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**A xanthine-based epithelium-dependent airway relaxant KMUP-3 increases respiratory performance and protects against TNF- $\alpha$ -induced tracheal contraction, involving NO release and expression of cGMP and PKG**

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- d) Abbreviations:** AC, adenylyl cyclase; 4-AP, 4-aminopyridine; cAMP, adenosine 3',5'-cyclic monophosphate; cGMP, guanosine 3',5'-cyclic monophosphate; ChTX, charybdotoxin; IBMX, 3-isobutyl-1-methylxanthine;  $K_{ATP}$  channels, ATP-sensitive potassium channels; KMUP-3, 7-[2-[4-(4-nitrobenzene)piperazinyl]ethyl]-1,3-dimethylxanthine; L-NAME, N<sup>0</sup>-nitro-L-Arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one; PDE, phosphodiesterase; PKA, protein kinase A; PKG, protein kinase G; sGC, soluble guanylyl cyclase, SQ 22536, 9-(terahydro-2-furanyl)-9H-purin-6-amine; SNP, sodium nitroprusside; TEA, tetraethylammonium; TNF- $\alpha$ , tumor necrosis factor-alpha; TSM, tracheal smooth muscle; TSMCs, tracheal smooth muscle cells
- e) Recommended Section:** Gastrointestinal, hepatic, pulmonary, and renal.

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### ABSTRACT

KMUP-3 (7-[2-[4-(4-nitrobenzene)piperazinyl]ethyl]-1,3-dimethylxanthine) was investigated in guinea-pig tracheal smooth muscle (TSM). Intra-tracheal instillation of TNF- $\alpha$  (0.01 mg/kg/300  $\mu$ l) induced bronchoconstriction, increases of lung resistance and decreases of dynamic lung compliance. Instillation of KMUP-3 (0.5-2.0 mg/kg) reversed this situation. In isolated trachea precontracted with carbachol, KMUP-3 (10-100  $\mu$ M) caused relaxations were attenuated by epithelium removal and by pretreatments with an inhibitor of K<sup>+</sup> channel, tetraethylammonium (TEA, 10 mM); K<sub>ATP</sub> channel, glibenclamide (1  $\mu$ M); voltage-dependent K<sup>+</sup> channel, 4-aminopyridine (4-AP, 100  $\mu$ M); Ca<sup>2+</sup>-dependent K<sup>+</sup> channel, charybdotoxin (ChTX, 0.1  $\mu$ M) or apamin (1  $\mu$ M); soluble guanylate cyclase (sGC), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1  $\mu$ M); nitric oxide (NO) synthase, N<sup>ω</sup>-nitro-l-arginine methyl ester (L-NAME, 100  $\mu$ M); adenylate cyclase, 9-(terahydro-2-furanyl)-9H-purin-6-amine (SQ 22536, 100  $\mu$ M). KMUP-3 (0.01-100  $\mu$ M) induced increases of cGMP and cAMP in primary culture of tracheal smooth muscle cells (TSMCs). The increase in cGMP by KMUP-3 was reduced by ODQ and L-NAME; the increase in cAMP was reduced by SQ 22536. Western blot analysis indicated that KMUP-3 (1  $\mu$ M) induced expression of PKA<sub>RI</sub> and PKG<sub>1 $\alpha$ 1 $\beta$</sub>  in TSMCs. SQ 22536 inhibited KMUP-3-induced expression of PKA<sub>RI</sub>. On the contrary, ODQ inhibited KMUP-3-induced expression of PKG<sub>1 $\alpha$ 1 $\beta$</sub> . In epithelium-intact trachea, KMUP-3 increased the NO release. Activation of sGC, NO release, and inhibition of phosphodiesterases (PDEs) in TSMCs by KMUP-3 may result in increases of intracellular cGMP and cAMP, which subsequently activate PKG and PKA, efflux of K<sup>+</sup> ion and associated reduction in Ca<sup>2+</sup> influx *in vitro*, indicating the action mechanism to protect against TNF- $\alpha$ -induced airway dysfunction *in vivo*.

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### Introduction

Xanthine derivatives, including theophylline, have long been recognized as bronchodilators, owing to their non-specific inhibition activities of phosphodiesterase (PDE) to increase cAMP and they have been widely used in the therapy of bronchospastic diseases, including acute asthmatic attacks, emphysema, and chronic bronchitis, which are often associated with immuno-responses (Caramori and Adcock, 2003). The immuno-pharmacologic effects of xanthine derivatives have recently become a particular subject of interest (Kobayashi et al., 2004). Nevertheless, several important drawbacks remain and a low therapeutic margin of safety, associated with a pharmacokinetic profile highly influenced by individual factors, results in the need for monitoring blood levels, which makes the drug hard to use widely in clinics (Barnes, 2003; Sohei et al., 2004).

Likewise, the properties of xanthine derivatives to increase epithelium-dependent cGMP and protein kinase G (PKG) expression, which suppress tumor necrosis factor-alpha (TNF- $\alpha$ )-induced response in tracheal smooth muscle cells (TSMCs), have not been explored thoroughly. TNF- $\alpha$  has been described to up-regulate 5-HT<sub>2A</sub>-mediated and B1/B2 receptor responses in airway inflammation (Adner et al., 2002; Zhang et al., 2005), to modulate tracheal responsiveness to G-protein-coupled receptor agonist (Chen et al., 2003) and to act as an inflammatory cytokine involved in asthmatics (Leung et al., 2005). Previously, 7-[2-[4-(2-chlorophenyl)piperazinyl] ethyl]-1,3-dimethyl xanthine (KMUP-1) has been stated to possess airway relaxation activities (Wu et al., 2004). In order to create a more hopeful xanthine-based tracheal smooth muscle (TSM) relaxant, 7-[2-[4-(4-nitrobenzene)piperazinyl] ethyl]-1,3-dimethylxanthine (KMUP-3) (Fig. 1) was synthesized (Wu et al., 2005) and investigated regarding its mechanism of TSM relaxing activity and the benefits of its intratracheal administration. In routine clinical practice, in addition to corticosteroids and  $\beta_2$ -adrenoreceptor agonist inhalants, however, no xanthine derivatives are inhaled for the treatment of bronchocontraction or airway inflammation.

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It has been reported that epithelial cells release various smooth muscle inhibitory mediators, such as nitric oxide (NO) and prostaglandin E<sub>2</sub>, as well as so-called epithelium-derived relaxing factor (EpDRF) detected by the coaxial bioassay system (Nijkamp et al., 1993; Folkerts and Nijkamp, 1998). Some reports regarding xanthine derivatives on tracheal epithelium, modulated by NO, have been published (Kanoh et al., 2000; Vajner et al., 2002). In the present study, we characterize the effects of KMUP-3 on TSM, associated specific epithelium-dependent action, soluble guanylate cyclase (sGC) activation, PDE inhibition, and reciprocation of cAMP- and cGMP-dependent protein kinase pathways, and opening of K<sup>+</sup>-channels. The combination of epithelium-derived action (including NO release), PDE inhibition, sGC stimulation and K<sup>+</sup>-channel opening activity is suggested to enhance the results achieved. Additive effects of those pathways result in increase of cGMP/cAMP, co-expression of PKG/PKA and protection activity against TNF- $\alpha$ -induced bronchoconstriction.

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### **Materials and Methods**

#### **Animals**

Male Dunkin Hartley guinea-pigs (350-450 g) were provided by the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and housed under conditions of constant temperature and controlled illumination. Food and water were available *ad libitum*. The study was approved by the Animal Care and Use Committee of the Kaohsiung Medical University.

#### **Pulmonary function**

Guinea-pigs (350-450g) were anesthetized with pentobarbital (40 mg/kg, i.p.) and maintained with further intravenous doses of 2 to 5 mg/kg when required according to methods previously described (Wu et al., 2004). The animal was tethered in a supine position and the trachea was cannulated below the larynx with a short tracheal cannula via a tracheotomy. Tracheal pressure was measured by a catheter connected to the side arm of the tracheal cannula. The carotid artery and jugular vein were cannulated to monitor blood pressure and heart rate and for drug administration, respectively. Throughout the experiment, the body temperature of the guinea-pig was maintained at 37°C. Measurements of pulmonary cardiac function were carried out as previously described (Hsu et al., 1998). Total lung resistance ( $R_L$ ) and dynamic lung compliance ( $C_{dyn}$ ) were measured on a breath-by-breath basis using a computer equipped with an A/D interface (DAS 1600, Buxco) and software (version 1.5.7, Buxco Electronics, Inc, USA). Results obtained from the computer analysis were checked for accuracy by comparison with those calculated manually.

Animals were allowed to stabilize for 10 min following surgical manipulations before the test agent was administered. Intratracheal instillation was performed using a catheter placed through the tracheal tube into the bronchial system. The catheter connected to a 1 ml syringe was inserted into the trachea cannula extending 1 cm beyond the tip of the cannula for

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instillation of drug or vehicle. The instillation was followed by rapid intratracheal injection of 0.5 ml of air to facilitate deposition of drug or vehicle into the lungs as in the modified methods of Chung et al., 2001. The animals were pretreated intratracheally with KMUP-3 (0.5, 1.0, 2.0 mg/kg/150  $\mu$ l saline) or an equivalent volume (150  $\mu$ l) of vehicle 15 min before TNF- $\alpha$  (0.01 mg/kg/300  $\mu$ l saline) was instilled to induce acute bronchoconstriction. In another experiments, KMUP-3 or KMUP-1 (1.0 mg/kg, i.v.) were used to compare the effects on respiratory performance, indicated by changes of tidal volume, total lung resistance ( $R_L$ ) and dynamic lung compliance ( $C_{dyn}$ ).

### **Isolated tracheal preparation and measurement of tension**

Guinea-pigs (350-450 g) were anesthetized with pentobarbital (70 mg/kg) intraperitoneally and killed by a sharp blow on the neck, followed by cervical dislocation. The trachea was excised, cleaned of adhering fat and connective tissue, cut transversely into 4 to 5 rings and then opened by cutting longitudinally through the cartilage rings diametrically opposite the tracheal smooth muscle (Hwang et al., 1999). Then trachea rings were suspended in a 10 ml organ bath containing Krebs solution (NaCl, 118 mM; NaHCO<sub>3</sub>, 25 mM; KCl, 4.7 mM; KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM; MgSO<sub>4</sub>, 1.2 mM; glucose, 11 mM; CaCl<sub>2</sub>, 2.5 mM; pH 7.3-7.4) maintained at 37°C and aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Each tracheal tissue sample was subjected to 2 g initial basal tension. All experiments were carried out in the presence of indomethacin (3  $\mu$ M) and propranolol (1  $\mu$ M) to prevent the formation of prostanoids and to inhibit beta-adrenergic responses, respectively. Isometric tension was recorded with a force displacement transducer (Model FT03, Grass Institute, West Warwick, RI, U.S.A.). Before the start of measurements, all preparations were allowed to equilibrate with frequent washing for 1 hr. They were first contracted with carbachol (1  $\mu$ M) to determine the contractility of preparations. This was also done at the end of each experiment. The preparations were then washed and allowed to equilibrate with Krebs solution for 50 min before being contracted a second time with



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carbachol (1  $\mu\text{M}$ ). When stable constriction to carbachol was reached, concentration-responses of KMUP-3 (10, 30, 100  $\mu\text{M}$ ) were obtained. Data were expressed as percentages of the maximum contractile response to carbachol (1  $\mu\text{M}$ ). In comparison with KMUP-3, theophylline, IBMX, milrinone, rolipram, zaprinast and levcromakalim were respectively examined at 100  $\mu\text{M}$ .

In experiments to examine the role of epithelium in tracheal relaxation, the epithelial cells were removed mechanically by rubbing the internal surface of trachea with a fine silver wire and the removal of the epithelial layer was confirmed by histological examination as previously described (Wu et al., 2004).

To examine the possible mechanism of tracheal relaxant effects of KMUP-3, the tracheal strips were pretreated with a  $\text{K}^+$  channel blocker, TEA (10 mM); a  $\text{K}_{\text{ATP}}$  channel blocker, glibenclamide (1  $\mu\text{M}$ ); voltage-dependent  $\text{K}^+$  channel blocker, 4-aminopyridine (4-AP, 100  $\mu\text{M}$ );  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channel blockers, apamin (1  $\mu\text{M}$ ) and charybdotoxin (ChTX, 0.1  $\mu\text{M}$ ); a sGC inhibitor, ODQ (1  $\mu\text{M}$ ); a NOS inhibitor, L-NAME (100  $\mu\text{M}$ ) and adenylyl cyclase (AC) inhibitor, SQ 22536 (100  $\mu\text{M}$ ) for 30 min prior to the addition of KMUP-3 (10, 30, 100  $\mu\text{M}$ ). In the other experiments, TSMs were precontracted with 60 mM KCl to examine the relaxation achieved by KMUP-3. The KCl solution was prepared by substituting NaCl with KCl (60 mM) in an equimolar amount.

### **Determination of nitrite**

To measure concentrations of nitrite ( $\text{NO}_2^-$ ) in Guinea-pigs TSM strips, TSM strips were pretreated with KMUP-3 (100  $\mu\text{M}$ ) in Krebs solution for 30 min. Then Guinea-pig TSM strips (2 ml/g tissue of methanol) were ultrasonically homogenized in an ice bath and then centrifuged at 10,000 g for 10 min at 4°C. Briefly, harvestable supernatants were reacted with an equal volume of Griess reagents (1% sulfonamide/0.1% N-1-naphthylethylenediamine

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dihydrochloride/5% H<sub>3</sub>PO<sub>4</sub>) for 10 min to form a colored azo dye. The absorbance at 540 nm was detected by a flow-through visible spectrophotometer and an equal volume of methanol was used for blank control.

### **Cell culture of tracheal smooth muscle**

Guinea-pigs (350-450 g) were injected intra-peritoneally with a lethal dose of pentobarbital. The tracheas were excised and cut longitudinally through the cartilage. Using a dissecting microscope, TSM strips were dissected from the surrounding parenchyma. The epithelium was removed from the luminal surface, and bands of TSM were gently separated from the underlying connective tissue. Then TSM strips were chopped into small sections (1 mm<sup>3</sup>) and incubated in Hank's balanced salt solution (NaCl, 138 mM; NaHCO<sub>3</sub>, 4 mM; KCl, 5 mM; KH<sub>2</sub>PO<sub>4</sub>, 0.3 mM; Na<sub>2</sub>HPO<sub>4</sub>, 0.3 mM; glucose, 1.0 mM) with 0.05% elastase type IV and 0.2% collagenase type IV (Invitrogen Co.) for 30 min at 37°C with gentle shaking. The solution of dissociated smooth muscle cells was centrifuged (6 min at 500 g) and the pellet was resuspended in 1:1 Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS), 0.244% NaHCO<sub>3</sub>, and 1% penicillin/streptomycin. Cells were cultured in 25-cm<sup>2</sup> flasks at 37°C in humidified air containing 5% CO<sub>2</sub>. Confluent cells were detached with 0.25% trypsin-0.02% EDTA at 37°C and then subcultured to establish secondary cultures. Cultures were maintained for no more than four passages. They were identified as smooth muscle cells by the typical hill-and-valley appearance, and cellular homogeneity was further confirmed by the presence of smooth muscle specific  $\alpha$ -myosin and  $\alpha$ -actin immunoreactivity. Indirect immunofluorescence staining for a variety of antigens was carried out by first plating the cells on chamber slides, fixing the cells in 3.7% formaldehyde-phosphate buffered saline for 10 minutes, and permeabilizing the cells with phosphate buffered saline 0.1% Triton X-100. Cells were then stained with either a mouse monoclonal antibody directed against the amino terminal 10 amino acids of  $\alpha$ -smooth muscle

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actin or  $\alpha$ -myosin (Boehringer Mannheim, Indianapolis, Indiana). All cells were stained with fluorescein-labeled goat anti-mouse IgG antibody (Boehringer Mannheim). Over 95% of the cell preparation was found to be composed of smooth muscle cells.

### **Phosphodiesterase activity**

PDE activity was determined by the method of Nicholson et al. (1991). PDE3 and PDE5 purified from human platelets and PDE4 purified from human U-937 pronocytic cells were used. Test compound (10  $\mu$ M) or vehicle was incubated with 0.2  $\mu$ g enzyme and 1  $\mu$ M cAMP containing 0.01  $\mu$ M [ $^3$ H]cAMP in Tris buffer pH 7.5 for 20 minutes at 25°C. The reaction was terminated by boiling for 2 minutes and the resulting cAMP was converted to adenosine by the addition of 10 mg/ml snake venom nucleotidase, which is followed by a further incubation at 37°C for 10 minutes. Un-hydrolyzed cAMP was bound to AG1-X2 resin, and the remaining [ $^3$ H]adenosine in the aqueous phase was quantitated by scintillation counting.

Another test compound (10  $\mu$ M) or vehicle was incubated with 3.5  $\mu$ g enzyme and 1  $\mu$ M cGMP containing 0.01  $\mu$ M [ $^3$ H]cGMP in Tris buffer pH 7.5 for 20 minutes at 25°C. The reaction was terminated by boiling for 2 minutes and the resulting GMP was converted to adenosine by the addition of 10 mg/ml snake venom nucleotidase, which was followed by a further incubation at 37°C for 10 minutes. Unhydrolyzed cGMP is bound to AG1-X2 resin, and the remaining [ $^3$ H]guanosine in the aqueous phase was quantitated by scintillation counting.

### **Measurement of cAMP and cGMP**

Intracellular concentrations of cAMP and cGMP in TSMCs were measured as in our previous reports (Wu et al., 2001; Lin et al 2002). In brief, cells were finally grown in 24-well plates (10<sup>5</sup> cells/well). At confluence, monolayer cells were washed with phosphate buffer solution (PBS) and then incubated with KMUP-3 (0.01-100  $\mu$ M) for 20 min at 37°C in medium. Milrinone, rolipram, zaprinast, theophylline or aminophylline (100  $\mu$ M) was added for 20 min.

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The reaction was terminated by replacement of medium with 1 ml of ice-cold 1N hydrochloric acid. Cell suspensions were sonicated and then centrifuged at 2500 g for 15 min at 4°C. Then, the supernatants were lyophilized and the cAMP and cGMP of each sample were determined using commercially available radioimmunoassay kits (Amersham Pharmacia Biotech, Buckinghamshire, England).

### **Expression of PKA and PKG**

The expression of protein kinases A (PKA) and G (PKG) were determined by Western blot as previously described by Wu et al. (2004) and Murthy et al. (2002). Guinea-pig TSMCs were incubated with KMUP-3 for 30 min. Each of the cell lysates, each containing 1 µg of cellular protein, was electrophoresed in 7.5% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA). The membrane was stained with Ponceau S to verify the integrity of the transferred proteins and to monitor the unbiased transfer of all protein samples. The membrane was then washed with 25 ml of TBS (100 mM NaCl, 0.1% and 10 mM Tris-HCl, pH 7.5) for 5 min at room temperature and incubated in 25 ml of Blocking Buffer (TBS plus 0.1% Tween 20 and 5% nonfat milk) overnight at 4°C. In the measurement of protein, the membrane was incubated with appropriate PKA<sub>RI</sub> or PKG<sub>1α1β</sub> primary antibody (diluted 1:250) in 2 ml of Blocking Buffer for 1 h at room temperature, washed three times for 5 min each with 15 ml of TBST (TBS plus 0.1% Tween 20), incubated with horseradish peroxidase (HRP) conjugated secondary antibody (1:10,000) in 15 ml of Blocking Buffer with gentle agitation for 1 h at room temperature, and finally washed three times for 5 min each time with 15 ml of TBST. The membrane was then subjected to chemiluminescence (ECL) (Amersham Life Sciences Inc., Arlington Heights, IL) for the detection of the specific antigen.

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### **Drugs and chemicals**

Levcromakalim was generously supplied by the GlaxoSmithKline Pharmaceuticals and dissolved in ethanol at 10 mM. 4-aminopyridine (4-AP), aminophylline, apamin, carbachol, charybdotoxin (ChTX), forskolin, glibenclamide, Griess reagents, 3-isobutyl-1-methylxanthine (IBMX), indomethacin, Krebs solution reagents, 6N<sup>o</sup>-nitro-L-arginine methyl ester (L-NAME), methylene blue, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), propranolol, 9-(terahydro-2-furanyl)-9H-purin-6-amine (SQ 22536), tetraethylammonium (TEA), theophylline and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were all obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). PKA<sub>RI</sub> and PKG<sub>1 $\alpha$ 1 $\beta$</sub>  antibodies were purchased from Calbiochem Co. (San Diego, CA, U.S.A.). All drugs and reagents were dissolved in distilled water unless otherwise noted. Apamin was dissolved in 0.05 M acetic acid; indomethacin was dissolved in 100 mM sodium carbonate; ChTX, glibenclamide, milrinone, rolipram, zaprinast, IBMX and ODQ were dissolved in DMSO at 10 mM and serially diluted with distilled water; KMUP-3, synthesized in our laboratory, was dissolved in 10% absolute alcohol, 10% propylene glycol and 2% 1N HCl at 10 mM. Serial dilutions were made in distilled water.

### **Statistical evaluation of data**

The results are expressed as mean  $\pm$  s.e. Statistical differences were determined by independent and paired Student's *t*-test in unpaired and paired samples, respectively. Whenever a control group was compared with more than one treated group, the one-way ANOVA or two-way repeated measures ANOVA was used. When the ANOVA manifested a statistical difference, the Dunnett's or Student-Newman-Keuls test was applied. A P value of less than 0.05 was considered to be significant in all experiments. Analysis of the data and plotting of the figures were performed with the aid of software (SigmaPlot Version 8.0 and SigmaStat Version 2.03, Chicago, IL, U.S.A.) run on an IBM-compatible computer.

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### Results

#### Pulmonary function

The baseline values of total lung resistance, dynamic lung compliance and tidal volume were  $0.25 \pm 0.02$  cmH<sub>2</sub>O ml<sup>-1</sup>s<sup>-1</sup>,  $0.31 \pm 0.02$  ml cmH<sub>2</sub>O<sup>-1</sup> and  $1.91 \pm 0.11$  ml, respectively. TNF- $\alpha$ -induced bronchocontraction by intratracheal (IT) instillation increased lung resistance ( $R_L$ ) and decreased dynamic compliance ( $C_{dyn}$ ), respectively (Fig. 2). KMUP-3 (0.5, 1.0, 2.0 mg/kg) instilled through trachea for 15 min prior to TNF- $\alpha$ -induced bronchocontraction could reverse the situation by decreasing  $R_L$  and by increasing  $C_{dyn}$  (Fig. 2). Blood pressure and heart rate were not significantly changed by instillation of KMUP-3 during the recording of lung function. In another experiment, intravenous KMUP-3 (1 mg/kg) more significantly decreased lung resistance ( $R_L$ ) and increased both dynamic compliance ( $C_{dyn}$ ) and respiratory tidal volume than KMUP-1 (Table 1).

#### NOS and epithelium-derived relaxation

As shown in Fig. 3A, KMUP-3 (10, 30, 100  $\mu$ M) produced concentration-dependent relaxations both in epithelium-denuded ( $E_p^-$ ) and epithelium-intact ( $E_p^+$ ) guinea-pig TSM, indicating that KMUP-3-induced epithelium-independent relaxation activity. However, KMUP-3 did demonstrate a significant decrease in the relaxation response after epithelium denudation, suggesting that at least part of the observed effect is epithelium-dependent. Obviously, KMUP-3 completely relaxed the TSM strips at 100  $\mu$ M, achieving even up to 130% relaxation. In addition, the relaxations of guinea-pig  $E_p^+$  TSM elicited by KMUP-3 were significantly inhibited by pretreatment with a NOS inhibitor L-NAME (Fig. 5). The NO<sub>2</sub><sup>-</sup> release reached  $160 \pm 15\%$  (n=3) by KMUP-3 (100  $\mu$ M) in comparison with the basal level ( $0.8 \pm 0.1$   $\mu$ M/mg) in epithelium-intact ( $E_p^+$ ) guinea-pig TSM.

KMUP-3, theophylline, IBMX, milrinone, rolipram and zaprinast (100  $\mu$ M) were all found to cause relaxations in epithelium-intact trachea. Both IBMX and KMUP-3 were more potent at

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100  $\mu\text{M}$  concentrations than theophylline-inducing tracheal relaxation (Fig. 3B). A potassium channel opener, levcromakalim (100  $\mu\text{M}$ ), also displayed relaxation effects on TSM ( $\text{E}_\text{P}^+$ ) (Fig. 3B).

### **$\text{K}^+$ channel activities**

KMUP-3 (10, 30, 100  $\mu\text{M}$ ) caused a concentration-dependent relaxation in carbachol- or high  $\text{K}^+$ -contracted guinea-pig  $\text{E}_\text{P}^+$  TSM. KMUP-3 against carbachol-induced contractions in TSM ( $\text{E}_\text{P}^+$ ) was inhibited by the  $\text{K}^+$  channel blocker, TEA; a  $\text{K}_{\text{ATP}}$  channel blocker, glibenclamide; a voltage-dependent  $\text{K}^+$  channel blocker, 4-AP and  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channel blockers, apamin and charybdotoxin (ChTX) (Fig. 4).

### **Involvement of sGC and adenylate cyclase**

The relaxations of guinea-pig  $\text{E}_\text{P}^+$  TSM elicited by KMUP-3 were attenuated by pretreatment with a sGC inhibitor, ODQ (1  $\mu\text{M}$ ); a NOS inhibitor, L-NAME (100  $\mu\text{M}$ ) or an adenylate cyclase inhibitor, SQ 22536 (100  $\mu\text{M}$ ) (Fig. 5). Additionally, KMUP-3-induced relaxations were dramatically reduced by the pretreatment with L-NAME + SQ 22536, ODQ + L-NAME or SQ 22536 + ODQ (Fig. 5).

### **Inhibition of phosphodiesterase**

The enzyme inhibitory activity of KMUP-3 on PDEs was measured and compared with KMUP-1. As shown in Table 1, KMUP-3 displays different inhibition activities on PDE3, PDE4 and PDE5. The result indicated that the enzyme inhibition activity of KMUP-3 appears to be more potent than KMUP-1, but it has no selectivity between PDE3, 4 or 5. We also observed that the inhibitory effects of theophylline (10  $\mu\text{M}$ ) on PDE3, 4 and 5 were  $8 \pm 1.2$ ,  $7.9 \pm 1.2$  and  $12 \pm 1.5$  % (n=3). The  $\text{IC}_{50}$  values of KMUP-3 for PDE3, PDE4 and PDE5 were  $8.5 \pm 1.5$ ,  $14.3 \pm 2.2$  and  $14.5 \pm 1.8$   $\mu\text{M}$  (n=3), respectively, whereas KMUP-1's  $\text{IC}_{50}$  values for

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those PDEs were  $> 100 \mu\text{M}$ . Under this condition, IBMX was used as a reference agent and its  $\text{IC}_{50}$  values for PDE3, PDE4 and PDE5 were  $6.4 \pm 1.6$ ,  $25.6 \pm 4.9$  and  $30.8 \pm 4.5 \mu\text{M}$  ( $n=3$ ), respectively.

### **Accumulation of cAMP and cGMP**

cAMP and cGMP levels were examined in guinea-pig primary TSMCs. The amounts of basal levels of cAMP and cGMP in the cells were  $62.6 \pm 6.4$  and  $1.18 \pm 0.06 \text{ pmol/mg protein}$ , respectively ( $n=3$ ). KMUP-3 (0.01, 0.1, 1, 10, 100  $\mu\text{M}$ ) significantly increased both cAMP ( $80.2 \pm 2.5$ ,  $91.3 \pm 3.4$ ,  $97.9 \pm 2.5$ ,  $110.7 \pm 4.5$ ,  $127.5 \pm 4.8 \text{ pmol/mg protein}$ ) and GMP ( $1.62 \pm 0.05$ ,  $1.73 \pm 0.01$ ,  $1.75 \pm 0.04$ ,  $1.86 \pm 0.05$ ,  $1.98 \pm 0.09 \text{ pmol/mg protein}$ ) levels as compared with each basal value in guinea-pig primary TSMCs. Moreover, we compared both cAMP and GMP levels of KMUP-3 to theophylline, aminophylline, milrinone, rolipram and zaprinast at 100  $\mu\text{M}$  (Fig. 6). KMUP-3, theophylline, aminophylline and zaprinast significantly enhanced the amounts of cGMP, but this was not observed in milrinone or rolipram for selective PDE3 and PDE4 inhibitions, respectively. On the other hand, KMUP-3, theophylline, aminophylline, milrinone and rolipram all elicited significant elevations of cAMP accumulation, but this was not so for the selective PDE5 inhibitor zaprinast (Fig. 6). The elevated cAMP levels induced by KMUP-3 and forskolin (100  $\mu\text{M}$ ), an adenylate cyclase activator, were inhibited by pretreatment with the adenylate cyclase inhibitor, SQ 22536 (100  $\mu\text{M}$ ) (Table 2). Correspondingly, the rise of cGMP accumulation by KMUP-3 was abolished by pretreatment with the NOS inhibitor L-NAME (100  $\mu\text{M}$ ) or the sGC inhibitor ODQ (10  $\mu\text{M}$ ) respectively (Table 2).

### **Expression of PKA and PKG**

Expression of immunoreactive  $\text{PKA}_{\text{RI}}$  is shown in Fig. 7A. Monoclonal antibody to  $\text{PKA}_{\text{RI}}$



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recognized a band at 49 KDa in extracts of tracheal smooth muscle. KMUP-3 (1  $\mu$ M) stimulated the expression of immunoreactive PKA<sub>RI</sub> protein. Treatment with SQ 22536 (100  $\mu$ M) 30 min prior to KMUP-3 (1  $\mu$ M) attenuated the expression of PKA<sub>RI</sub> (Fig. 7A). Both 8-Br-cAMP and KMUP-3 (1  $\mu$ M) increased the expression of PKA<sub>RI</sub>; however, pretreatment with ODQ (10  $\mu$ M) 30 min prior to KMUP-3 did not significantly affect the PKA protein expression by KMUP-3 (unpublished data). Expression of immunoreactive PKG<sub>1 $\alpha$ 1 $\beta$</sub>  is shown in Fig. 7B. Polyclonal antibody to PKG<sub>1 $\alpha$ 1 $\beta$</sub>  recognized a band at 75 KDa in extracts of tracheal smooth muscle. Both 8-Br-cGMP and KMUP-3 (1  $\mu$ M) increased the expression of PKG<sub>1 $\alpha$ 1 $\beta$</sub> , and pretreatment with ODQ (10  $\mu$ M) 30 min prior to KMUP-3 (1  $\mu$ M) significantly reduced the PKG protein induced by KMUP-3 (Fig. 7B).

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### Discussion

Relaxation of smooth muscles are through activation of AC and sGC and accumulation of cAMP and cGMP (Ignarro and Kadowitz, 1985). cAMP and cGMP are degraded by PDE, which catalyzes the conversion of cAMP to 5'-AMP and cGMP to 5'-GMP and therefore leads to decreases in intracellular cAMP or cGMP levels. Among them, PDE3 is inhibited by cGMP, indirectly leading to the increase of cAMP. Accumulations of cAMP and cGMP further induced the increases of PKG and PKA. In this study, KMUP-3 significantly increases the expression of PKA and PKG and thus confirms the contents of cAMP and GMP in TSMCs. The rank order of relaxant activity at 100  $\mu$ M was KMUP-3 > KMUP-1 (Wu et al., 2004) > IBMX > milrinone > rolipram > zaprinast, theophylline and levcromakalim (Fig. 3B). This result is adaptable to enzyme inhibition activity and IC<sub>50</sub> of KMUP-3 on PDE3, 4 and 5, in comparison with previous KMUP-1. We suggest that the higher inhibition (%) of KMUP-3 than KMUP-1 on PDE3, 4 and 5 favors better respiratory performance as shown by increased respiratory tidal volume, decreased R<sub>L</sub> and increased C<sub>dyn</sub> (Table 1).

Inhibition of PDE3 with specific inhibitors has resulted in relaxation of TSM, either on spontaneous or carbachol-induced tone (Torphy and Cieslinski, 1990). In contrast, the PDE4 inhibitor rolipram did not reduce spontaneous tone but relaxed carbachol-induced tone. To date, some of the highly selective PDE inhibitors have been disappointing. One reason for this may be the presence of significant amounts of other PDE isoenzymes, existing in target tissues (Giembycz et al., 1996). Combination inhibition on PDE3 and PDE4 may thus be more effective than either alone in relaxing TSM, suggesting that the additive effects of PDE3 and PDE4 inhibition are able to optimize the bronchodilatory benefits (Giembycz and Barnes, 1991; de Boer et al., 1992). KMUP-3 is found to have PDE3 and PDE4 inhibitions, and therefore we suggest that it can effectively relax TSM. KMUP-3 showed a stronger inhibition on PDE3 and PDE4 than KMUP-1. Indeed, inhibitors with PDE3 and PDE4 inhibition have attracted considerable interest as potential anti-asthmatic agents because they possess both

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bronchodilatory and anti-inflammatory activities (Barnes, 1996). However, in addition to PDE inhibition, previous epithelium-dependent cGMP enhancing activity of KMUP-1 encourages us to further investigate the benefits of KMUP-3, administered by intratracheal instillation, for the treatment of airway dysfunction caused by bronchocontraction.

In guinea-pig trachea, the NO donor has been proven to increase cGMP and it enhances the increase of cAMP induced by the PDE4 inhibitor rolipram, (Turner et al., 1994). Moreover, the PDE5 inhibitors sildenafil and zaprinast, with their cGMP increasing activities, have been proven to induce relaxation in TSM (Hirose et al., 2001; Ellis and Conanan, 1995). KMUP-1, with NO-independent sGC activation and PDE inhibition activities, also leads to cGMP-mediated inhibition of TSMs (Wu et al., 2004). In the present study, KMUP-3 is further suggested to have PDE inhibition and action of epithelium-derived NO to increase cGMP and cAMP. KMUP-3 retains partial PDE4 inhibition activity, which increases cAMP, and subsequently the expression of PKA in TSMs. Inhibition of PDE5 activity by KMUP-3 may increase cGMP, which has proven to inhibit PDE3 activity, resulting increase of cAMP, and subsequently the expression of PKG and partial PKA (Fig. 8).

NO is capable of stimulating  $K^+$  channels in smooth muscle cells, such as those which are  $Ca^{+2}$ -activated (Bolotina et al., 1994; Koh et al., 1995), leading to hyperpolarization and consequently muscle relaxation (Lincoln and Cornwell, 1991; Robertson et al., 1993), including in airway (Abderrahmane et al., 1998).  $K^+$  channel openers, acting by liberation of NO, have been shown to relax TSM and to produce bronchodilation. Based on these results, we sought to explore whether the opening of  $K^+$  channels and associated expression of cGMP-dependent protein kinase accounts for the relaxation caused by KMUP-3 in the guinea-pig trachea. Evidence indicates that KMUP-3-induced relaxation was sensitive to various  $K^+$  channel blockers. Therefore, it is likely that KMUP-3-induced relaxation is partly mediated by NO-induced effects, including stimulating  $K^+$  efflux resulting in hyperpolarization (Wu et al., 2005) and thereafter accumulation of cGMP leading to the expression of PKG and

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reduction of  $\text{Ca}^{2+}$  influx. KMUP-3-induced TSM relaxation was significantly reduced by pretreatment with ODQ. This fact suggests activation of sGC and stimulation of cGMP-dependent mechanism could be involved in KMUP-3-induced relaxation. Basically, the cAMP pathway was involved in KMUP-3-induced TSM relaxations, which were inhibited by a selective AC inhibitor SQ 22536 (Fig. 5). However, in addition to PDE4 inhibition by KMUP-3 to increase cAMP, we can not neglect the contribution from cGMP-induced inhibition on PDE3. Furthermore, SQ 22536 abolished the cAMP accumulation in response to both KMUP-3 and forskolin in TSMCs (Table 2). These facts indicate that KMUP-3-induced relaxation is partially mediated by activation of AC. PKG is the action target of cGMP (Komalavilas and Lincoln, 1994). It is reasonable that the expression of PKG accompanies the increased cGMP. Since KMUP-3 induced TSM relaxation and increased cGMP, the expression of PKG is thus realizable. Pretreatment with ODQ abolished the KMUP-3-induced  $\text{PKG}_{1\alpha\beta}$  expression; moreover, the  $\text{PKA}_{\text{RI}}$  expression caused by KMUP-3 also showed similar responses but was inhibited by AC inhibitor SQ 22536 (Fig. 7A). Notably, KMUP-3-induced TSM relaxation is associated with  $\text{K}^+$  channel opening activity (Somlyo and Somlyo, 1994), resulting in increased expression of PKA and PKG (Fig. 8).

TNF- $\alpha$  has been shown to induce hyper-responsiveness and bronchocontraction of TSM (Wu et al., 2004). As a member of cytokines, TNF- $\alpha$  attracts and activates nonspecific inflammatory macrophages and neutrophils during occurrence of type IV hypersensitivity, usually synthesized in response to infection, including that found in airway system. Since KMUP-3 has PDE3, PDE4 and PDE5 inhibition activities, we thus investigated the benefits of KMUP-3 to protect against intratracheal TNF- $\alpha$ -induced bronchoconstriction. In the present study, exogenous TNF- $\alpha$  certainly increased  $R_L$  components and decreased  $C_{\text{dyn}}$  factor. However, these situations are significantly reversed by KMUP-3 (Fig. 2). These facts imply that KMUP-3 has the potential to protect against TNF- $\alpha$ -induced tracheal over-contractility in the airway.

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KMUP-3 is suggested to accumulate cAMP/cGMP and enhance  $K^+$  efflux, leading to reduction of  $Ca^{2+}$  influx-associated contractility in TSM (Fig. 8). Whether accumulation of cAMP or cGMP caused by KMUP-3 is attributable to  $K^+$  efflux in TSMC remains to be resolved. In this study, the expression of  $PKG_{1\alpha\beta}$  and  $PKA_{RI}$ , able to be activated by cGMP/cAMP, was suggested to mediate the  $K^+$  channel opening activity through their cGMP/cAMP increasing activities.

The combination of the multiple actions of KMUP-3 above may thus contribute to significant relaxation of carbachol-induced TSM contraction *in vitro* and protect against TNF- $\alpha$ -induced increases of  $R_L$  and decreases of  $C_{dyn}$  *in vivo*. It is suggested that PDE inhibition, cAMP/cGMP increasing, and  $K^+$  channel activities of KMUP-3 are the crucial determinants for its relaxation effects on TSM. Particularly, more PDE4 inhibiting activity by KMUP-3 than by KMUP-1 may favor the former as useful in increasing respiratory performance. We also suggest that the *in vivo* activities of KMUP-3 in airway are consistent with the proposed mode of action derived from the *in vitro* experiments. Intratracheal instillation with KMUP-3, leading to increase of respiratory performance with protection activity against TNF- $\alpha$ -induced tracheal contractility may indicate its considerable usefulness and may be the challenge for future inhalation research.

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### **Footnotes**

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### Legends for Figures

**Fig. 1.** Chemical structures of KMUP-3 and KMUP-1.

**Fig. 2.** Lung resistance ( $R_L$ ) and dynamic compliance ( $C_{dyn}$ ) effects of KMUP-3 on TNF- $\alpha$ -induced (0.01 mg/kg) bronchoconstriction by intratracheal instillation. Each value represents the mean  $\pm$  S.E., from 6 independent experiments. \* $P < 0.05$  as compared with the KMUP-3 solvent + TNF- $\alpha$  (ANOVA followed by Student-Newman-Keuls test).

**Fig. 3.** (A) Effects of KMUP-3 on carbachol (1  $\mu$ M)-precontracted guinea-pig trachea in the epithelium intact ( $Ep^+$ ) and denuded ( $Ep^-$ ) tissues. \* $P < 0.05$ ,  $n=8$ , as compared with the  $Ep^+$  (ANOVA followed by Tukey test). (B) Effects of KMUP-3, theophylline, IBMX, milrinone, rolipram, zaprinast and levcromakalim (100  $\mu$ M) on carbachol (1  $\mu$ M)-precontracted guinea-pig epithelial-intact trachea.

**Fig. 4.** Effects of KMUP-3 on carbachol (1  $\mu$ M)-precontracted guinea-pig epithelial-intact trachea in the absence or presence of various potassium channel blockers. \* $P < 0.05$ ,  $n=8$ , as compared with the control (ANOVA followed by Tukey test).

**Fig. 5.** Effects of KMUP-3 on carbachol (1  $\mu$ M)-precontracted guinea-pig epithelial-intact trachea in the absence or presence of L-NAME (100  $\mu$ M), ODQ (1  $\mu$ M) or SQ 22536 (100  $\mu$ M). \* $P < 0.05$ ,  $n=8$ , as compared with the control and # $P < 0.05$ ,  $n=4$ , as compared with the L-NAME, ODQ or SQ 22536, respectively (ANOVA followed by Tukey test).

**Fig. 6.** Effects of KMUP-3, milrinone, rolipram, zaprinast, theophylline and aminophylline (100  $\mu$ M) on cGMP and cAMP levels in guinea-pig tracheal smooth muscle cells. Each value

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represents the mean  $\pm$  S.E., from 3 independent experiments. \* $P < 0.05$  as compared with the vehicle (ANOVA followed by Dunnett's test).

**Fig. 7.** Representative Western blots and the corresponding group data depicting PKA<sub>RI</sub> and PKG<sub>I $\alpha$ 1 $\beta$</sub>  protein expression in culture guinea-pig tracheal smooth muscle cells incubated for 30 minutes in the absence (vehicle) and presence of (A) 8-Br-cAMP (1  $\mu$ M), SQ 22536 (100  $\mu$ M), SQ 22536 + KMUP-3 (1  $\mu$ M) or KMUP-3 (1  $\mu$ M); (B) 8-Br-cGMP (1  $\mu$ M), ODQ (10  $\mu$ M), ODQ + KMUP-3 or KMUP-3. SQ 22536 or ODQ was treated 30 minutes prior to KMUP-3. \* $P < 0.05$  as compared with the vehicle, # $P < 0.05$  as compared with the SQ 22536 + KMUP-3 and + $P < 0.05$  as compared with the ODQ + KMUP-3 (n=3) (ANOVA followed by Dunnett's test).

**Fig. 8.** Proposed mechanism of action of KMUP-3 on the intracellular cGMP and cAMP synthesis and metabolism, K<sup>+</sup> channels opening and phosphodiesterase inhibition. Increased cAMP and cGMP then activate the protein kinases PKA and PKG cascades, and thus enhance the K<sup>+</sup> efflux, leading to attenuation of Ca<sup>2+</sup> influx-associated contractility in tracheal smooth muscle cells. AC indicates adenylate cyclase. Cycles symbols indicate as KMUP-3 effects.

**Table 1.** Effects of KMUP-3 and KMUP-1 on phosphodiesterases (PDEs) inhibitory activity, and lung resistance, dynamic compliance and tidal volume maximum changes.

Compound	PDE3	PDE4	PDE5	Lung resistance ( $R_L$ )	Dynamic compliance		Tidal volume
	inhibition	inhibition	inhibition	(max. change of baseline)	( $C_{dyn}$ )	(max. change of baseline)	(max. change of baseline)
<b>KMUP-1</b>	23 ± 2.2%	18 ± 2.3%	29 ± 2.5%	-20 ± 3.5%		35 ± 4.5%	2.8 ± 0.2 %
<b>KMUP-3</b>	<sup>#</sup> 55 ± 2.5%	<sup>#</sup> 48 ± 2.1%	<sup>#</sup> 48 ± 1.9%	<sup>#</sup> -55 ± 4.5%		<sup>#</sup> 50 ± 5.5%	<sup>#</sup> 21.7 ± 0.5%

The enzyme inhibitory activity were measured by 10  $\mu$ M KMUP-1 and KMUP-3 (n=3) on various phosphodiesterases (PDEs). Lung resistance ( $R_L$ ), dynamic compliance ( $C_{dyn}$ ) and tidal volume were measured after i.v. injection of KMUP-1 or KMUP-3 (1 mg/kg). <sup>#</sup>P<0.05 as compared with KMUP-1, respectively (n = 3).

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**Table 2.** Effects of KMUP-3 and Forskolin (100  $\mu$ M) on cyclic AMP and cyclic GMP levels in TSMCs, in the absence and presence of SQ 22536 (100  $\mu$ M), L-NAME (100  $\mu$ M) or ODQ (10  $\mu$ M).

Treatment	Cyclic AMP (pmol/mg protein)
Vehicle	62.60 $\pm$ 6.40
SQ 22536	74.32 $\pm$ 7.56
Forskolin	119.15 $\pm$ 5.11*
Forskolin plus SQ 22536	94.33 $\pm$ 3.42 <sup>#</sup>
KMUP-3	128.72 $\pm$ 4.57*
KMUP-3 plus SQ 22536	74.51 $\pm$ 3.18 <sup>#</sup>

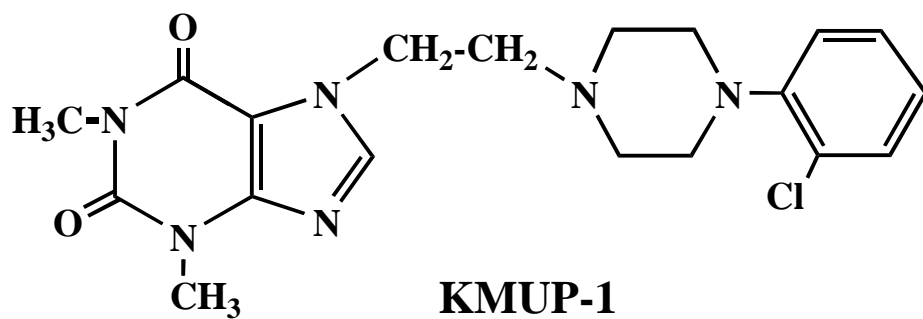
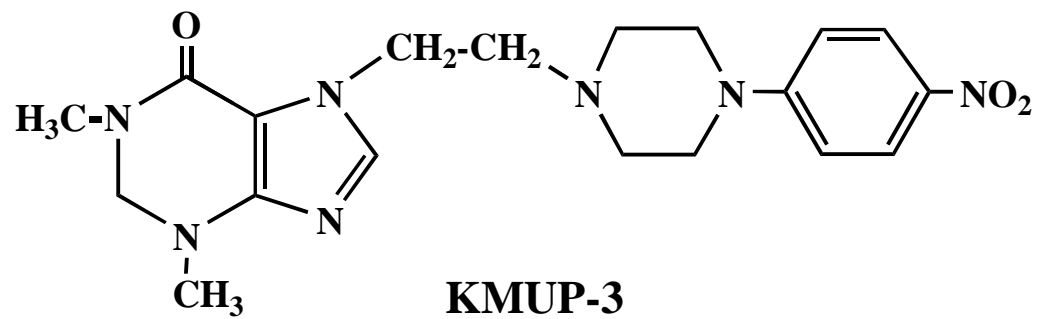
	Cyclic GMP (pmol/mg protein)
Vehicle	1.18 $\pm$ 0.06
KMUP-3	1.98 $\pm$ 0.08*
L-NAME	1.12 $\pm$ 0.02
KMUP-3 plus L-NAME	1.28 $\pm$ 0.01 <sup>#</sup>
ODQ	1.12 $\pm$ 0.03
KMUP-3 plus ODQ	1.35 $\pm$ 0.04 <sup>#</sup>

Each value represents the mean  $\pm$  SEM of 3 independent experiments.

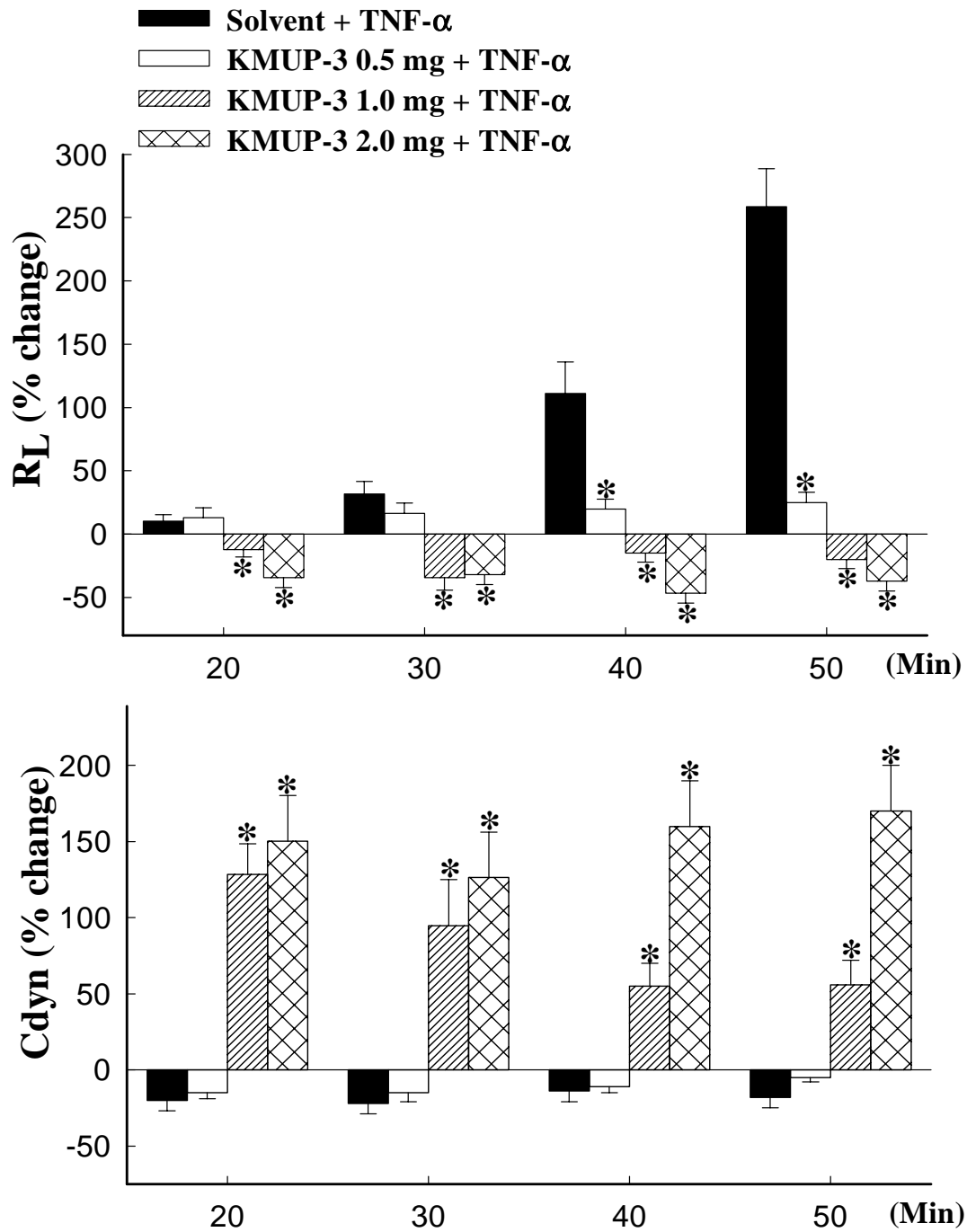
\*indicates  $P < 0.05$  as compared with the vehicle, ANOVA followed by Dunnett's test.

<sup>#</sup>indicates  $P < 0.05$  as compared with Forskolin or KMUP-3.

Figure 1

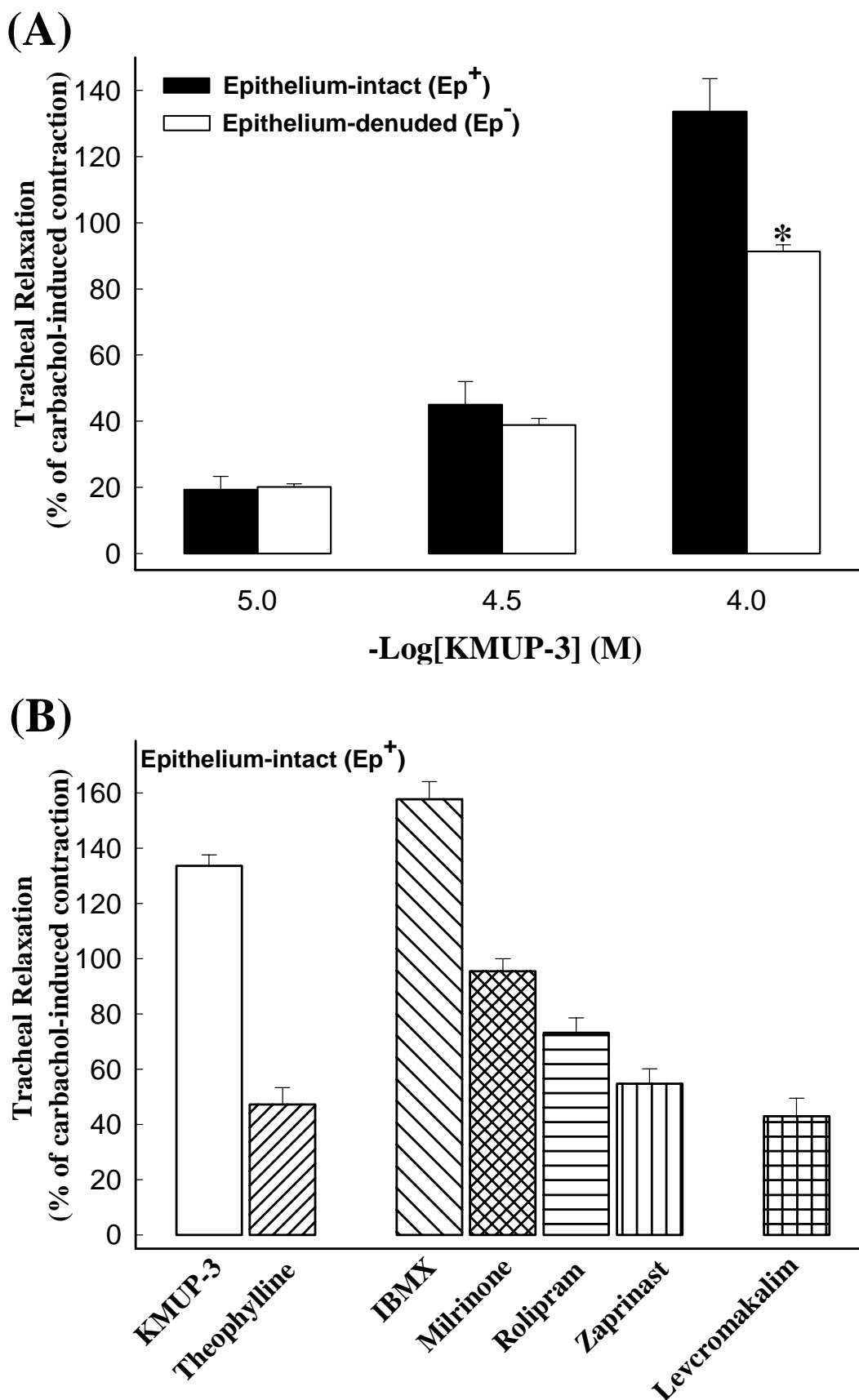


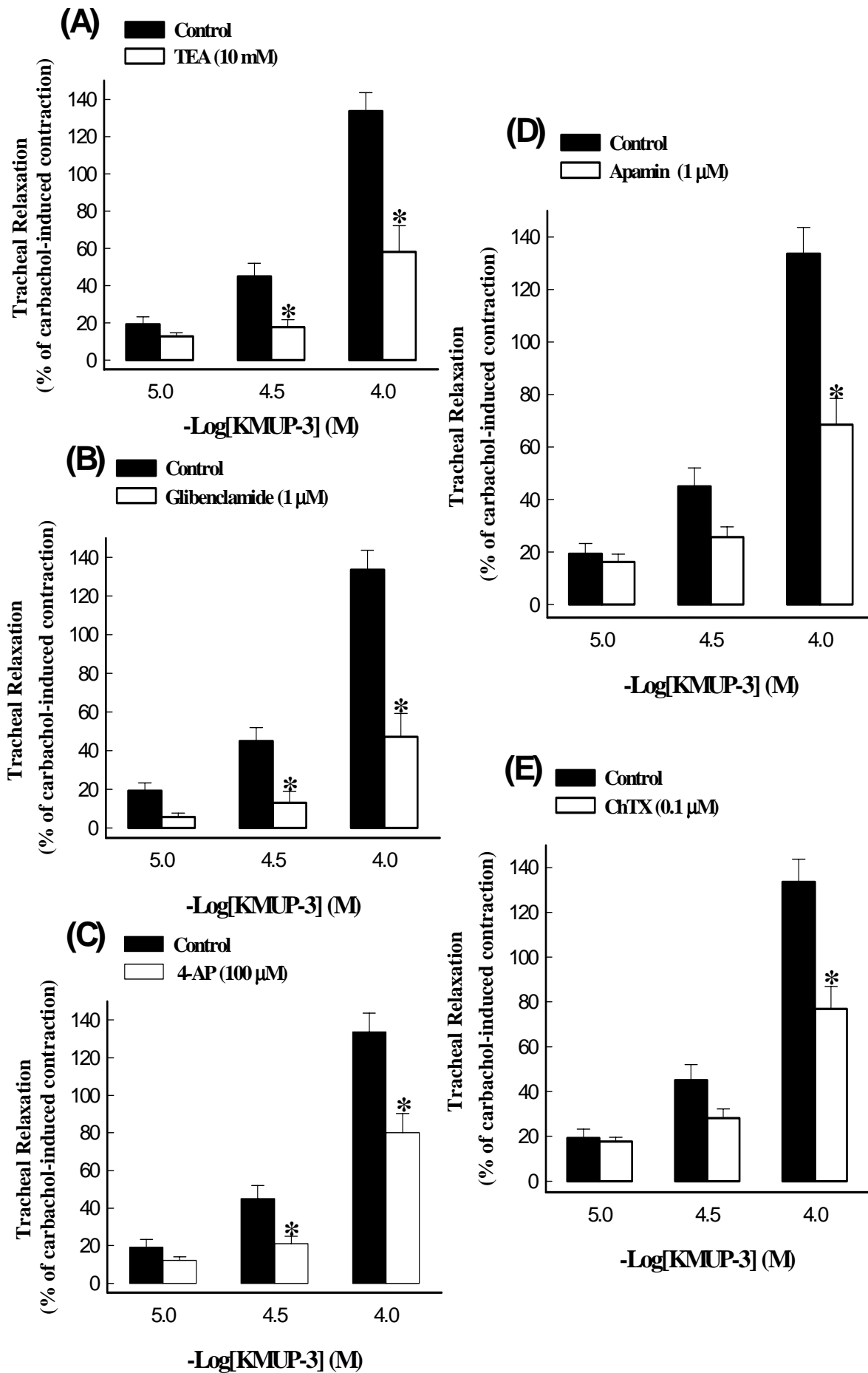
**Figure 2**





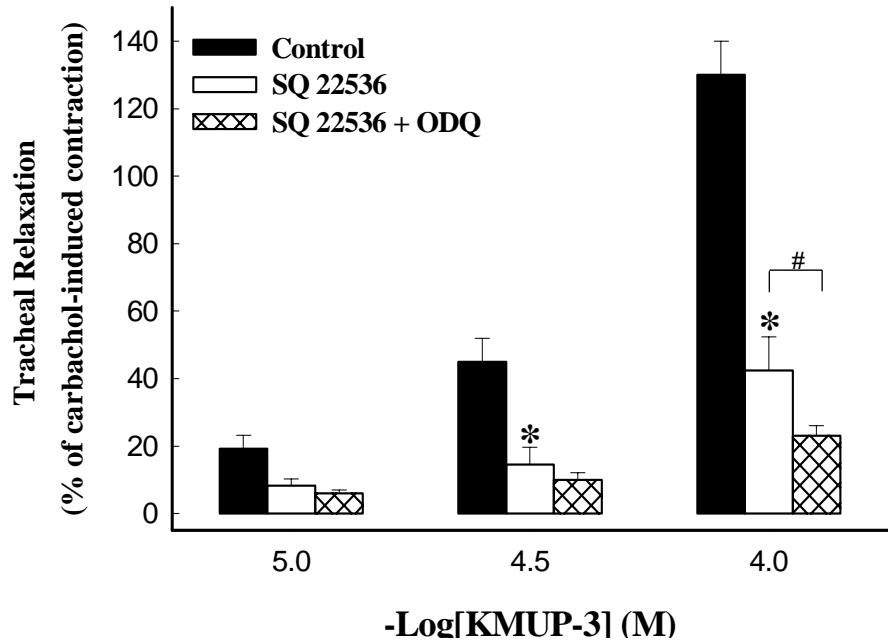
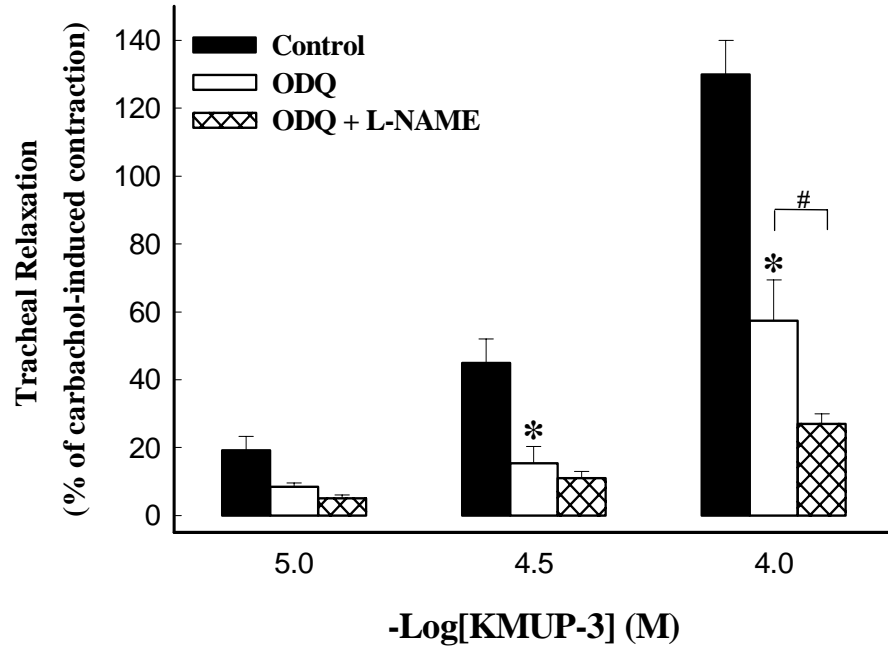
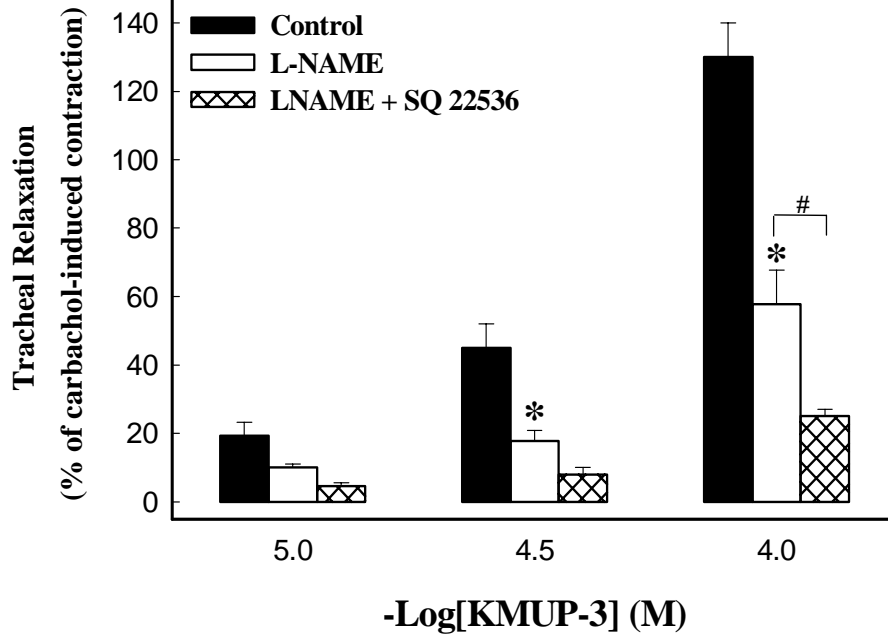
**Figure 3**



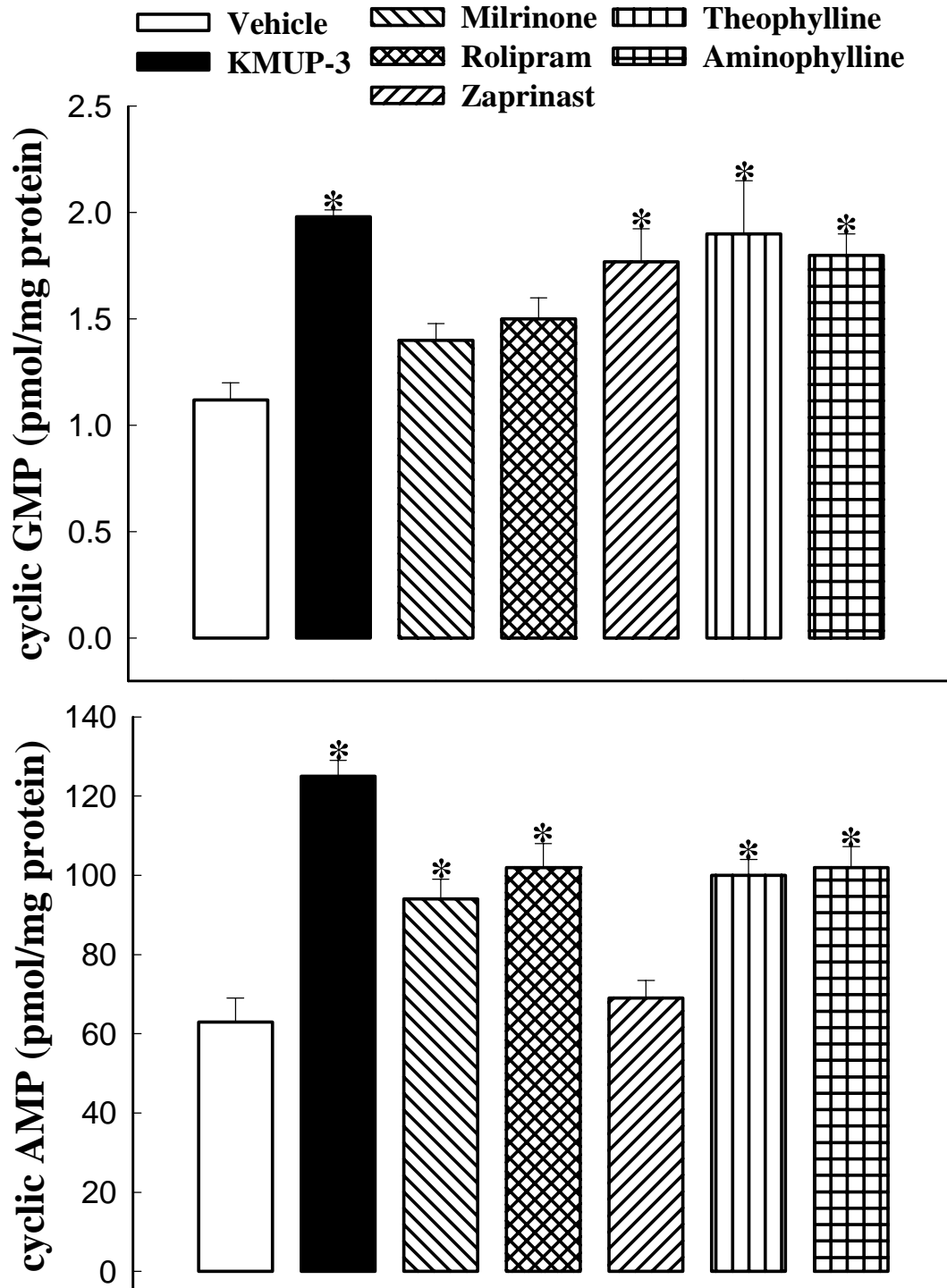


# Figure 5

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**Figure 6**



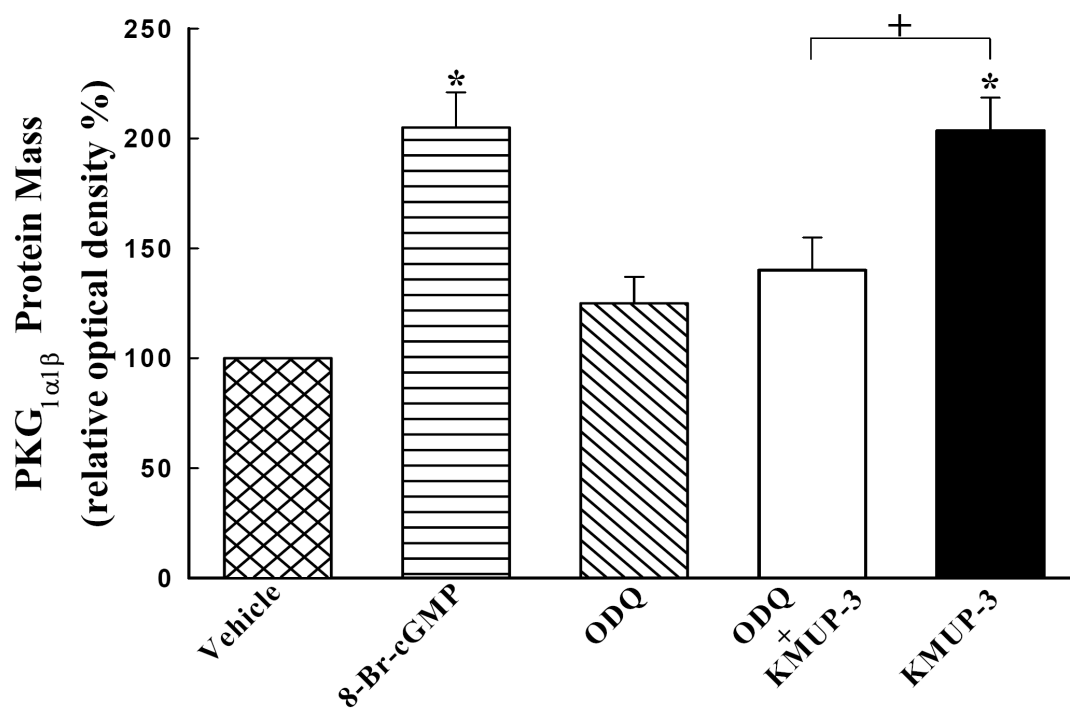
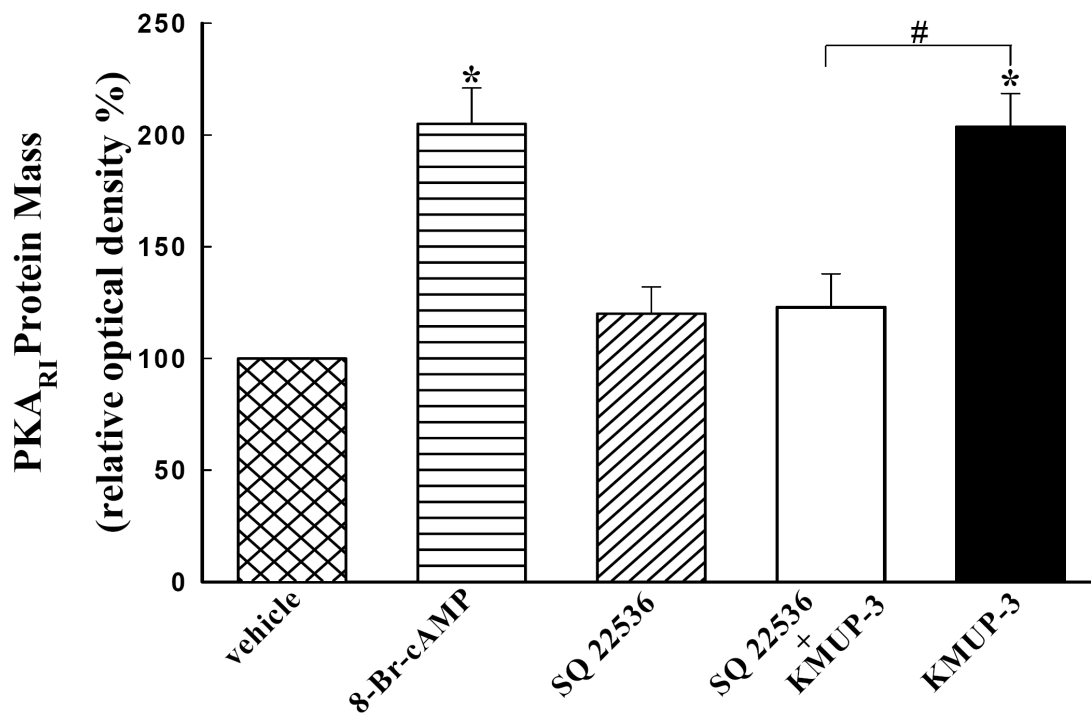
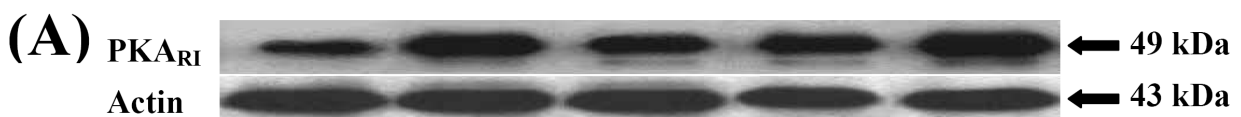


Figure 8

