

Wun-Chang Ko¹
Chwen-Ming Shih²
Mei-Chun Chen¹
Ya-Hsin Lai¹
Jun-Hao Chen¹
Chien-Ming Chen³
Chun-Nan Lin⁴

Suppressive Effects of 3-O-Methylquercetin on Ovalbumin-Induced Airway Hyperresponsiveness

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 K_m 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 $\mu\text{mol/kg}$, *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 $\mu\text{mol/kg}$, *i.p.*) also significantly suppressed total inflammatory cells, macrophages, neutrophils, and eosinophils, but not lymphocytes. In addition, 3-MQ (3 $\mu\text{mol/kg}$, *i.p.*) significantly decreased the secretion of TNF- α , and at the highest dose (30 $\mu\text{mol/kg}$, *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 $\mu\text{g/mL}$)-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. How-

ever, neither 3-MQ nor milrinone significantly affected baseline beating rate in the right atria. 3-MQ (3 – 30 $\mu\text{mol/kg}$, *i.p.*) did not significantly affect systolic pressure 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 affecting blood pressure.

Key words

3-O-Methylquercetin · *Rhamnus nakaharai* · Rhamnaceae · ovalbumin-sensitized mice · inflammatory cells · cytokines · 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

Affiliation

¹ Graduate Institute of Pharmacology, College of Medicine, Taipei Medical University, Taipei, Taiwan, ROC

² Department of Biochemistry, College of Medicine, Taipei Medical University, Taipei, Taiwan, ROC

³ Department of Medical Technology, College of Medicine, Taipei Medical University, Taipei, Taiwan, ROC

⁴ School of Pharmacy, Kaoshiung Medical University, Kaoshiung, Taiwan, ROC

Correspondence

Prof. Dr. Wun-Chang Ko · Graduate Institute of Pharmacology · College of Medicine · Taipei Medical University · 250 Wu-Hsing St. · Taipei 110 · Taiwan · R.O.C. · Fax: +886-2-2377-7639 · E-mail: wc_ko@tmu.edu.tw

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Introduction

Besides the 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 lipoxygenase of platelets, vasodilation of the rabbit central ear artery, anti-arrhythmia of the left atria, and a potentiation of the 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 ($< 60 \mu\text{M}$) were not significant. Recently, we reported that 3-MQ ($7.5 - 15 \mu\text{M}$) concentration-dependently potentiated the relaxant effects of forskoline and nitroprusside in guinea pig trachealis [4]. The IC_{50} values of 3-MQ on total cAMP- and cGMP-phosphodiesterase (PDE) activities were 13.8 and $14.3 \mu\text{M}$, respectively. Recently, 3-MQ was reported to more selectively inhibit PDE3 (IC_{50} : $1.6 \mu\text{M}$) than PDE4 (IC_{50} : $28.5 \mu\text{M}$) in our laboratory [5]. 3-MQ is a main constituent of *Rhamnus nakaharai* Hayata (Rhamnaceae) 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 [7], and identified by spectral methods, including UV, IR, mass, 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, and dimethyl sulfoxide (DMSO) 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. Polyethylene glycol 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

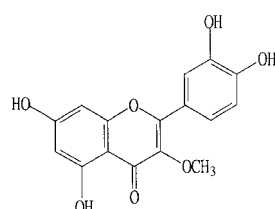


Fig. 1 Chemical structure of 3-O-methylquercetin (3-MQ).

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 of 8 to 12 weeks of age were obtained from the Animal Center of the National Science Council, ROC. Mice were sensitized by an intraperitoneal (*i.p.*) injection of $20 \mu\text{g}$ of OVA emulsified in 2.25 mg aluminum hydroxide gel in a total volume of $100 \mu\text{L}$ 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 $30 \mu\text{mol/kg}$ of 3-MQ 2 h before and 6 and 24 h after OVA provocation. The vehicle, a mixture of alcohol:polyethylene glycol 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 ($6.25 - 50 \text{ mg/mL}$) 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 ($1 \times 1.0 \text{ mL}$, 37°C). 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°C until the determination of cytokines, including interleukin (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, Taiwan, 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 ($3 \mu\text{M}$), gassed with a 95% O_2 plus 5% CO_2 mixture at 37°C , and attached by the other end of each segment to force displacement transducers (Grass FT03) for the isometric recording of tension changes on a polygraph (Gould RS3200). The composition of the normal Krebs solution was (mM): NaCl 118, KCl 4.7, MgSO_4 1.2, KH_2PO_4 1.2, CaCl_2 2.5, NaHCO_3 25, and dextrose 10.1. The 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 ($0.1 - 100 \mu\text{g/mL}$) 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 $10 \mu\text{M}$) 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°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 3-MQ ($1 - 30 \mu\text{M}$) or milrinone ($1 - 10 \mu\text{M}$) was cumulatively added, and the baseline contractions and beating rates were allowed to reach a steady state at each contraction. The changes in the baselines of contractile tension and beating rate by 3-MQ or milrinone were measured.

Determination of systolic pressure in conscious mice

Female BALB/c mice 8 to 12 weeks 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 $30 \mu\text{mol/kg}$ of 3-MQ. The vehicle, a mixture of alcohol:polyethylene glycol 400:saline (1:14.5:14.5), or 3-MQ was injected at a volume of 0.01 mL/g 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.

Statistical analysis

All values are given as the mean $\pm$ 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 ($6.25 - 50 \text{ mg/mL}$) 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 ($3 - 30 \mu\text{mol/kg}$, *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 ($30 \mu\text{mol/kg}$, *i.p.*) significantly attenuated secretions of IL-4, IL-5, and $\text{TNF-}\alpha$, and at a lower dose of $3 \mu\text{mol/kg}$ (*i.p.*) also significantly decreased secretion of $\text{TNF-}\alpha$ (Fig. 2C). OVA ($0.1 - 100 \mu\text{g/mL}$) concentration-dependently enhanced tension from the baseline to $102.9 \pm 4.3\%$ of 60 mM KCl. The vehicle (0.1% alcohol) of 3-MQ or Ro 20-1724 did not affect OVA ($100 \mu\text{g/mL}$)-induced maximal contractions. However, 3-MQ ($1 - 10 \mu\text{M}$), as well as Ro 20-1724 ($3 - 30 \mu\text{M}$), significantly inhibited OVA ($100 \mu\text{g/mL}$)-induced contractions when compared to their vehicle (Figs. 3A,B). Also, 3-MQ ($10 \mu\text{M}$), as well as Ro 20-1724 ($3 \mu\text{M}$), significantly attenuated OVA ($10 \mu\text{g/mL}$)-induced contractions (Figs. 3A,B). 3-MQ ($30 \mu\text{M}$) as well as milrinone ($1 - 10 \mu\text{M}$, data not shown) also significantly enhanced baseline contractions in isolated guinea pig left (Fig. 4A) and right (Fig. 4B) atria. However, neither 3-MQ nor milrinone (data not shown) significantly affected the baseline beating rate in right atria (Fig. 4C). 3-MQ ($3 - 30 \mu\text{mol/kg}$, *i.p.*) did not significantly affect systolic pressure (data not shown).

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 [15]. 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 [10]. In the present results, 3-MQ ($3 - 30 \mu\text{mol/kg}$, *i.p.*) significantly inhibited total inflammatory cells, macrophages, neutrophils, and eosinophils, but not lymphocytes. In contrast, rolipram ($0.03 - 0.3 \text{ mg/kg}$, *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,

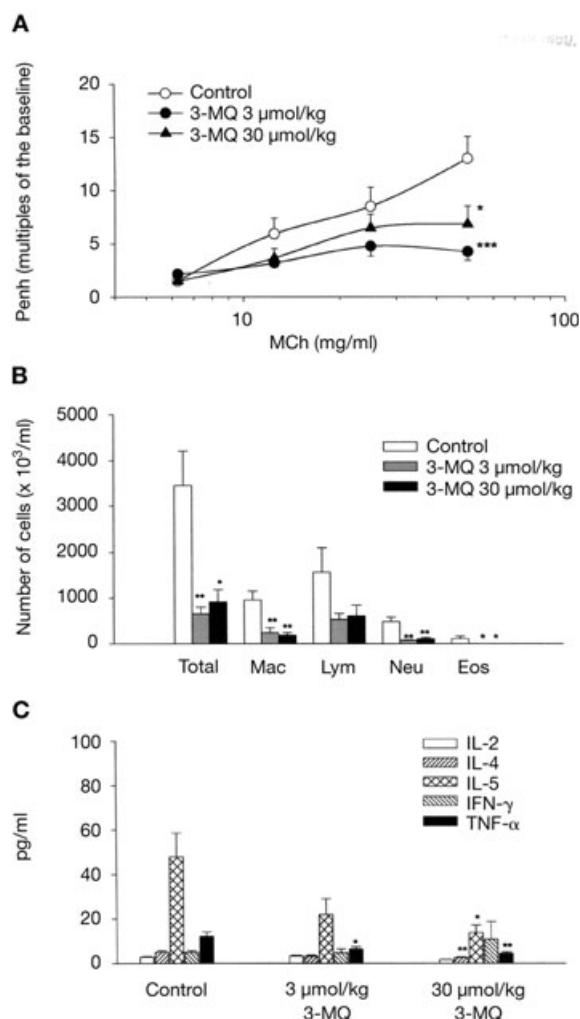


Fig. 2 Effects of 3-MQ (3–30 $\mu\text{mol/kg}$, *i.p.*) on Penh (**A**), inflammatory cells (**B**), and cytokines (**C**) in sensitized mice which received aerosolized methacholine (MCh, 6.25–50 mg/ml) 2 days after secondary allergen challenge. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$ when compared with the vehicle (control). The number of each group of mice was 9–10. Total: total cells; Mac: macrophages; Lym: lymphocytes; Neu: neutrophils; Eos: eosinophils

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], which may activate adenylate cyclase and cause increase of cAMP. In our present results, 3-MQ (3–30 $\mu\text{mol/kg}$, *i.p.*), a dual PDE3/4 inhibitor [5], significantly inhibited Penh values (Fig. 2A). Therefore, 3-MQ may cause an increase of cAMP and have both anti-inflammatory [18] and bronchodilating effects. The dose of 3-MQ (3–30 $\mu\text{mol/kg}$) 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 $\mu\text{g/mL}$)-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 (Fig. 4). Therefore, 3-MQ (3–30 $\mu\text{mol/kg}$, *i.p.*), similar to milrinone, may have a positive inotropic effect and result in increased cardiac output. This result is consistent with the previous report that milrinone, a selective PDE3 inhibitor, had a positive inotropic effect [21]. 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 $\mu\text{mol/kg}$, *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 cAMP, which may dilate the arterioles and offset its cardiac tonic effect.

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 affecting blood pressure.

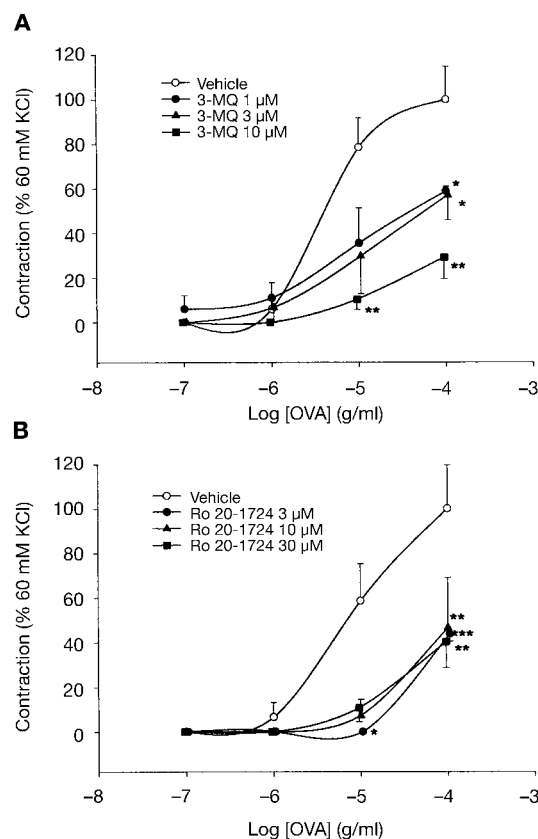


Fig. 3 Effects of 3-MQ (**A**) and Ro 20-1724 (**B**) on cumulative ovalbumin (OVA)-induced contractions in isolated sensitized guinea pig trachealis. The number of experiments was 3–7. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$ when compared with the vehicle (control).

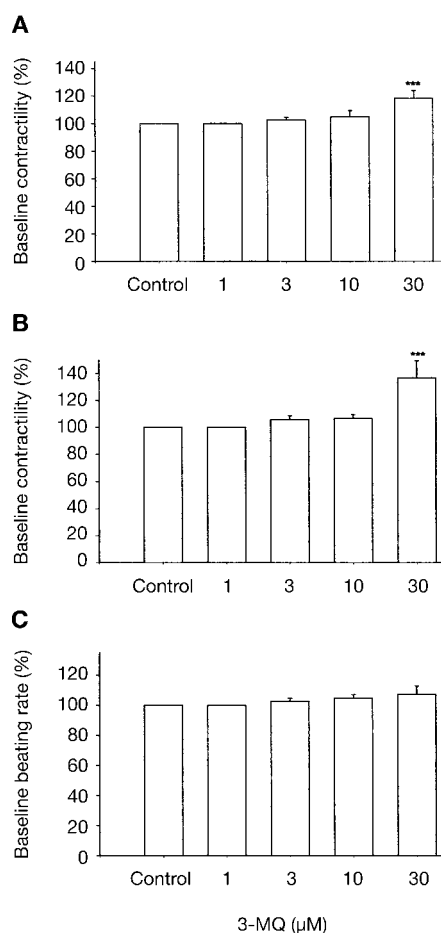


Fig. 4 Effects of 3-MQ on the baseline of contractility in isolated guinea pig left **(A)** and right atria **(B)**, and of the beating rate in right atria **(C)**. The number of experiments was 4–7. *** $P < 0.001$ when compared with the vehicle (alcohol).

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