A Xanthine-Based Epithelium-Dependent Airway Relaxant KMUP-3 (7-[2-[4-(4-Nitrobenzene)piperazinyl]ethyl]-1,3-dimethylxanthine) Increases Respiratory Performance and Protects against Tumor Necrosis Factor-α-Induced Tracheal Contraction, Involving Nitric Oxide Release and Expression of cGMP and Protein Kinase G

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ABSTRACT

KMUP-3 (7-[2-[4-(4-nitrobenzene)piperazinyl]ethyl]-1,3-dimethyl-xanthine) was investigated in guinea pig tracheal smooth muscle. Intratracheal instillation of tumor necrosis factor (TNF)-α (0.01 mg/kg/300 μl) 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 μM)-caused relaxations were attenuated by epithelium removal and by pretreatments with an inhibitor of K^+ channel, tetraethylammonium (10 mM); K_{ATP} channel, glibenclamide (1 μM); voltage-dependent K^+ channel, 4-aminopyridine (100 μM); Ca^{2+}-dependent K^+ channel, charybdotoxin (0.1 μM) or apamin (1 μM); soluble guanylate cyclase (sGC), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1 μM); nitric-oxide (NO) synthase, N^ω-nitro-L-arginine methyl ester (L-NAME, 100 μM); and adenylate cyclase, SQ 22536 [9-(terahydro-2-furanyl)-9H-purin-6-amine] (100 μM). KMUP-3 (0.01–100 μM) 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 μM) induced expression of protein kinase A $(PKA)_{RI}$ and protein kinase G $(PKG)_{1\alpha 1\beta}$ in TSMCs. SQ 22536 inhibited KMUP-3-induced expression of PKA_{RI}. On the contrary, ODQ inhibited KMUP-3-induced expression of $PKG_{1\alpha 1\beta}$. In epithelium-intact trachea, KMUP-3 increased the NO release. Activation of sGC, NO release, and inhibition of phosphodiesterases in TSMCs by KMUP-3 may result in increases of intracellular cGMP and cAMP, which subsequently activate PKG and PKA, efflux of K^+ ion, and associated reduction in Ca^{2+} influx in vitro, indicating the action mechanism to protect against TNF-α-induced airway dysfunction in vivo.

Xanthine derivatives, including theophylline, have long been recognized as bronchodilators because of their nonspecific 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 immunoresponses (Caramori and Adcock, 2003). The immunopharmacologic effects of xanthine derivatives have recently become a particular subject of interest (Kobayashi et al., 2004). Nevertheless, several important

ABBREVIATIONS: PDE, phosphodiesterase; PKG, protein kinase G; TNF, tumor necrosis factor; TSMC, tracheal smooth muscle cell; KMUP-1, 7-[2-[4-(2-chlorophenyl)piperazinyl] ethyl]-1,3-dimethyl xanthine; TSM, tracheal smooth muscle; KMUP-3, 7-[2-[4-(4-nitrobenzene)piperazinyl]-ethyl]-1,3-dimethylxanthine; NO, nitric oxide; sGC, soluble guanylyl cyclase; PKA, protein kinase A; IBMX, 3-isobutyl-1-methylxanthine; TEA, tetraethylammonium; 4-AP, 4-aminopyridine; ChTX, charybdotoxin; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; NOS, nitric-oxide synthase; L-NAME, N^ω-nitro-L-arginine methyl ester; AC, adenylate cyclase; SQ 22536, 9-(terahydro-2-furanyl)-9H-purin-6-amine; TBS, Tris-buffered saline; ANOVA, analysis of variance; 8-Br-cGMP; 8-bromo-cGMP.

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).

Likewise, the properties of xanthine derivatives to increase epithelium-dependent cGMP and protein kinase G (PKG) expression, which suppress tumor necrosis factor-α (TNF-α)-induced response in tracheal smooth muscle cells (TSMCs), have not been explored thoroughly. TNF-α has been described to up-regulate 5-hydroxytryptamine$_{2A}$-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, KMUP-1 has been stated to possess airway relaxation activities (Wu et al., 2004). To create a more hopeful xanthine-based tracheal smooth muscle (TSM) relaxant, 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 β_2-adrenoreceptor agonist inhalants, however, no xanthine derivatives are inhaled for the treatment of bronchocontraction or airway inflammation.

It has been reported that epithelial cells release various smooth muscle inhibitory mediators, such as nitric oxide (NO) and prostaglandin E$_2$, as well as so-called epithelium-derived relaxing factor 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$^+$-channels. The combination of epithelium-derived action (including NO release), PDE inhibition, sGC stimulation, and K$^+$-channel opening activity is suggested to enhance the results achieved. Additive effects of those pathways result in increase of cGMP/cAMP, coexpression of PKG/protein kinase A (PKA), and protection activity against TNF-α-induced bronchoconstriction.
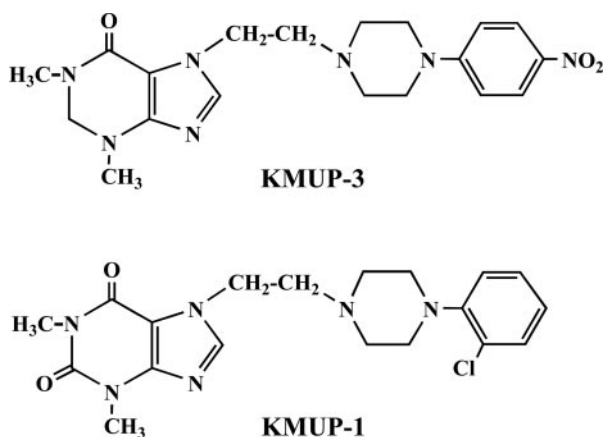

Fig. 1. Chemical structures of KMUP-3 and KMUP-1.

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 (350–450 g) were anesthetized with pentobarbital (40 mg/kg i.p.) and maintained with further i.v. 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 Electronics, Inc., Wilmington, NC) and software (version 1.5.7; Buxco Electronics, Inc.). 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 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, and 2.0 mg/kg/150 μl of saline) or an equivalent volume (150 μl) of vehicle 15 min before TNF-α (0.01 mg/kg/300 μl 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 R_L, and C_{dyn}.

Isolated Tracheal Preparation and Measurement of Tension. Guinea pigs (350–450 g) were anesthetized with pentobarbital (70 mg/kg) i.p. 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 four to five 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 (118 mM NaCl, 25 mM NaHCO$_3$, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 11 mM glucose, and 2.5 mM CaCl$_2$, pH 7.3–7.4) maintained at 37°C and aerated with 95% O$_2$ and 5% CO$_2$. Each tracheal tissue sample was subjected to 2 g of initial basal tension. All experiments were carried out in the presence of indomethacin (3 μM) and propranolol (1 μM) to prevent the formation of prostanoids and to inhibit β-adrenergic responses, respectively. Isometric tension was recorded with a force displacement transducer (model FT03; Grass Instruments, Quincy, MA). Before the start of measurements, all preparations were allowed to equilibrate with frequent washing for 1 h. They were first contracted with carbachol (1 μM) 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 carbachol (1 μM). When stable constriction to carbachol was reached, concentration responses of KMUP-3 (10, 30, and 100 μM) were obtained. Data were expressed as percentages of the maximum contractile response to carbachol (1 μM). In comparison with KMUP-3, theophylline,

3-isobutyl-1-methylxanthine (IBMX), milrinone, rolipram, zaprinast, and levcromakalim, respectively, were examined at 100 μM.

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 K^+ channel blocker, tetraethylammonium (TEA; 10 mM); a K_{ATP} channel blocker, glibenclamide (1 μM); voltage-dependent K^+ channel blocker, 4-aminopyridine (4-AP, 100 μM); Ca^{2+}-dependent K^+ channel blockers, apamin (1 μM) and charybdotoxin (ChTX; 0.1 μM); a sGC inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 1 μM); an NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME; 100 μM); and an adenylate cyclase (AC) inhibitor, SQ 22536 (100 μM) for 30 min prior to the addition of KMUP-3 (10, 30, and 100 μM). 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 (NO_2^-) in guinea pig TSM strips, TSM strips were pretreated with KMUP-3 (100 μM) 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 10,000g for 10 min at 4°C. Briefly, harvestable supernatants were reacted with an equal volume of Griess reagents (1% sulfonamide/0.1% N-L-naphthylethylenediamine dihydrochloride/5% H_3PO_4) 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 i.p. 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^3) and incubated in Hanks' balanced salt solution (138 mM NaCl, 4 mM NaHCO$_3$, 5 mM KCl, 0.3 mM KH$_2$PO$_4$, 0.3 mM Na$_2$HPO$_4$, and 1.0 mM glucose) with 0.05% elastase type IV and 0.2% collagenase type IV (Invitrogen, Carlsbad, CA) for 30 min at 37°C with gentle shaking. The solution of dissociated smooth muscle cells was centrifuged (6 min at 500g), and the pellet was resuspended in 1:1 Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% fetal bovine serum, 0.244% NaHCO$_3$, and 1% penicillin/streptomycin. Cells were cultured in 25-cm^2 flasks at 37°C in humidified air containing 5% CO$_2$. 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 α-myosin and α-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 min, and permeabilizing the cells with phosphate-buffered saline and 0.1% Triton X-100. Cells were then stained with either a mouse monoclonal antibody directed against the amino-terminal 10 amino acids of α-smooth muscle actin or α-myosin (Roche Diagnostics, Indianapolis, IN). All cells were stained with fluorescein-labeled goat anti-mouse IgG antibody (Roche Diagnostics). 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 μM) or vehicle was incubated with 0.2 μg of enzyme and 1 μM cAMP containing 0.01 μM [^{3}H]cAMP in Tris buffer, pH 7.5, for 20 min at 25°C. The reaction was terminated by boiling for 2 min, 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 min. Unhydrolyzed 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 μM) or vehicle was incubated with 3.5 μg of enzyme and 1 μM cGMP containing 0.01 μM [^{3}H]cGMP in Tris buffer, pH 7.5, for 20 min at 25°C. The reaction was terminated by boiling for 2 min, and the resulting GMP 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 min. Unhydrolyzed cGMP was 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 (10^5 cells/well). At confluence, monolayer cells were washed with phosphate-buffered saline and then incubated with KMUP-3 (0.01–100 μM) for 20 min at 37°C in medium. Milrinone, rolipram, zaprinast, theophylline, or aminophylline (100 μM) was added for 20 min. The reaction was terminated by replacement of medium with 1 ml of ice-cold 1 N hydrochloric acid. Cell suspensions were sonicated and then centrifuged at 2500g 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 (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Expression of PKA and PKG. The expression of PKA and PKG was 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-polyacrylamide gel electrophoresis and then transferred to polyvinylidene diflouride membrane (Millipore Corporation, Billerica, 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$_{RI}$ or PKG$_{1\alpha1\beta}$ 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 Tris-buffered saline/Tween 20 (TBS plus 0.1% Tween 20), incubated with horseradish peroxidase-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 Tris-buffered saline/Tween 20. The membrane was then subjected to enhanced chemiluminescence (GE Healthcare) for the detection of the specific antigen.
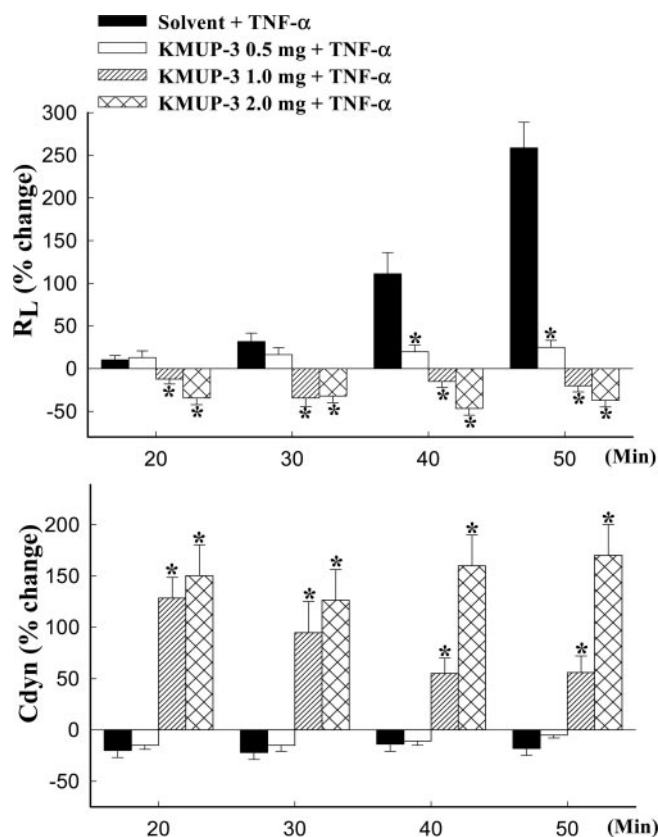
Drugs and Chemicals. Levcromakalim was generously supplied by the GlaxoSmithKline (Uxbridge, Middlesex, UK) and dissolved in ethanol at 10 mM. 4-AP, aminophylline, apamin, carbachol, ChTX, forskolin, glibenclamide, Griess reagents, IBMX, indomethacin, Krebs' solution reagents, L-NAME, methylene blue, ODQ, propranolol, SQ 22536, TEA, theophylline, and TNF-α were all obtained from Sigma-Aldrich (St. Louis, MO). PKA$_{RI}$ and PKG$_{1\alpha1\beta}$ antibodies were purchased from Calbiochem (San Diego, CA). 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; and KMUP-3, synthesized in our laboratory, was dissolved in 10% absolute alcohol, 10% pro-

pylene glycol, and 2% 1 N HCl at 10 mM. Serial dilutions were made in distilled water.

Statistical Evaluation of Data. The results are expressed as mean ± 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. $P < 0.05$ 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) run on an IBM-compatible computer.

Results

Pulmonary Function. The baseline values of total lung resistance, dynamic lung compliance, and tidal volume were 0.25 ± 0.02 cmH$_2$O ml^{-1} s^{-1}, 0.31 ± 0.02 ml cmH$_2$O^{-1}, and 1.91 ± 0.11 ml, respectively. TNF-α-induced bronchocontrac-

Fig. 2. R_L and C_{dyn} effects of KMUP-3 on TNF-α-induced (0.01 mg/kg) bronchoconstriction by intratracheal instillation. Each value represents the mean ± S.E. from six independent experiments. ∗, $P < 0.05$ compared with the KMUP-3 solvent + TNF-α (ANOVA followed by Student-Newman-Keuls test).

tion by intratracheal instillation increased R_L and decreased C_{dyn}, respectively (Fig. 2). KMUP-3 (0.5, 1.0, and 2.0 mg/kg) instilled through trachea for 15 min prior to TNF-α-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, i.v. KMUP-3 (1 mg/kg) more significantly decreased R_L and increased both C_{dyn} and respiratory tidal volume than KMUP-1 (Table 1).
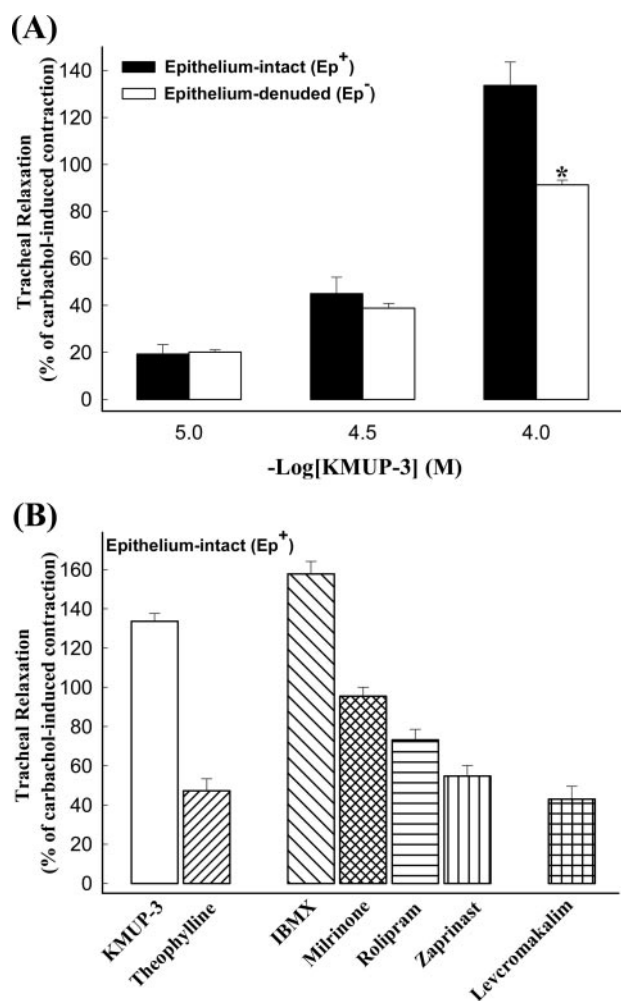
NOS and Epithelium-Derived Relaxation. As shown in Fig. 3A, KMUP-3 (10, 30, and 100 μM) produced concentration-dependent relaxations both in epithelium-denuded and epithelium-intact (E_P^+) guinea pig TSM, indicating that KMUP-3 induced epithelium-independent relaxation activity. However, KMUP-3 did 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 μM, 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 an NOS inhibitor L-NAME. The NO$_2^-$ release reached $160 \pm 15\%$ ($n = 3$) by KMUP-3 (100 μM) in comparison with the basal level (0.8 ± 0.1 μM/mg) in E_P^+ guinea pig TSM.

KMUP-3, theophylline, IBMX, milrinone, rolipram, and zaprinast (100 μM) were all found to cause relaxations in epithelium-intact trachea. Both IBMX and KMUP-3 were more potent at 100 μM concentrations than theophylline-inducing tracheal relaxation (Fig. 3B). A potassium channel opener, levcromakalim (100 μM), also displayed relaxation effects on TSM (E_P^+) (Fig. 3B).

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

Involvement of sGC and Adenylate Cyclase. The relaxations of guinea pig E_P^+ TSM elicited by KMUP-3 were attenuated by pretreatment with a sGC inhibitor, ODQ (1 μM); an NOS inhibitor, L-NAME (100 μM); or an adenylate cyclase inhibitor, SQ 22536 (100 μM) (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

TABLE 1
Effects of KMUP-3 and KMUP-1 on PDE inhibitory activity and lung resistance, dynamic compliance, and tidal volume maximum changes
The enzyme inhibitory activity was measured by 10 μM KMUP-1 and KMUP-3 ($n = 3$) on various PDEs. R_L, C_{dyn}, and tidal volume were measured after i.v. injection of KMUP-1 or KMUP-3 (1 mg/kg).

Compound	PDE3 Inhibition	PDE4 Inhibition	PDE5 Inhibition	R_L	C_{dyn}	Tidal Volume
	%			maximum change of baseline (%)		
KMUP-1	23 ± 2.2	18 ± 2.3	29 ± 2.5	-20 ± 3.5	35 ± 4.5	2.8 ± 0.2
KMUP-3	$55 \pm 2.5^*$	$48 \pm 2.1^*$	$48 \pm 1.9^*$	$-55 \pm 4.5^*$	$50 \pm 5.5^*$	$21.7 \pm 0.5^*$

∗ $P < 0.05$ compared with KMUP-1, respectively ($n = 3$).

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

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 among PDE3, 4, or 5. We also observed that the inhibitory effects of theophylline (10 μM) on PDE3, 4, and 5 were 8 ± 1.2, 7.9 ± 1.2, and $12 \pm 1.5\%$ ($n = 3$). The IC_{50} values of KMUP-3 for PDE3, PDE4, and PDE5 were 8.5 ± 1.5, 14.3 ± 2.2, and 14.5 ± 1.8 μM ($n = 3$), respectively, whereas KMUP-1's IC_{50} values for those PDEs were >100 μM. Under this condition, IBMX was used as a reference agent, and its IC_{50} values for PDE3, PDE4, and PDE5 were 6.4 ± 1.6, 25.6 ± 4.9, and 30.8 ± 4.5 μM ($n = 3$), 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 ± 6.4 and 1.18 ± 0.06 pmol/mg protein, respectively ($n = 3$). KMUP-3 (0.01, 0.1, 1, 10, and 100 μM) significantly increased both cAMP (80.2 ± 2.5, 91.3 ± 3.4, 97.9 ± 2.5, 110.7 ± 4.5, and 127.5 ± 4.8 pmol/mg protein) and GMP (1.62 ± 0.05, 1.73 ± 0.01, 1.75 ± 0.04, 1.86 ± 0.05, and 1.98 ± 0.09 pmol/mg protein) levels compared with each basal value in guinea pig primary TSMCs. Moreover, we compared both

cAMP and GMP levels of KMUP-3 with theophylline, aminophylline, milrinone, rolipram, and zaprinast at 100 μM (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 μM), an adenylate cyclase activator, were inhibited by pretreatment with the adenylate cyclase inhibitor, SQ 22536 (100 μM) (Table 2). Correspondingly, the rise of cGMP accumulation by KMUP-3 was abolished by pretreatment with the NOS inhibitor L-NAME (100 μM) or the sGC inhibitor ODQ (10 μM), respectively (Table 2).

Expression of PKA and PKG. Expression of immunoreactive PKA_{RI} is shown in Fig. 7A. Monoclonal antibody to PKA_{RI} recognized a band at 49 kDa in extracts of tracheal smooth muscle. KMUP-3 (1 μM) stimulated the expression of immunoreactive PKA_{RI} protein. Treatment with SQ 22536 (100 μM) 30 min prior to KMUP-3 (1 μM) attenuated the expression of PKA_{RI} (Fig. 7A). Both 8-Br-cAMP and KMUP-3 (1 μM) increased the expression of PKA_{RI}; however, pretreatment with ODQ (10 μM) 30 min prior to KMUP-3 did not significantly affect the PKA protein expression by KMUP-3 (B. N. Wu and I. J. Chen, unpublished data). Expression of immunoreactive $PKG_{1\alpha1\beta}$ is shown in Fig. 7B. Polyclonal antibody to $PKG_{1\alpha1\beta}$ recognized a band at 75 kDa in extracts of tracheal smooth muscle. Both 8-Br-cGMP and KMUP-3 (1 μM) increased the expression of $PKG_{1\alpha1\beta}$, and pretreatment with ODQ (10 μM) 30 min prior to KMUP-3 (1 μM) significantly reduced the PKG protein induced by KMUP-3 (Fig. 7B).

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 μM 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 IC50 of KMUP-3 on PDE3, 4, and 5, in comparison with previous KMUP-1. We suggest that the higher inhibition (percentage) of KMUP-3 than KMUP-1 on PDE3, 4 and 5 favors better respiratory performance as shown by increased respiratory tidal volume, decreased R_L, and increased C_{dyn} (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
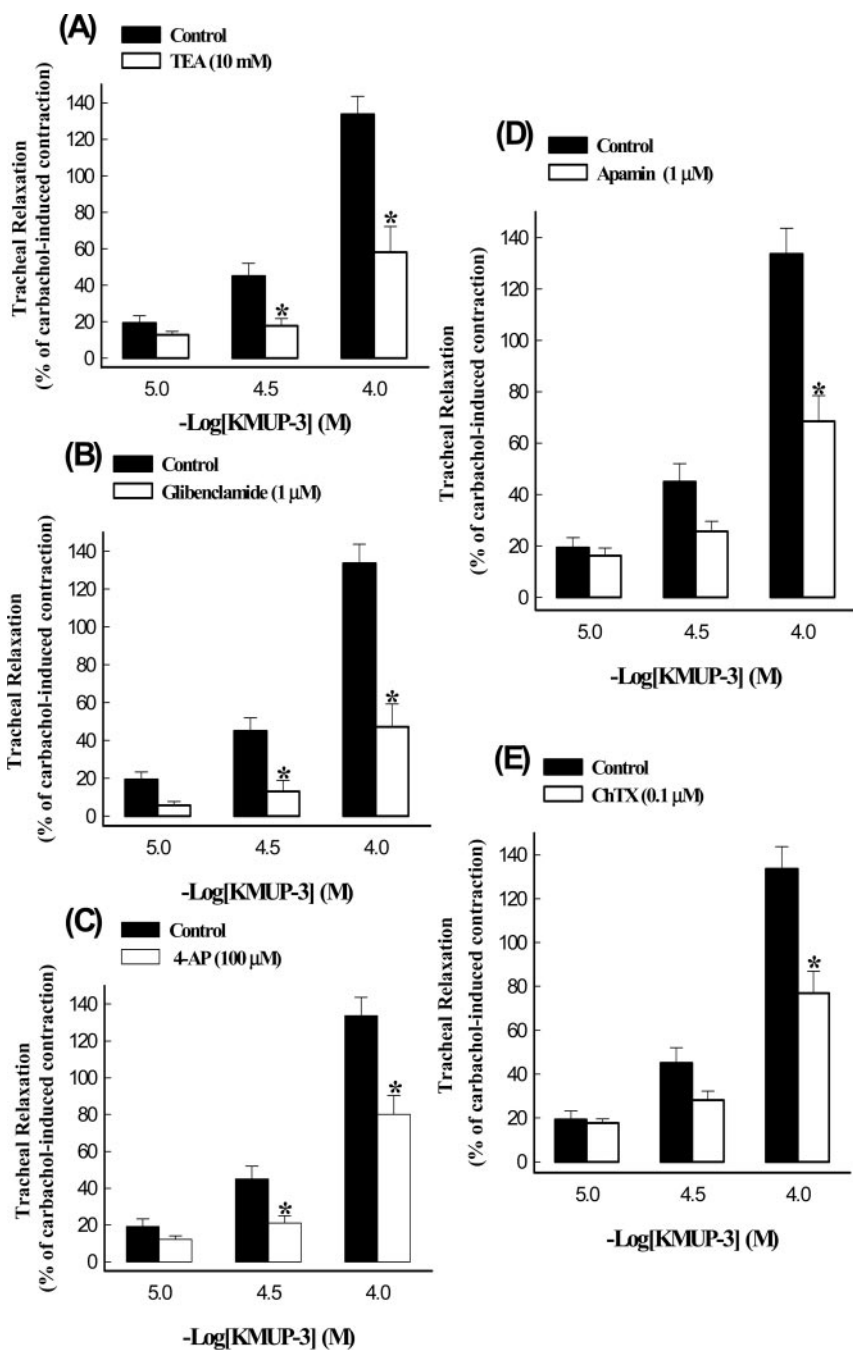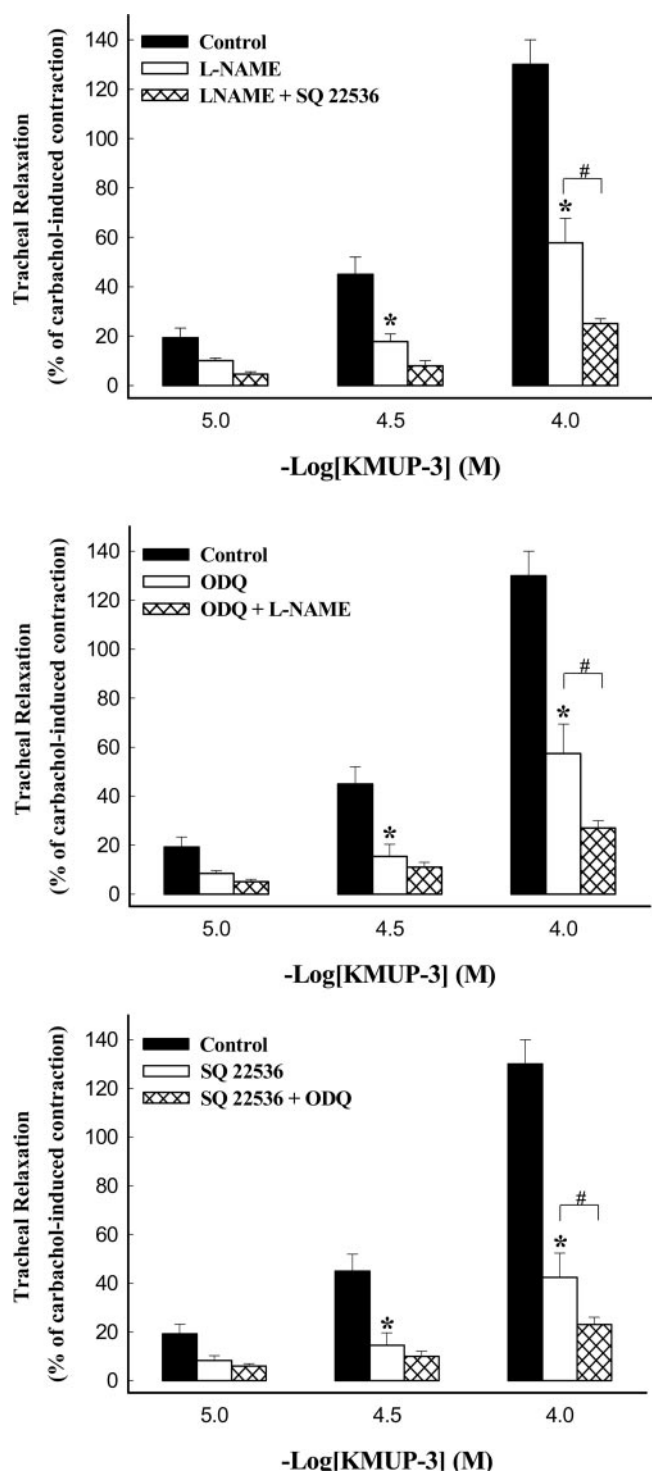
Fig. 4. Effects of KMUP-3 on carbachol (1 μM)-pre-contracted guinea pig epithelial-intact trachea in the absence or presence of various potassium channel blockers. *, $P < 0.05$, $n = 8$, compared with the control (ANOVA followed by Tukey test).

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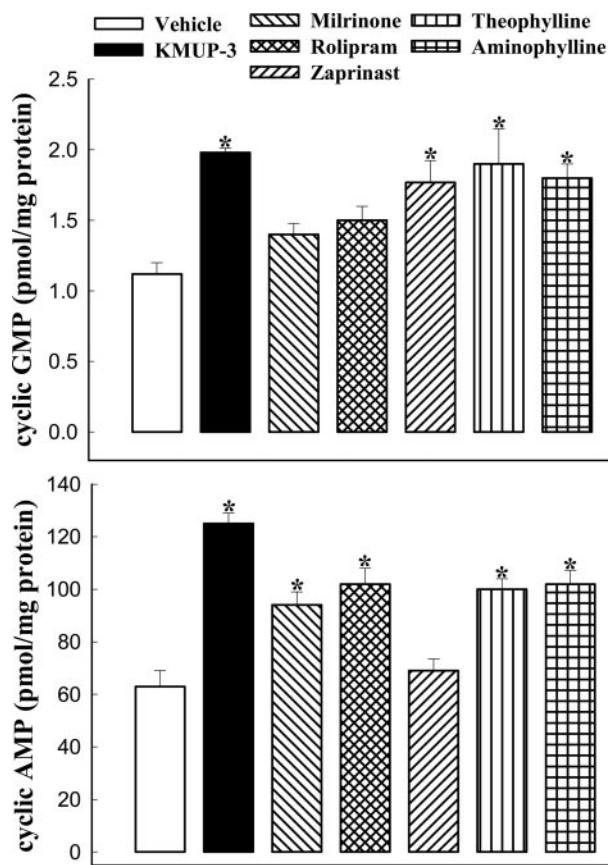
to PDE inhibition, previous epithelium-dependent cGMP enhancing activity of KMUP-1 encourages us to further investigate the benefits of KMUP-3, administered by intratracheal instillation, for the treatment of airway dysfunction caused by bronchocontraction.

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 (Ellis and Conanan, 1995; Hirose et al., 2001). 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

Fig. 5. Effects of KMUP-3 on carbachol (1 μM)-precontracted guinea pig epithelial-intact trachea in the absence or presence of L-NAME (100 μM), ODQ (1 μM), or SQ 22536 (100 μM). ∗, $P < 0.05$, $n = 8$, compared with the control; #, $P < 0.05$, $n = 4$, 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 μM) on cGMP and cAMP levels in guinea pig tracheal smooth muscle cells. Each value represents the mean ± S.E. from three independent experiments. ∗, $P < 0.05$ compared with the vehicle (ANOVA followed by Dunnett's test).

TABLE 2
Effects of KMUP-3 and forskolin (100 μM) on cAMP and cGMP levels in TSMCs in the absence and presence of SQ 22536 (100 μM), L-NAME (100 μM), or ODQ (10 μM)
Each value represents the mean ± S.E.M. of three independent experiments.

Treatment	
cAMP (pmol/mg protein)	
Vehicle	62.60 ± 6.40
SQ 22536	74.32 ± 7.56
Forskolin	119.15 ± 5.11*
Forskolin plus SQ 22536	94.33 ± 3.42**
KMUP-3	128.72 ± 4.57*
KMUP-3 plus SQ 22536	74.51 ± 3.18**
Cyclic GMP (pmol/mg protein)	
Vehicle	1.18 ± 0.06
KMUP-3	1.98 ± 0.08*
L-NAME	1.12 ± 0.02
KMUP-3 plus L-NAME	1.28 ± 0.01**
ODQ	1.12 ± 0.03
KMUP-3 plus ODQ	1.35 ± 0.04**

∗ $P < 0.05$ compared with the vehicle, ANOVA followed by Dunnett's test.
∗∗ $P < 0.05$ compared with forskolin or KMUP-3.

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 that 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

(A) PKA$_{RI}$ — 49 kDa
Actin — 43 kDa

(B) PKG$_{1\alpha1\beta}$ — 75 kDa
Actin — 43 kDa
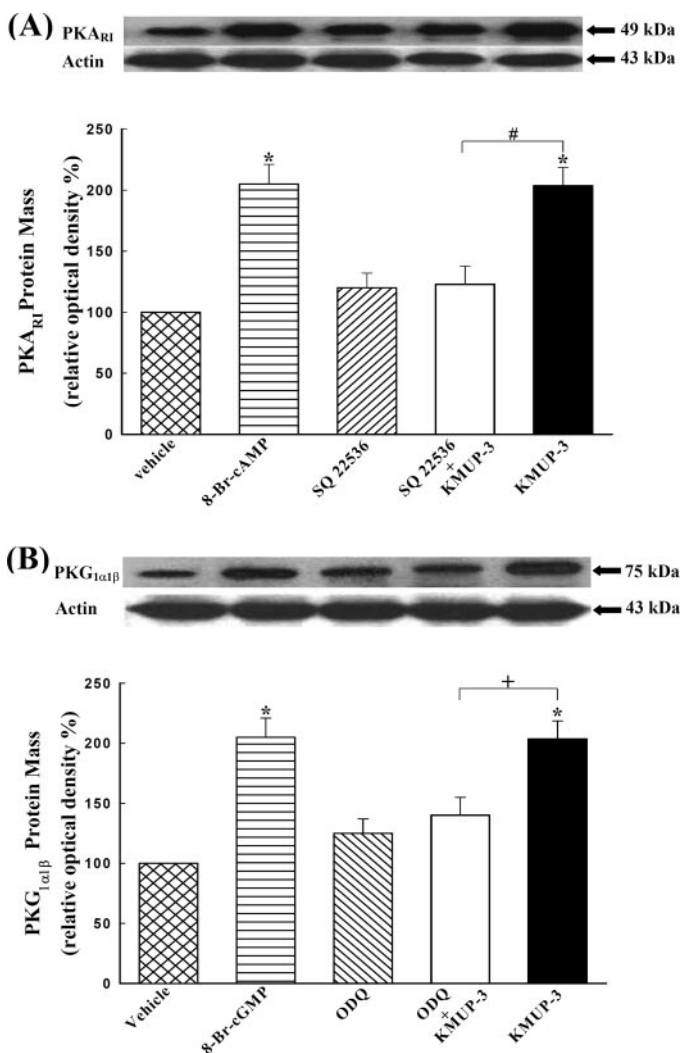
Fig. 7. Representative Western blots and the corresponding group data depicting PKA$_{RI}$ and PKG$_{1\alpha1\beta}$ protein expression in cultured guinea pig tracheal smooth muscle cells incubated for 30 min in the absence (vehicle) and presence of: A, 8-Br-cAMP (1 μM), SQ 22536 (100 μM), SQ 22536 + KMUP-3 (1 μM), or KMUP-3 (1 μM); or B, 8-Br-cGMP (1 μM), ODQ (10 μM), ODQ + KMUP-3, or KMUP-3. SQ 22536 or ODQ was treated 30 min prior to KMUP-3. $*$, $P < 0.05$ compared with the vehicle; #, $P < 0.05$ compared with the SQ 22536 + KMUP-3; +, $P < 0.05$ compared with the ODQ + KMUP-3 ($n = 3$) (ANOVA followed by Dunnett's test).

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 reduction of 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 cannot neglect the contribution from cGMP-induced inhibition on PDE3.
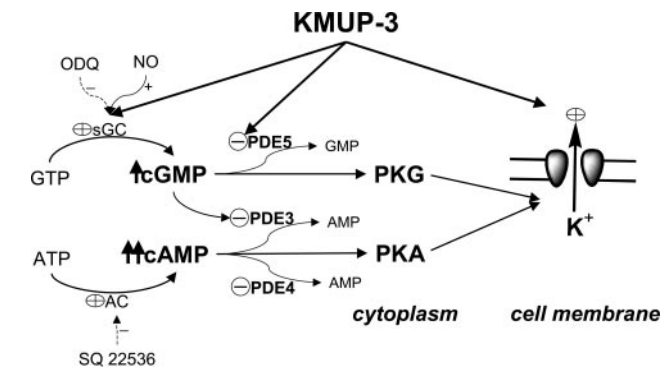
Fig. 8. Proposed mechanism of action of KMUP-3 on the intracellular cGMP and cAMP synthesis and metabolism, K$^+$ channel opening, and phosphodiesterase inhibition. Increased cAMP and cGMP then activate the PKA and PKG cascades and thus enhance the K$^+$ efflux, leading to attenuation of Ca^{2+} influx-associated contractility in tracheal smooth muscle cells. Cycle symbols indicated as KMUP-3 effects.

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 PKG$_{1\alpha1\beta}$ expression; moreover, the PKA$_{R1}$ expression caused by KMUP-3 also showed similar responses but was inhibited by the AC inhibitor SQ 22536 (Fig. 7A). Notably, KMUP-3-induced TSM relaxation is associated with K$^+$ channel opening activity (Somlyo and Somlyo, 1994), resulting in increased expression of PKA and PKG (Fig. 8).

TNF-α has been shown to induce hyper-responsiveness and bronchocontraction of TSM (Wu et al., 2004). As a member of cytokines, TNF-α 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-α-induced bronchoconstriction. In the present study, exogenous TNF-α certainly increased R_L components and decreased C_{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-α-induced tracheal overcontractility in the airway.

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$_{1\alpha1\beta}$ 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 thus may contribute to significant relaxation of carbachol-induced TSM contraction in vitro and protect against TNF-α-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-α-induced tracheal contractility, may indicate its considerable usefulness and may be the challenge for future inhalation research.

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