 inhibitors were reported to have a potential in the treatment of asthma [1], [6]. *Rhamnus nakaharai* (Hayata) Hayata has been used as a folk medicine, similar to other *Rhamnus* species in Taiwan, for treating constipation, inflammation, tumors, and asthma [7]. 3-*O*-Methylquercetin (3-MQ), a main constituent of the plant, has been reported to potently inhibit total cAMP- and cGMP-PDE of guinea pig trachealis [8]. Therefore we are interested in investigating the inhibitory effect of 3-MQ on various PDE1~5 isozymes from guinea pig lungs and hearts.

Materials and Methods

Reagents and drugs

3-MQ (Fig. **1**) was isolated from of *Rhamnus nakaharai* (Hayata) Hayata [9], and identified by spectral methods, including UV, IR, MS, and NMR spectroscopic techniques (spectral data of 3-MQ are obtainable on request from the author of correspondence). The purity of 3-MQ was 99%. Fresh stem bark of the plant was collected at Ali, Wu-Tai Shian, Ping-Tung Hsien, Taiwan in July of 1990 and identified by Professor Chung-Nan Lin, School of Pharmacy, Kaoshiung Medical University, Kaoshiung, Taiwan. Voucher specimens (no. 9001) are deposited in the herbarium of the School of Pharmacy, Kaoshiung Medical University.

Bistris base, Trizma base, d,l-dithiothreitol, benzamidine, Zaprinase, EDTA, EGTA, PMSF, BSA, cyclic AMP, cyclic GMP, calmodulin, Dowex resin, DMSO, and *Ophiophagus hannah* snake venom, etc., were purchased from Sigma Chemical, St. Louis, MO, USA. [^{3}*H*]cAMP, [^{3}*H*]cGMP, Q-sepharose, and calmodulin-agarose were purchased from Amersham Pharmacia Biotech, Buckinghamshire, UK. Vinpocetin, EHNA, milrinone, Ro 20-1724, and zaprinast were purchased from Biomol, Plymouth Meeting, PA, USA. Ethyleneglycol was purchased from Merck, Darmstadt, Germany. Other reagents, such as $CaCl_2$, $MgCl_2$, and NaCl, were of analytical grade. 3-MQ, vinpocetin, EHNA, Ro 20-1724, and PMSF were dissolved in ethyl alcohol. Milrinone, and zaprinast were dissolved in DMSO. EGTA was dissolved in 3N NaOH. Other drugs were dissolved in distilled water. The final concentration of ethyl alcohol or DMSO was less 0.1% and did not significantly affect the activities of PDE isozymes.

Separation of cyclic nucleotide PDE isozymes

Under a protocol approved by the Animal Care and Use Committee of Taipei Medical University, five male guinea pigs (Hartley), weighing 500-600g, were sacrificed. Their lungs (15 g) or hearts (4 g) were chopped into small pieces and homogenized with a glass/teflon homogenizer (Glas-Col, Terre Haute, IN, USA) in 10 volumes of cold medium (pH 7.5) containing 20 mM Tris-HCl, 2 mM benzamidine, 2 mM EDTA, 50 mM sodium chloride, 0.1 mM phenylmethanesulfonyl fluoride (PMSF) and 1 mM dithiothreitol. At 4 ℃, the homogenate was centrifuged at 170 g for 5 min, and the supernatant was then re-centrifuged at 40,000g for 30 min. The final supernatant fraction was filtered through 0.22 μm filters and applied to a Q-sepharose fast flow column (2.2 × 28 cm) pre-equilibrated in homogenization buffer. The column was washed with two bed volumes of homogenate buffer to remove unbound material. Proteins bound to Q-sepharose were eluted with various concentrations of NaCl, 0.23, 0.34, 0.44, 0.69 and 1.00 M in homogenate buffer (40 mL each concentration) at a flow rate of 30 mL/h. Fractions (3 mL) were collected, added ethylene glycol to a final concentration of 30 % (v/v), and frozen at –70 ℃. Under these conditions, the enzyme activity was stable for at least 3 months. In order to eliminate possible contamination of PDE5 by PDE1, the second peak of activity was further purified on a calmodulin-agarose column. The column (1.6 × 4 cm) was pre-equilibrated by a buffer containing 20 mM Bistris, 1 mM dithiothreitol, 2 mM benzamidine, 50 mM NaCl, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM PMSF, pH 6.5 (buffer A). The sample, which concentration of $CaCl_2$ was adjusted to 2 mM, was loaded and allowed to be absorbed on the gel for 30 min. The PDE isoezymes were eluted by stepwise elution using 20 mL buffer A followed by 20 mL buffer A with 1 M NaCl and 20 ml buffer A with 1 M NaCl plus 1 mM EGTA. PDE5 was collected first followed by PDE1.

Cyclic nucleotide PDE assay

The activities of PDE1~5 in the homogenate were measured by a two-step procedure according to the method of Thompson and Appleman [10], using cAMP with [^3H]-cAMP or cGMP with [^3H]-cGMP as substrates. The enzyme preparation (25 μL) was incubated for 30 min at 37 ℃ in a total assay volume of 100 μL containing 50 mM Tris/HCl (pH 7.4), 3 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% BSA, 1 μM cAMP with 0.2 μCi [^3H]-cAMP as a substrate alone or in the presence of 0.1 unit calmodulin with 10 μM $CaCl_2$ or 5 μM cGMP, and 1 μM cGMP with 0.2 μCi [^3H]-cGMP as another substrate alone or in the presence of 0.1 unit calmodulin with 10 μM $CaCl_2$. In tests of enzyme inhibition, the reaction mixture contained 10 μL of vehicle or inhibitors, at various concentrations of 3-MQ or selective PDE1~5 inhibitors, such as vinpocetin [11], EHNA [12], milrinone [13], Ro 20-1724 [14] and zaprinast [15], as reference drugs, respectively. The reagents and homogenate were mixed on ice, and the reaction was initiated by transferring the mixture to a water bath at 37 ℃. 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 *Ophiophagus hannah* venom were added to the reaction mixture, and the mixture was incubated at 37 ^oC 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. The total protein in each fraction used was assayed according to the method described by Bradford [16]. The PDE activities were showed as nmol /mg /min in Lineweaver-Burk analysis, but showed as cpm of radiation in the primary fractionation of these enzymes.

Statistical analysis

The IC_{50} values of 3-MQ and various reference drugs were calculated from non-linear regression by a software of SigmaPlot 4.0 (Sigma Chemical, St. Louis, MO, USA), and K_i values were determined from the equation, K_M/slope. Where K_M is Michaelis constant, and slope is relationship between apparent Km values and inhibitor concentrations used in Lineweaver-Burk analysis. All values are shown as means ± SEM. The differences among these values were statistically calculated by one-way analysis of variance (ANOVA), and 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

There were four major peaks separated from guinea pig lungs (Fig. **2A**). PDE1 appeared from fraction 10-14, PDE5 from fraction 22-28, PDE2 from fraction 34-36, and PDE4 from fraction 44-50, respectively. There were three major peaks separated from guinea pig hearts. PDE3 appeared in the third peak from fraction 40-46 (Fig. **2B**). The –log concentration-inhibition curves of 3-MQ on various PDE isozymes are shown in Fig. **3**. The IC_{50} values of 3-MQ and their reference drugs for PDE1~5 are shown in Table **1.** Among them, the IC_{50} value of 3-MQ for PDE3 was significantly less than the others, but the IC_{50} value of 3-MQ for PDE4 was only significantly less than that for PDE5. From Lineweaver-Burk analysis, it was found that 3-MQ (0.3-3 μM) competitively inhibited PDE3 (Fig. **5A**), and the K_i value was not significantly different from that of milrinone, a selective PDE3 inhibitor (Fig. **5B**). Also, 3-MQ (10-100 μM) competitively inhibited PDE4 (Fig. **6A**), and its K_i value was not significantly different from that of Ro 20-1724, a selective PDE4 inhibitor (Fig. **6B**). However, 3MQ (10-100 μM) non-competitively inhibited PDE2 (Fig. **4A**), and its K_i value was significantly greater than that of EHNA, a selective PDE2 inhibitor (Fig. **4B**). The inhibitory modes of 3-MQ on PDE 2~4 are summarized in Table **1**.

Discussion

Zardaverine, a dual PDE3/4 inhibitor, and a combination of siguazodan, a selective PDE3 inhibitor, and rolipram, a selective PDE4 inhibitor, clearly enhanced bronchodilating activity in guinea pig and successfully antagonized both histamine- and leukotriene D_4-induced bronchospasm *in vivo* and *in vitro* [6]. It seems that the inhibitors of PDE3 and PDE4 additively or synergistically act to inhibit histamine- or leukotriene D_4-induced bronchospasm in guinea pigs [6]. However, treatment with a single PDE

isozyme-selective inhibitor, siguazodan or rolipram, provides little or no inhibition of bronchospasm elicited by histamine or leukotriene D_4 [6], although PDE4 inhibitors have been well developed by many pharmaceutical companies [1]. The present results revealed that 3-MQ competitively inhibited both PDE3 (IC_{50}, 1.6 μM) and PDE4 (IC_{50}, 28.5 μM). Therefore, 3-MQ may have a potential to be a bronchodilator in the treatment of asthma. Also, 3-MQ (10 μM) has been reported to inhibit 48.1 % of nitrite release from RAW 264.7 cells and to have anti-inflammatory effect [17]. The inhibition of nitric oxide is mainly due to its scavenging property, which has been observed in our laboratory. In 2001, Cimanga et al. also reported that 3-MQ inhibited xanthine oxidase (IC_{50}, 27.3 μM) and scavenged superoxide anions (IC_{50}, 15.7 μM). They also suggested that only those compounds where the IC_{50} as a radical scavenger was lower than the IC_{50} as an inhibitor of xanthine oxidase had an additional scavenging effect and can be considered as true superoxide anion scavengers [18]. In addition, 3-MQ (150-300 μM) was reported to significantly inhibit lipoxygenase and cyclooxygenase activities, although the potency of 3-MQ was less than that of indomethacin [19]. Therefore, 3-MQ seems to have an anti-inflammatory effect, and to have a potential to inhibit bronchospasm in the treatment of asthma. The present results showed that Ki values of 3-MQ was similar to that of milrinone in PDE3, and was not significantly different from that of Ro 20-1724 in PDE4, respectively. In conclusion, 3-MQ proved to be a selective and competitive PDE3/PDE4 inhibitor, although its inhibitory effect on PDE4 was not potent. Therefore, 3-MQ besides its antiviral activity [20] may have a potential in the treatment of asthma.

Acknowledgements

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Table **1** IC_{50} (μM) values and inhibitory modes of 3-MQ on various PDE isozymes

Inhibitors	PDE isozymes				
	1	2	3	4	5
3-MQ	31.9 ± 16.9 (3)*	18.6 ± 5.7 (3)*	1.6 ± 0.7 (5)[#]	28.5 ± 5.8 (4)*	86.9 ± 17.7 (8)*,[#]
	ND	Non-competitive	Competitive	Competitive	ND
Reference drugs[+]	58.8 ± 12.8 (3)	4.4 ± 2.5 (3)	0.8 ± 0.4 (3)	5.5 ± 1.6 (5)	2.5 ± 0.9 (6)

[+] Reference drugs for PDE isozymes 1, 2, 3, 4, and 5 were vinpocetine, EHNA, milrinone, Ro 20-1724 and zaprinast, respectively. *P <0.05 when compared with the corresponding value of PDE isozyme type 3. [#]P < 0.05 when compared with the corresponding value of PDE isozyme type 4.
ND: not determined.

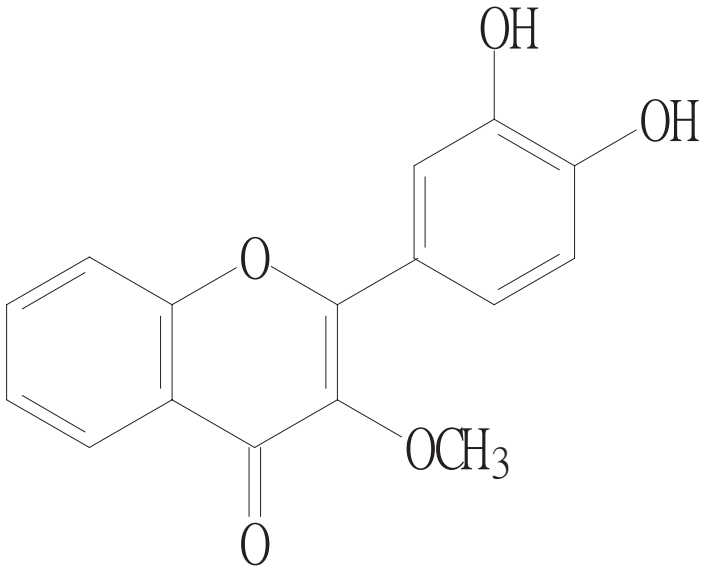

Fig. **1** Chemical structure of 3-*O*-methylquercetin (3-MQ).

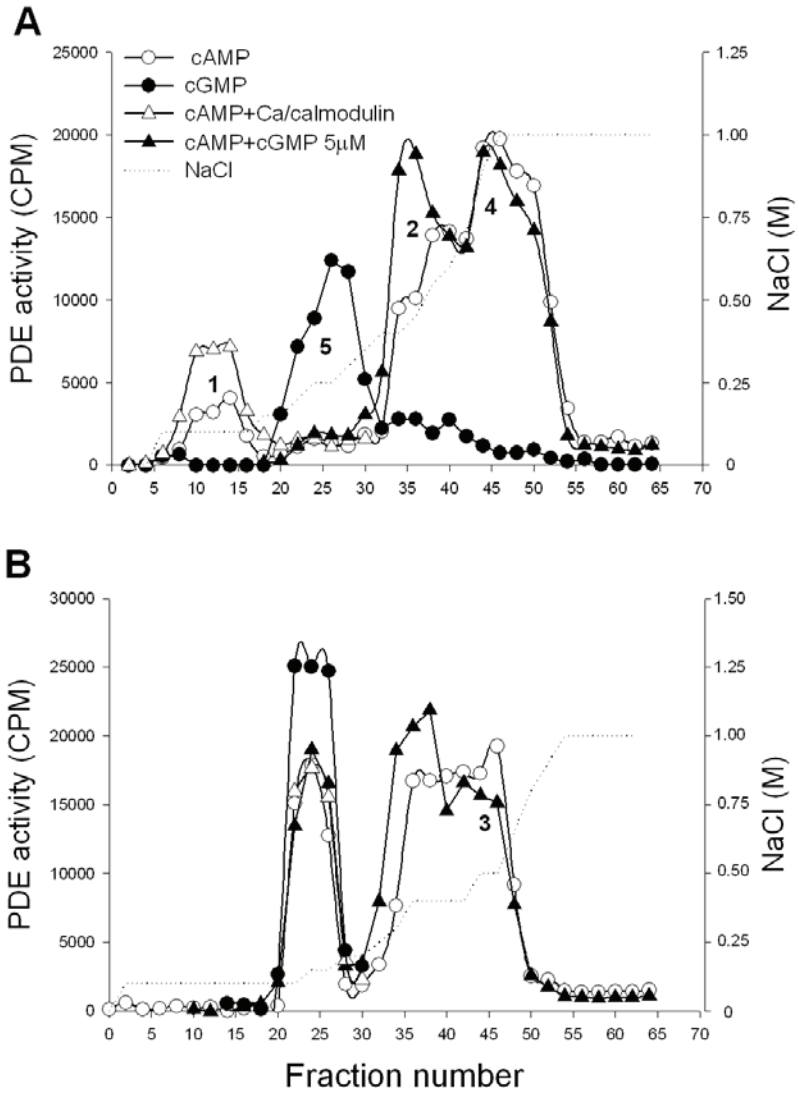

Fig. **2** Elution profiles of PDE isozymes from guinea pig lungs (A) and hearts (B). The Arabic number shows the respective PDE isozyme subtype.

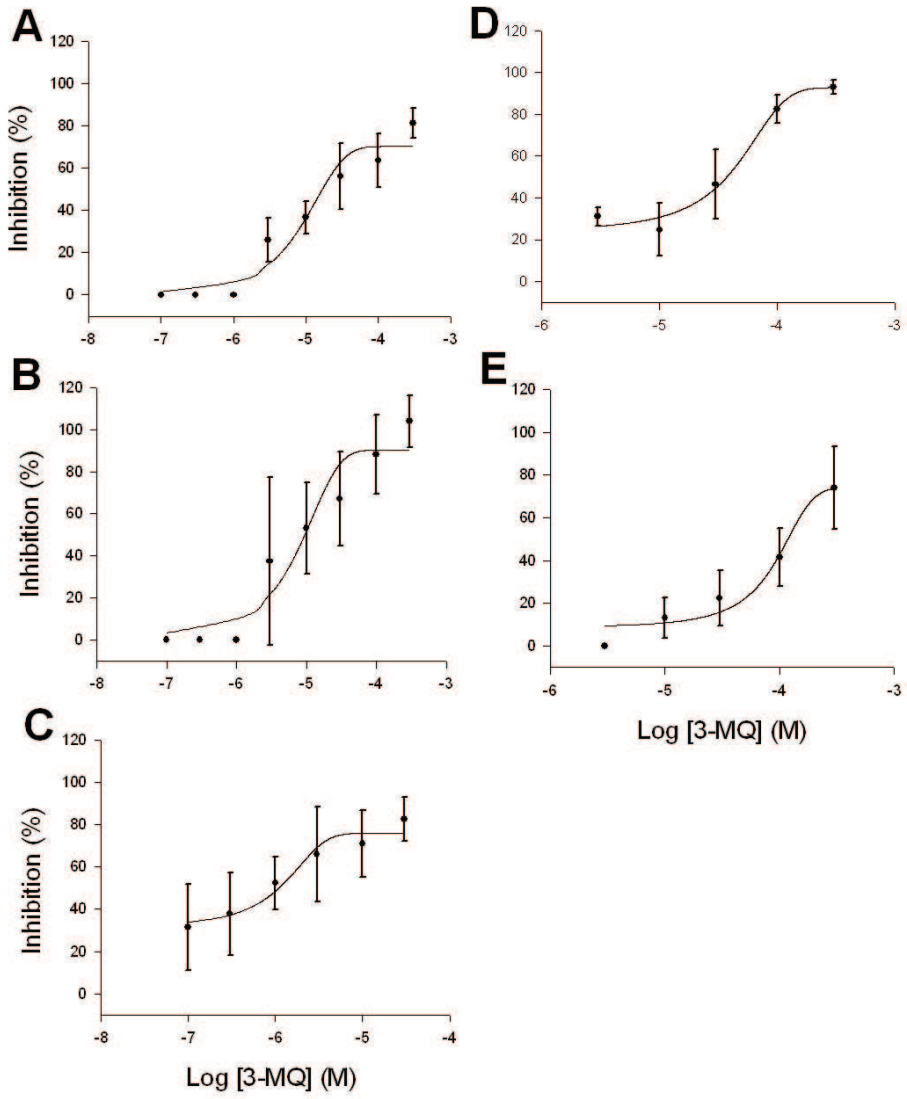

Fig. **3** Log concentration-inhibition curves of 3-MQ on PDE1 (A), 2 (B), 3 (C), 4 (D), and 5 (E). The number of experiments was 3-4.

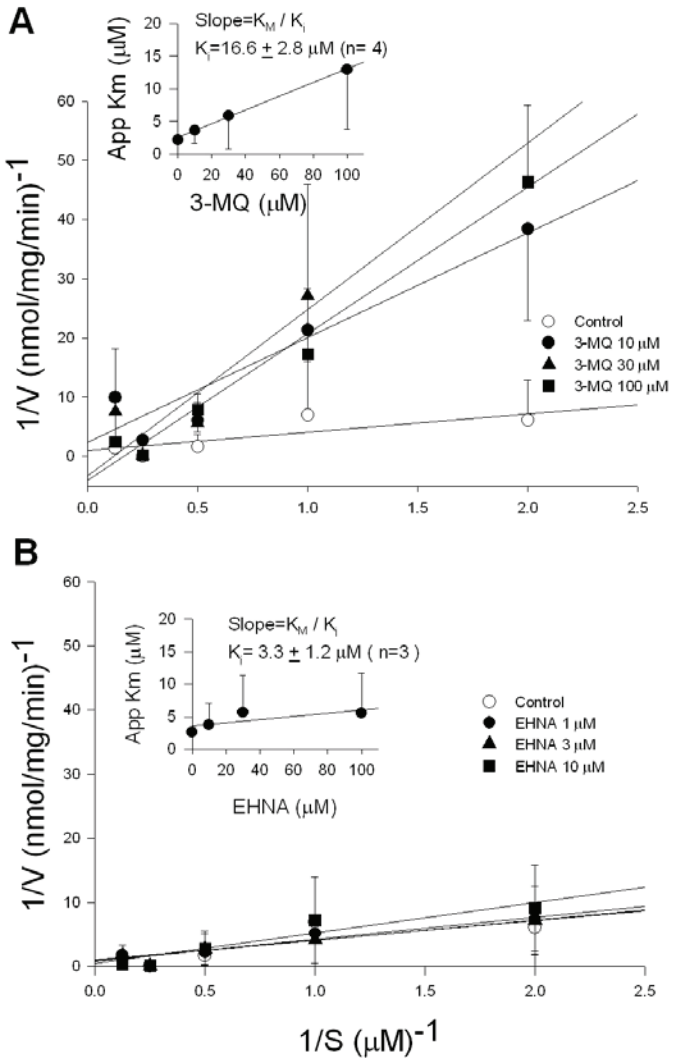

Fig. **4** Inhibition by 3-MQ (**A**) and EHNA (**B**) on PDE2-induced cAMP hydrolysis. The activities of PDE2 in the presence of various concentrations of 3-MQ or EHNA, and substrate (cAMP) were plotted according to Lineweaver-Burk. K_i value was determined from the equation of apparent Km as a function of the inhibitor concentration (insert in **A** and **B**, respectively). The number of experiments was 3-4.

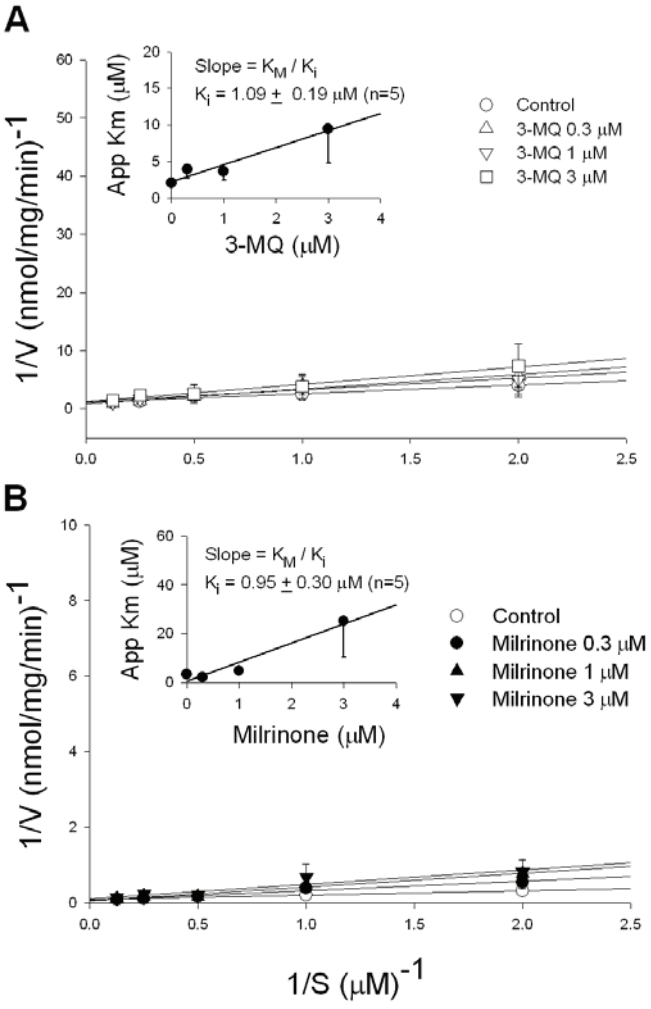

Fig. **5** Inhibition by 3-MQ (**A**) and milrinone (**B**) on PDE3-induced cAMP hydrolysis. The activities of PDE3 in the presence of various concentrations of 3-MQ or milrinone, and substrate (cAMP) were plotted according to Lineweaver-Burk. K_i value was determined from the equation of apparent Km as a function of the inhibitor concentration (insert in **A** and **B**, respectively). The number of experiments was 5.

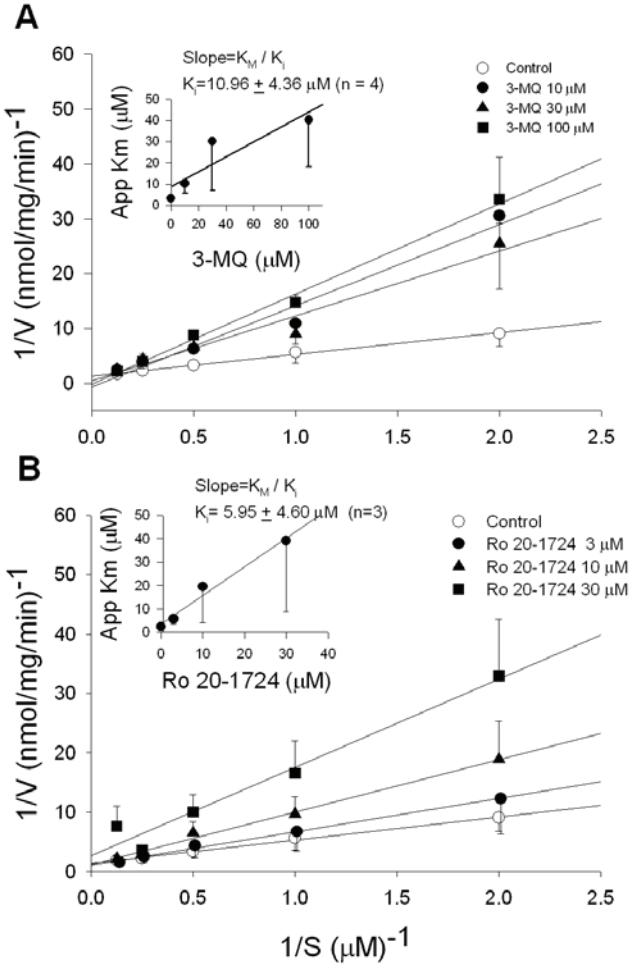

Fig. **6** Inhibition by 3-MQ (**A**) and Ro 20-1724 (**B**) on PDE4-induced cAMP hydrolysis. The activities of PDE4 in the presence of various concentrations of 3-MQ or Ro 20-1724, and substrate (cAMP) were plotted according to Lineweaver-Burk. K_i value was determined from the equation of apparent Km as a function of the inhibitor concentration (insert in **A** and **B**, respectively). The number of experiments was 3-4.

Relaxant Action Mechanisms of 3-*O*-Methylquercetin in Isolated Trachea (3/3)

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Abstract

Rhamnus nakaharai Hayata (Rhamnaceae), has been used as a folk medicine in Taiwan for treating constipation, inflammation, tumors, and asthma. 3-*O*-Methylquercetin (3-MQ), a main constituent of the plant, has been reported to inhibit total cAMP- and cGMP-phosphodiesterase (PDE) of guinea pig trachealis at low concentrations. 3-MQ has been also reported to more selectively inhibit PDE3 than PDE4 with a low Km value. Therefore we were interested in investigating its suppressive effects on ovalbumin (OVA)-induced airway hyperresponsiveness in vivo and in vitro. 3-MQ (3-30 μmol/kg, i.p.) significantly suppressed the enhanced pause (Penh) value induced by aerosolized methacholine (50 mg/ml) in sensitized mice after secondary allergen challenge. 3-MQ (3-30 μmol/kg, i.p.) also significantly suppressed total inflammatory cells, marcrophages, neutrophils, and eosinophils, but not lymphocytes. In addition, 3-MQ (3 μmol/kg, i.p.) significantly decreased the secretion of TNF-α, and at the highest dose (30 μmol/kg, i.p.) even decreased the secretions of IL-4, IL-5, and TNF-α. 3-MQ (1-10 μM) as well as Ro 20-1724 (3-30 μM), a selective PDE4 inhibitor, significantly attenuated OVA (100 μg/ml)-induced contractions. 3-MQ (30 μM) as well as milrinone (1-10 μM), a selective PDE3 inhibitor, significantly enhanced baseline contractions in isolated guinea pig left and right atria. However, neither 3-MQ nor milrinone significantly affected baseline beating rate in the right atria, and nor did they significantly shift the log concentration-response curves of isoproterenol in either inotropic or chronotropic effects. 3-MQ (3-30 μmol/kg, i.p.) did not significantly affect systolic pressure or step-through latency of passive avoidance behavior in conscious mice. In conclusion, 3-MQ has both anti-inflammatory and bronchodilating effects, and has the potential for use in the treatment of asthma at a dose without obvious central side effects, which often appear in many PDE4 inhibitors.

Key words
3-*O*-Methylquercetin · *Rhamnus nakaharai* · ovalbumin-sensitized mice · inflammatory cells · cytokines · airway hyperresponsiveness · asthma

Abbreviations
3-MQ: 3-*O*-methylquercetin
Penh: enhanced pause
AHR: airway hyperresponsiveness
OVA: ovalbumin
PDE: phosphodiesterase
BALF: bronchoalveolar lavage fluid
IL: interleukine
TNF-α: tumor necrosis factor-α
IFN-γ: interferon-γ
CBA: cytometric bead array
STL: step-through latency

Introduction

Besides antiviral activity [1], [2] of 3-*O*-methylquercetin (3-MQ), Laekeman et al. reported some cardiovascular effects, including anti-aggregation of rabbit platelets, inhibition of both cyclooxygenase and lipooxygenase of platelets, vasodilation of the rabbit central ear artery, anti-arrhythmia of the left atria, and potentiation of a positive chronotropic effect of isoproterenol in guinea pig right atria [3]. However, they concluded that the above effects of 3-MQ at a lower concentration (< 60 μM) were not significant. Recently we reported that 3-MQ (7.5-15 μM) concentration-dependently potentiated the relaxant effects of forskoline and nitroprusside in guinea pig trachealis [4]. The IC_{50} value of 3-MQ on total cAMP- and cGMP-phosphodiesterase (PDE) activities was 13.8 and 14.3 μM, respectively. Recently, 3-MQ was reported to more selectively inhibit PDE3 (IC_{50}, 1.6 μM) than PDE4 (IC_{50}, 28.5 μM) in our laboratory [5]. 3-MQ, a main constituent of *Rhamnus nakaharai* (Hayata) Hayata which has been used as a folk medicine in Taiwan for treating constipation, inflammation, tumors, and asthma [6]. Therefore, we were interested in investigating the suppressive effects of the compound on ovalbumin (OVA)-induced airway hyperresponsiveness (AHR).

Materials and Methods

Reagents and drugs

3-MQ (Fig. **1**) was isolated from *Rhamnus nakaharai* (Hayata) Hayata [7], and identified by spectral methods, including UV, IR, MS, and NMR spectroscopic techniques (spectral data of 3-MQ are obtainable on request from the author of correspondence). The purity of 3-MQ was 99%. Fresh stem bark of the plant was collected at Ali, Wu-Tai Shian, Ping-Tung Hsien, Taiwan in July of 1990 and identified by Professor Chung-Nan Lin, School of Pharmacy, Kaoshiung Medical University, Kaoshiung, Taiwan. Voucher specimens (9001) are deposited in the herbarium of the School of Pharmacy, Kaoshiung Medical University. Ovalbumin, methacholine, dimethylsulfoxide (DMSO), and isoproterenol were purchased from Sigma Chemical, St. Louis, MO, USA. Freund's adjuvant (*Mycobacterium butyricum*) was purchased from Pierce Biotechnology, Rockford, IL, USA. Mouse Th1/Th2 cytokine CBA kits were purchased from Pharmingen, San Diego, CA, USA. Milrinone, a selective PDE3 inhibitor [8], and Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone, a selective PDE4 inhibitor [9], were purchased from Biomol, Plymouth Meeting, PA, USA. Polyethyleneglycol 400 and ethyl alcohol were purchased from Merck, Darmstadt, Germany. Other reagents, such as $CaCl_2$, $MgCl_2$, and NaCl, were of analytical grade. 3-MQ and Ro 20-1724 were dissolved in ethyl alcohol. Milrinone was dissolved in DMSO. Other drugs were dissolved in distilled water. The final concentration of ethyl alcohol or DMSO was less than or equal to 0.1%, and did not significantly affect the contraction or beating rate of tissues, such as the trachea and atria. Under a protocol approved by the Animal Care and Use Committee of Taipei Medical University, the following *in vivo* and *in vitro* experiments were performed.

Airway hyperresponsiveness in vivo

Female BALB/c mice at 8 to 12 wk of age were obtained from the Animal Center of the

National Science Council, ROC. Mice were sensitized by an intraperitoneal (i.p.) injection of 20 μg of OVA emulsified in 2.25 mg aluminum hydroxide gel in a total volume of 100 μl on days 0 and 14. Mice were challenged via the airway by OVA (1% in saline) for 30 min on days 28, 29, and 30 by ultrasonic nebulization. Six weeks after the last of the three primary OVA challenges, mice were exposed to 1% OVA for 30 min by nebulization as a secondary challenge [10]. AHR was assessed on day 74 (48 h after 1% OVA provocation) for each group. Each group of mice was intraperitoneally injected vehicle, or 3 or 30 μmol/kg of 3-MQ 2 h before and 6 and 24 h after OVA provocation. The vehicle, a mixture of alcohol: polyethyleneglycol 400: saline (1: 14.5: 14.5), or 3-MQ was injected at a volume of 0.01 ml/g of body weight. The AHR was measured in unrestrained animals by barometric plethysmography [11] using a whole-body plethysmograph (WBP) (Buxco, Troy, NY, USA). Mice were placed into the main chamber of the WBP and nebulized first with phosphate buffer solution (PBS), then with increasing doses (6.25-50 mg/ml) of methacholine (MCh) for 3 min for each nebulization, followed by readings of breathing parameters for 3 min after each nebulization with determination of enhanced pause (Penh) values. Twenty-four hours after the Penh determination, these mice were anesthetized with pentobarbital (50 mg/kg, i.p.), and lavaged via a tracheal tube with PBS (1 × 1.0 ml, 37 ℃). The collected bronchoalveolar lavage fluid (BALF) was centrifuged at 500 rpm for 5 min, and the pellet was resuspended in ACK lysing buffer to lyse residual erythrocytes in each sample. The number of inflammatory cells was counted using a hemacytometer (Hausser Sci., Horsham, PA, USA). Cytospin slides were stained and differentiated in a blinded fashion by counting at least 100 cells under light microscopy. However after centrifugation, the supernatant was stored at –20 ℃ until the determination of cytokines, including interleukine (IL)-2, IL-4, IL-5, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ by flow cytometric methods [12] using mouse Th1/Th2 cytokine CBA kits (Pharmingen, San Diego, CA, USA) according to the recommendations of the manufacturer.

Airway hyperresponsiveness in vitro

Male Hartley guinea pigs (500-600 g), obtained from the Animal Center of the National Science Council, ROC, were sensitized by intramuscular injections of 0.7 ml of 5% (w/v) OVA in saline on days 1, 4, and 43, and in adjuvant on days 25 and 39 into each thigh, respectively, according to the method described by Underwood et al. [13] and modified by us. Three days after the last injection, the sensitized guinea pigs were sacrificed 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 sutures, placed in 5 ml of normal Krebs solution containing indomethacin (3 μM), gassed with a 95% O_2 plus 5% CO_2 mixture at 37 ^{o}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 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 KCl (60 mM) and washed with normal Krebs solution, OVA (0.1-100 μg/ml) was cumulatively added, and contractions were allowed to reach a steady state at each concentration. To evaluate the suppressive effect of 3-MQ on OVA-induced contractions, each tissue was preincubated with each concentration (1, 3, or 10 μM) of 3-MQ or its vehicle (alcohol) for 15 min and then challenged with cumulative OVA again. Therefore, the log concentration-response curves of OVA were constructed in the absence and presence of 3-MQ. The tension of precontraction induced by KCl was set as 100%.

Isolated guinea pig atria

Male Hartley guinea pigs weighing 500 to 600 g were obtained from the Animal Center of the National Science Council, ROC, and sacrificed by cervical dislocation. The hearts were rapidly excised, and the right and left atria were dissected and mounted in a 5-ml organ bath containing Tyrode solution of the following composition (mM): NaCl 137.0, KCl 5.4, $MgCl_2$ 1.1, $NaHCO_3$ 11.9, NaH_2PO_4 0.33, $CaCl_2$ 2.0, and dextrose 11.0. The solutions were gassed with 95% O_2 plus 5% CO_2 and kept at 32 ^{o}C. Contractions of spontaneously beating right atria and electrically driven left atria were measured by connecting one end of the preparations with a fine silk thread to a force-displacement transducer (Gould UC2) and isometrically recording the tension changes on a polygraph (Gould RS3200). To obtain the maximal developed tension, an optimal preload (0.5 to 1 g) was used. The contractile signals of spontaneously beating atria were connected to another amplifier (Gould Biotach), and thereby heart rates were simultaneously recorded on a polygraph. The left atria were stimulated at a frequency of 2 Hz through bipolar platinum electrodes with rectangular pulses (1-ms duration, twice threshold strength) delivered by a Grass S88 stimulator. The preparations were equilibrated for 60 min, and then incubated with vehicle (alcohol) of 3-MQ or vehicle (DMSO) of milrinone, a reference drug, for 15 minutes. The baseline contraction of spontaneously beating right atria and electrically driven left atria, and the baseline heart rates of right atria were set as 100%. Then a control cumulative concentration-response curve of isoproterenol was developed for each preparation. Following incubation with 3-MQ or milrinone for another 15 minutes, a new concentration-response curve of isoproterenol was obtained. During incubation, changes in the baselines of contractile tension and heart rate by 3-MQ or milrinone or their vehicles in right and left atria were measured.

Determination of systolic pressure in conscious mice

Female BALB/c mice 8 to 12 wk of age were obtained from the Animal Center of the National Science Council, ROC. Each group of mice was intraperitoneally injected with vehicle, or 3 or 30 μmol/kg of 3-MQ. The vehicle, a mixture of alcohol: polyethyleneglycol 400: saline (1: 14.5: 14.5), or 3-MQ was injected at a volume of 0.01 ml/g of body weight. Thirty minutes after the injection, the systolic pressures of these mice were determined according to the indirect tail-cuff method [14]. Briefly, a pneumatic sensor (International Biomedical, Houston, TX, USA) and a pneumatic pulse transducer (Narco Bio-system, Houston, TX, USA) were used for this determination.

Passive avoidance behavior with a brief shock

Female BALB/c mice 8 to 12 wk of age were obtained from the Animal Center of the National Science Council, ROC. Each group of ten mice was intraperitoneally injected with vehicle, or 3 or 30 μmol/kg of 3-MQ. The vehicle, a mixture of alcohol: polyethyleneglycol 400: saline (1: 14.5: 14.5), or 3-MQ was injected at a volume of 0.01 ml/g of body weight. In the training trial, the guillotine door between the light and dark compartment (9 × 9 × 13.5 cm) was closed. When each mouse was placed in the light compartment with its back to the guillotine door, the door was opened and simultaneously the time (step-through latency, STL) was measured with a stopwatch until the mouse entered the dark compartment. Thirty minutes after injection of vehicle or 3-MQ, the mouse was put back the light compartment. After the mouse entered the dark compartment, the door was closed. An inescapable scrambled footshock (1 mA for 2 s) was delivered through the steel-rod flood (10 parallel steel rods, 0.5 cm in diameter set 0.3 cm apart). The mouse was removed from the dark compartment 5 s after the shock, and the STL was determined again. Then the mouse was put back into the home cage until the retention trial. Eight and twenty-four hours later, the retention trials were carried out. The mouse was placed in the compartment again, as on the training trial, the guillotine door was opened and the STL was recorded and used as a measure of retention [15]. The upper cut-off time was set to 300 s.

Statistical analysis

All values are given as the mean ± SEM. Differences among values were statistically calculated by one-way analysis of variance (ANOVA), and then determined by least significant difference (LSD). The difference between two values, however, was determined by use of Student′s *t*-test. Differences were considered statistically significant if the *P*-value was less than 0.05.

Results

In conscious mice, MCh (6.25-50 mg/ml) concentration-dependently increased Penh values from 1.5 ± 0.2 to 13.0 ± 2.1 times that of the baseline, as standardized by aerosol PBS. 3-MQ (3-30 μmol/kg, i.p.) significantly suppressed the maximum Penh value (Fig. **2A**), and also significantly suppressed total inflammatory cells, macrophages, neutrophils, and eosinophils, but not lymphocytes (Fig. **2B**). In addition, 3-MQ (30 μmol/kg, i.p.) significantly attenuated secretions of IL-4, IL-5, and TNF-α, and at a lower dose of 3 μmol/kg (i.p.) also significantly decreased secretion of TNF-α (Fig. **2C**). OVA (0.1-100 μg/ml) concentration-dependently enhanced tension from the baseline to 102.9 ± 4.3% of 60 mM KCl. The vehicle (0.1% alcohol) of 3-MQ or Ro 20-1724 did not affect OVA (100 μg/ml)-induced maximal contractions. However, 3-MQ (1-10 μM), as well as Ro 20-1724 (3-30 μM), significantly inhibited OVA (100 μg/ml)-induced contractions when compared to their vehicle (Fig. **3A**, **B**). Also, 3-MQ (10 μM), as well as Ro 20-1724 (3 μM), significantly attenuated OVA (10 μg/ml)-induced contractions (Fig. **3A**, **B**). 3-MQ

(30 μM) as well as milrinone (1-10 μM) also significantly enhanced baseline contractions in isolated guinea pig left (Figs. **4A, 5A right**) and right (Figs. **4B, 5B right**) atria. However, neither 3-MQ nor milrinone significantly affected the baseline beating rate in right atria (Figs **4C, 5C right**), or shifted the log concentration-response curves of isoproterenol for either the inotropic or chronotropic effects (Figs. **4, 5 left**). 3-MQ (3-30 μmol/kg, i.p.) did not significantly affect systolic pressure (Table **1**) or STL of passive avoidance (Table **2**) in conscious mice.

Discussion

Bronchial asthma is a syndrome associated with allergen-induced AHR and chronic airway inflammation. Airway mucosal inflammation is characterized by an influx of activated eosinophils and T lymphocytes. Eosinophils are thought to be a major cell in the development of increased airway reactivity by releasing eosinophil granule proteins such as major basic protein and eosinophil cationic protein, and other mediators, including leukotrienes that damage the airway epithelium and induce airway smooth muscle contraction and vascular leakage. In the present results, 3-MQ (3-30 μmol/kg, i.p.) significantly inhibited total inflammatory cells, macrophages, neutrophils, and eosinophils, but not lymphocytes. In contrast, rolipram (0.03-0.3 mg/kg, i.p.), a selective PDE4 inhibitor [16], significantly inhibited lymphocytes and eosinophils, but not total inflammatory cells and macrophages [10]. Rolipram at the highest dose, 0.3 mg/kg, i.p., also significantly inhibited neutrophils [10]. The reason for the difference between 3-MQ and rolipram is unclear, but may be due to their selectivity for PDE. In our laboratory, we have detected that 3-MQ more selectively inhibits PDE3 than PDE4. In addition, it has been reported that PDE4 appears to be present throughout the whole bronchial tree, while PDE3 may be present predominantly in smaller airways [17]. Hamelmann et al. reported that increases in Penh values were inhibited by pretreatment of mice with a β_2-agonist [11]. In our present results, 3-MQ (3-30 μmol/kg, i.p.), a tracheal relaxant [4], significantly inhibited Penh values (Fig. **2A**). Therefore, 3-MQ seems to have both anti-inflammatory and bronchodilating effects. In RAW 264.7 cells, 3-MQ (10 μM) revealed scavenger properties; therefore it has been reported to have an anti-inflammatory effect [18]. The dose of 3-MQ (3-30 μmol/kg) was approximately equal to 1-10 mg/kg. The present results revealed that 3-MQ at the lowest dose significantly decreased the secretion of TNF-α, and at the highest dose even decreased the secretions of IL-4, IL-5, and TNF-α (Fig. **2C**). Rolipram (0.1-10 μM) or zardaverine, a dual PDE3/4 inhibitor [19], but not siguazodan, a selective PDE3 inhibitor [20], inhibited OVA-induced contractions of isolated guinea pig trachea in a concentration-dependent manner [13]. Rolipram or siguazodan alone (10 μM) were ineffective against histamine- or leukotriene D_4-induced contractions. Zardaverine alone or the combination of rolipram and siguazodan (10 μM each) markedly antagonized the contractions elicited by both spasmogens [13]. In our present results, 3-MQ (1-10 μM) significantly inhibited OVA (100 μg/ml)-induced contractions of isolated guinea pig trachea. Our previous report revealed that 3-MQ antagonized histamine-induced contractions in isolated guinea pig trachea with a pD'_2 value of 5.07 [4]. Therefore, 3-MQ seems to be able to antagonize endogenous and exogenous mediators, such as histamine or leukotriene D_4.

The present results revealed that 3-MQ (30 μM), as well as milrinone (1-10 μM), significantly enhanced baseline contractility in both atria, but not the beating rate (Figs. **4, 5**). Therefore, 3-MQ (3-30 μmol/kg, i.p.), similar to milrinone, may have a positive inotropic effect and result in increased cardiac output. However, 3-MQ (1-30 μM) did not significantly affect the log concentration-response curves of isoproterenol for either the inotropic or chronotropic effects, although 3-MQ (60 μM) was reported to potentiate the positive chronotropic effect of isoproterenol [3]. A single application of 3-MQ (0.32-0.64 μmol) has been reported to lower arterial pressure in isolated rabbit ear arterial perfusion [3]. However, 3-MQ (3-30 μmol/kg, i.p.) did not affect systolic pressure in conscious mice. A reasonable explanation is that 3-MQ inhibits PDE3/4 and causes an increase in c-AMP, which may dilate the arterioles and result in hypotension, which may offset its cardiac tonic effect. Also, 3-MQ (3-30 μmol/kg, i.p.) did not affect the STL of passive avoidance in conscious mice. This reveals that 3-MQ does not affect learning and memory. In our observations, 3-MQ (3-30 μmol/kg, i.p.) also did not affect naive behavior, right reflex, traction, and rotarod tests (data not shown).

In conclusion, 3-MQ, with both anti-inflammatory and bronchodilating effects, has the potential for use in the treatment of asthma at a dose without obvious central side effects which often appear in many PDE4 inhibitors.

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Table **1** Effects on systolic pressure (mmHg) before and 30 min after intraperitoneal (i.p.) administration of 3-MQ in conscious mice

Dose (μmol/kg, i.p.)	Before	After
Vehicle	85.6 ± 8.7 (10)	90.1 ± 6.5 (10)
3	92.6 ± 11.4 (5)	88.5 ± 3.3 (5)
30	81.2 ± 1.7 (5)	90.8 ± 5.8 (5)

There was no significant difference between values before and after drug administration. The number of determinations is given in parentheses.

Table **2** Effects of 3-MQ on step-through latency (s) of passive avoidance in mice

Footshock	Vehicle	3-MQ (3 μmol/kg, i.p.)	3-MQ (30 μmol/kg, i.p.)
Before	31.9 ± 7.2 (10)	44.0 ± 9.0 (5)	24.2 ± 6.1 (5)
After 0 h	246.7 ± 32 (10)	196.2 ± 48.0 (5)	139.6 ± 50.5 (5)
8 h	192.6 ± 38.7 (10)	130.0 ± 69.7 (5)	105.8 ± 52.4 (5)
24 h	164.9 ± 36.5 (10)	124.6 ± 57.4 (5)	149.2 ± 61.3 (5)

There was no significant difference between the control (vehicle) and test groups within 24 h after the footshock. The number of determinations is given in parentheses.

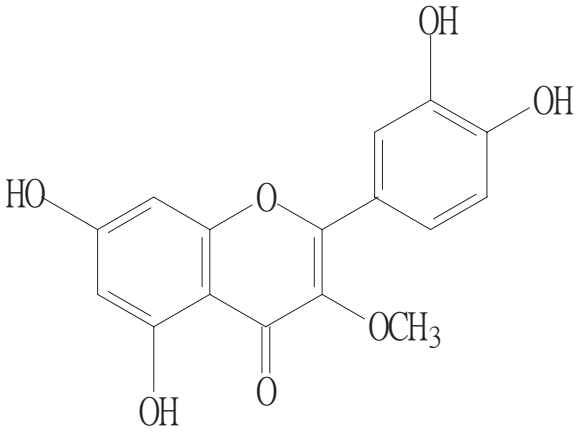

Fig. **1** Chemical structure of 3-*O*-methylquercetin (3-MQ).

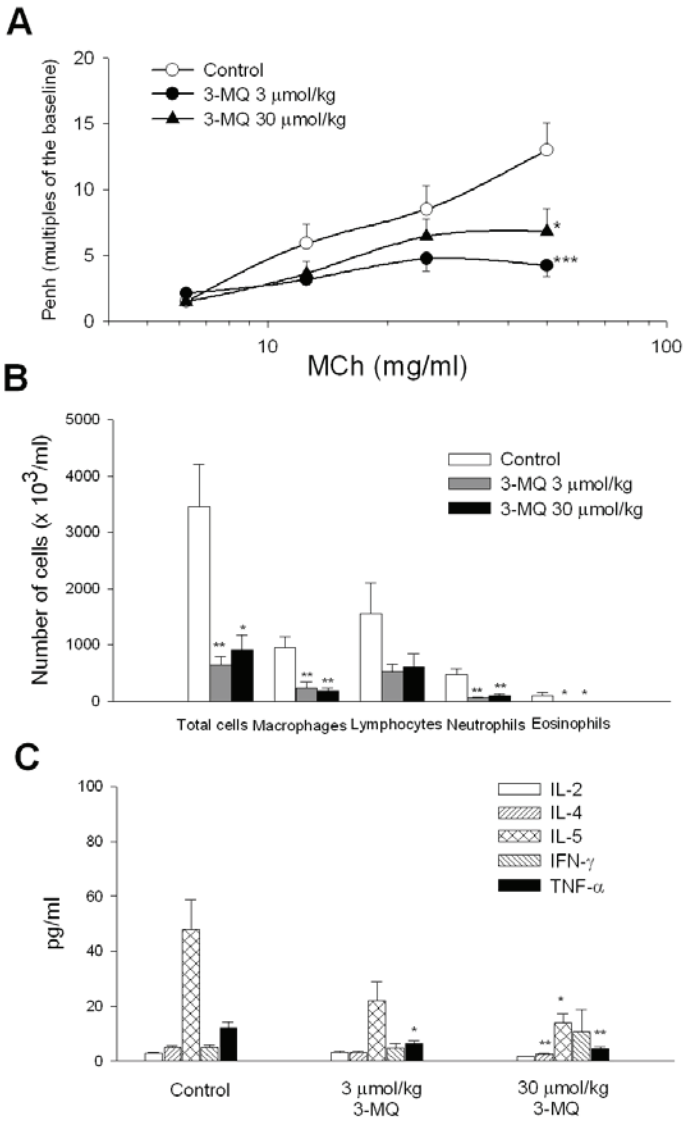

Fig. **2** Effects of 3-MQ (3-30 μmol/kg, i.p.) on Penh (**A**), inflammatory cells (**B**), and cytokines (**C**) in sensitized mice which received aerosolized methacholine (MCh, 6.25-50 mg/ml) 2 days after secondary allergen challenge. * P <0.05, ** P < 0.01, *** P < 0.01 when compared with the vehicle (control). The number of each group of mice was 9-10.

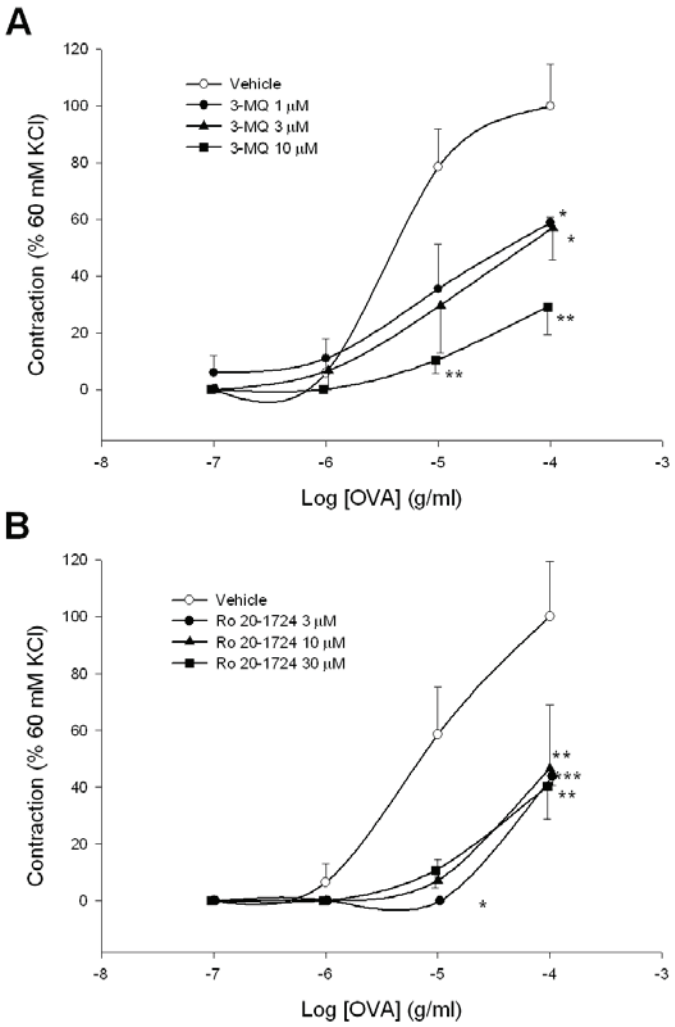

Fig. **3** Effects of 3-MQ (**A**) and Ro 20-1724 (**B**) on cumulative ovalbumin (OVA)-induced contractions in isolated sensitized guinea pig trachealis. The number of experiments was 3-7. * P <0.05, ** P < 0.01, *** P < 0.01 when compared with the vehicle (control).

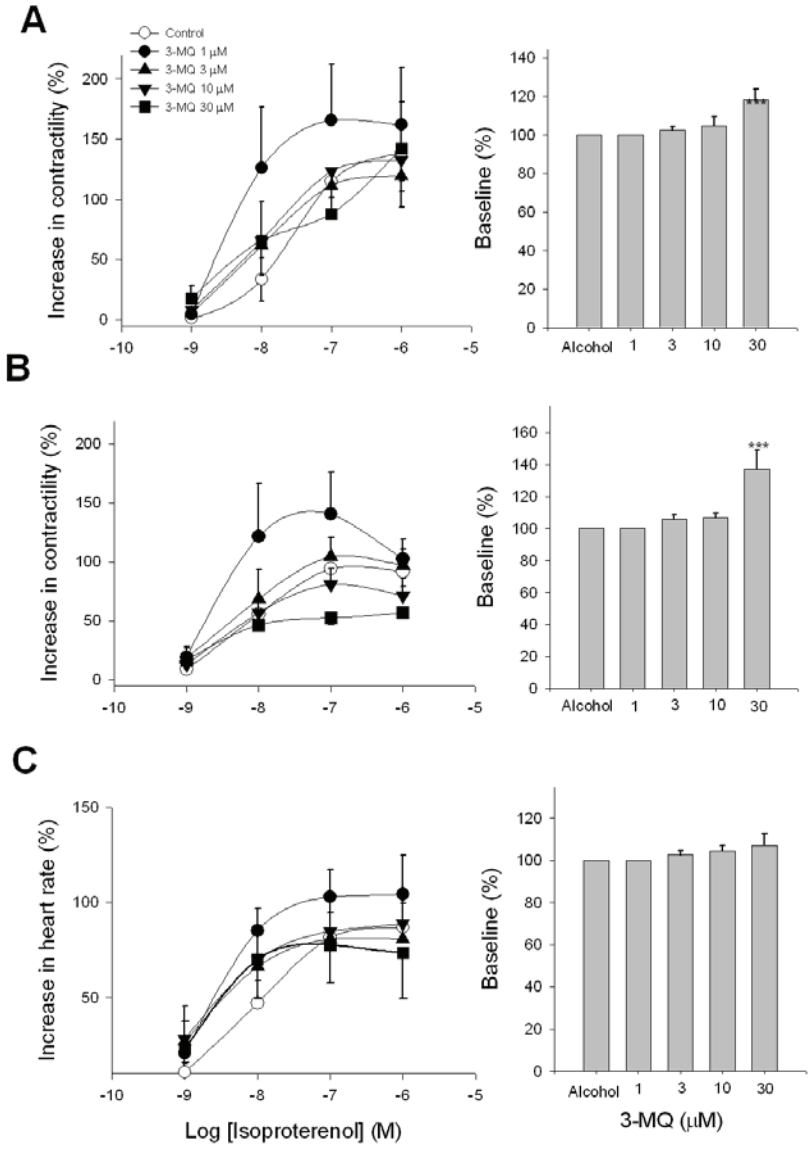

Fig. **4** Effects of 3-MQ on the log concentration-response curves of isoproterenol (**left**) and on the baseline (**right**) of contractility in isolated guinea pig left (**A**) and right atria (**B**), and of the beating rate in right atria (**C**). The number of experiments was 4-7. *** $P < 0.001$ when compared with the vehicle (alcohol).

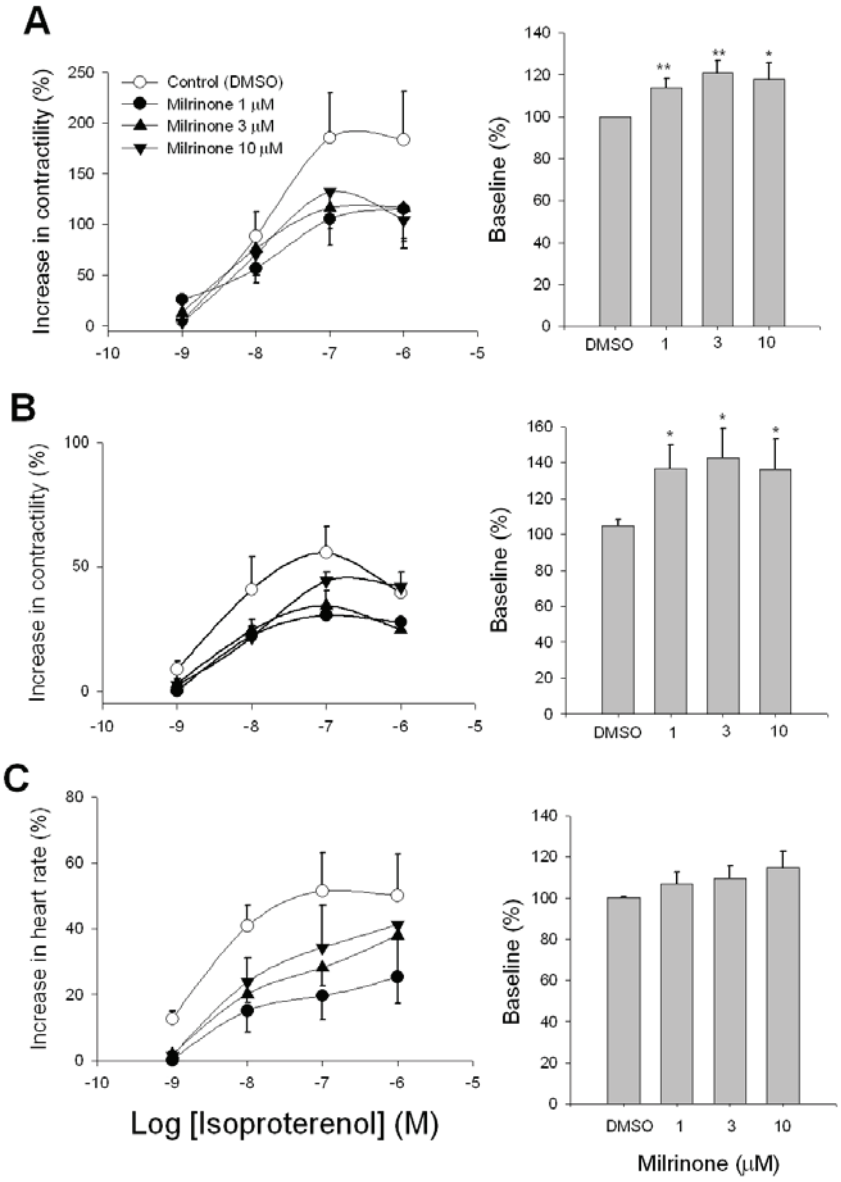

Fig. **5** Effects of milrinone on the log concentration-response curves of isoproterenol (**left**) and on the baseline (**right**) of contractility in isolated guinea pig left (**A**) and right atria (**B**), and of the beating rate in right atria (**C**). The number of experiments was 3-7. * $P < 0.05$, ** $P < 0.01$ when compared with the vehicle (DMSO).