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ORIGINAL ARTICLE

Phosphodiesterase inhibitor KMUP-3 displays cardioprotection via protein kinase G and increases cardiac output via G-protein-coupled receptor agonist activity and Ca^{2+} sensitization

Chung-Pin Liu [a], Jwu-Lai Yeh [b], Shu-Fen Liou [c], Bin-Nan Wu [b], Ing-Jun Chen [b,d,*]

[a] *Department of Internal Medicine, Yuan's General Hospital, Kaohsiung, Taiwan*
[b] *Department of Pharmacology, School of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[c] *Department of Pharmacy, Chia-Nan University of Pharmacy and Science, Tainan, Taiwan*
[d] *Department of Medicine and Education, Pingtung Christian Hospital, Pingtung, Taiwan*

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Abstract KMUP-3 (7-{2-[4-(4-nitrobenzene) piperazinyl]ethyl}-1, 3-dimethylxanthine) displays cardioprotection and increases cardiac output, and is suggested to increase cardiac performance and improve myocardial infarction. To determine whether KMUP-3 improves outcomes in hypoperfused myocardium by inducing Ca^{2+} sensitization to oppose protein kinase (PK)G-mediated Ca^{2+} blockade, we measured left ventricular systolic blood pressure, maximal rates of pressure development, mean arterial pressure and heart rate in rats, and measured contractility and expression of PKs/RhoA/Rho kinase (ROCK)II in beating guinea pig left atria. Hemodynamic changes induced by KMUP-3 (0.5–3.0 mg/kg, intravenously) were inhibited by Y27632 [(*R*)-(+)-*trans*-4-1-aminoethyl)-N-(4-Pyridyl) cyclohexane carboxamide] and ketanserin (1 mg/kg, intravenously). In electrically stimulated left guinea pig atria, positive inotropy induced by KMUP-3 (0.1–100μM) was inhibited by the endothelial NO synthase (eNOS) inhibitors *N*-nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole, cyclic AMP antagonist SQ22536 [9-(terahydro-2-furanyl)-9*H*-purin-6-amine], soluble guanylyl cyclase (sGC) antagonist ODQ (1*H*-[1,2,4] oxadiazolo[4,3-*a*] quinoxalin-1-one), RhoA inhibitor C3 exoenzyme, β-blocker propranolol, 5-hydroxytryptamine 2A antagonist ketanserin, ROCK inhibitor Y27632 and KMUP-1 (7-{2-[4-(2-chlorobenzene) piperazinyl]ethyl}-1, 3-dimethylxanthine) at 10μM. Western blotting assays indicated that KMUP-3 (0.1–10μM) increased PKA, RhoA/ROCKII, and PKC translocation and CIP-17 (an endogenous 17-kDa inhibitory protein) activation. In spontaneous right

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* Corresponding author. Department of Pharmacology, School of Medicine, Kaohsiung Medical University, 100 Shih-Chuan First Road, Kaohsiung 807, Taiwan.
E-mail address: ingjun@kmu.edu.tw (I.-J. Chen).

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atria, KMUP-3 induced negative chronotropy that was blunted by 7-nitroindazole and atropine. In neonatal myocytes, L-NAME inhibited KMUP-3-induced eNOS phosphorylation and RhoA/ROCK activation. In H9c2 cells, Y-27632 (50μM) and PKG antagonist KT5823 [2,3,9,10,11,12-hexahydro-10R- methoxy-2,9-dimethyl-1-oxo-9S,12R-epoxy-1H-diindolo(1,2,3-fg:3′,2′,1′-kl) pyrrolo(3,4-i)(1,6)benzodiazocine-10-carboxylic acid, methyl ester] (3μM) reversed KMUP-3 (1—100μM)-induced Ca^{2+}-entry blockade. GPCR agonist activity of KMUP-3 appeared opposed to KMUP-1, and increased cardiac output via Ca^{2+} sensitization, and displayed cardioprotection via cyclic GMP/PKG-mediated myocardial preconditioning in animal studies.

Introduction

Xanthine-based KMUP-3 (7-{2-[4-(4-nitrobenzene) piperazinyl]ethyl}-1, 3-dimethylxanthine) has shown the benefits in myocardiac infarction [1]. Here, the mechanisms involved in cardioprotection and associated with the mechanistic action of KMUP-3 were further addressed *in vitro*. By contrast, KMUP-1 (7-{2-[4-(2-chlorobenzene) piperazinyl]ethyl}-1, 3-dimethylxanthine), with similar chemical structure, was also explored because it shows the opposite G-protein-coupled receptor (GPCR) agonist activity to KMUP-3 [2—5]. This study aimed to investigate the role of Ca^{2+} sensitization in improving cardiac performance via GPCR and phosphodiesterase (PDE). Cardioprotection and vasorelaxation via NO/cyclic GMP (cGMP)/protein kinase (PK)G are required to prevent pressure overloading in patients with hypoperfused myocardium [6]. Treatment with a vasodilatory NO/cGMP enhancer with Ca^{2+} sensitization activity to increase cardiac output would be beneficial for hypoperfused myocardium.

This study investigated whether KMUP-3, a PDE-3/PDE-4/PDE-5 inhibitor that increases left ventricular systolic blood pressure (LVSP) and atrial inotropy, is therapeutically preferable to each class of PDE inhibitor for improving hypoperfused myocardium, in addition to its proven beneficial effects on the airways [7—9]. GPCR antagonism and PDE inhibition in the cardiovascular and lipid systems have been reported previously for KMUP-1 [10—17]. Here, we explored the use of KMUP-3 as a GPCR agonist for the first time, and found that it had the opposite effects of CGRP antagonist KMUP-1. Whether KMUP-3 increases the principal cyclic AMP (cAMP)/cGMP signal transduction pathways via GPCRs or cardiac PDE inhibition remains to be proven [7]. Since RhoA/Rho kinase (ROCK) mediates Ca^{2+} sensitization, we explored the activation of RhoA/ROCK by KMUP-3 [16—18]. Overstimulation of cardiac myocytes by cAMP/Ca^{2+} is associated with excessive workloads, which increases mortality in patients with hypoperfused myocardium. Ca^{2+} sensitization, attributable to PKs, including PKA, PKC, and RhoA/ROCK expression, the upstream signaling of myosin light chain phosphatase (MLCP), requires a protective strategy to avoid cardiac overstimulation [19]. This realization has caused a paradigm shift in pharmacotherapy for congestive heart failure (CHF) from cardiotonic to cardioprotective therapy [6—12]. ROCK activation/Ca^{2+}-sensitization activity of KMUP-3, in contrast to ROCK inactivation/Ca^{2+}-desensitization activity of KMUP-1, could be a potential mechanism for cardioprotection via increasing cardiac perfusion [10,20].

RhoA is a small GTP-binding protein controlled by guanine exchange factors, which catalyzes the exchange of GDP for GTP [8]. RhoA/ROCK is involved in cardiac contraction and may be an interesting drug target [22—24]. ROCK has also been shown to cause cardiac hypertrophy by raising workload [23]. Although there is much evidence that activation of RhoA is deleterious to the heart, RhoA may have the ability to protect cardiomyocytes by signaling through Akt [24]. Theoretically, Ca^{2+} sensitization by RhoA/ROCK to increase inotropy does not require much intracellular Ca^{2+}, in contrast to the requirements for Ca^{2+}-binding to troponin [6].

GPCR agonists/antagonists are composed of flexible structures and have been difficult to evaluate [25]. To date, GPCRs have been activated by thrombin and lysophosphatidic acid in vascular smooth muscles [26]. Cardiac GPCRs, including adrenergic and serotonergic receptors, are innervated by the nervous system. To examine the benefits of increasing cardiac output with GPCR agonist activity and increasing cardioprotection with PKG in CHF, we investigated the effects of KMUP-3 on hemodynamic/inotropic activity, Ca^{2+} entry, cGMP/PKG enhancement, and protein expression. The possibility of overcoming the heart failure by means of the GPCR-mediated positive inotropic action of KMUP-3, PDE inhibition, and cGMP/PKG-mediated cardioprotection were explored in this study.

Methods

Animals

Hartley guinea pigs of both sexes (350—500 g) and Wistar rats (200—250 g) were provided by the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and housed under constant temperature and controlled illumination at Kaohsiung Medical University, Kaohsiung, Taiwan. Neonatal rats were obtained from copulation between healthy male and female rats. Food and water were available *ad libitum*. This study was approved by the Animal Care and Use Committee at Kaohsiung Medical University.

Measurement of Ca^{2+} entry in H9c2 cells

To measure calcium current through L-type Ca^{2+} channels, whole cell patch-clamp electrophysiology was used in rat

heart-derived H9c2 cells as previously described [23,24]. H9c2 cells were placed in a recording dish and perfused with a bath solution containing 135mM tetraethylammonium chloride, 1.8mM $CaCl_2$, 2mM $MgCl_2$, 10mM glucose, and 10mM HEPES (pH 7.4, Tris). A recording electrode was pulled from borosilicate glass (resistance: 4—7 MW), and the pipette was coated with sticky wax close to the tip to reduce capacitance, backfilled with pipette solution containing 140mM CsCl, 1mM EGTA, 1mM $MgCl_2$, 5mM Na_2ATP, and 5mN HEPES (pH 7.2, Tris) and gently lowered onto a smooth muscle cell. Negative pressure was briefly applied to rupture the membrane and a giga-ohm seal was obtained.

H9c2 cells were clamped at −80 mV with step depolarizations (300 milliseconds) from −80 mV to 0 mV to evoke whole-cell calcium current. Voltage-clamped cells were equilibrated for 15 minutes prior to experimentation. Membrane currents were recorded on an Axopatch 700A amplifier (Axon Instruments, Union City, CA, USA), filtered at 1 kHz using a low-pass Bessel filter, digitized at 5 kHz, and stored on a computer for subsequent analysis with Clampfit 9.0 (SUNNY, CA, USA). Following equilibration, calcium current was monitored in the presence and absence of KMUP-3 (1μM, 10μM, and 100μM). To ascertain whether PKG or ROCK signaling was involved in the KMUP-3-induced decreases in calcium current, H9c2 cells were incubated for 15 minutes with a combination of KMUP-1 (10μM) and PKG antagonist KT5823 [2,3,9,10,11,12- hexahydro-10R-methoxy-2,9-dimethyl-1-oxo-9S,12R- epoxy-1H-diindolo(1,2,3-fg:3′,2′,1′-kl) pyrrolo(3,4-i)(1,6)benzodiazocine-10-carboxylic acid, methyl ester] (3μM) or ROCK inhibitor Y27632 [(*R*)-(+)-*trans*-4-1-aminoethyl)-N-(4-Pyridyl) cyclohexane carboxamide] (50μM). All electrical recordings were performed at room temperature [21] and recordings of calcium current each group were performed in triplicate.

Hemodynamic measurements

Hemodynamic measurements were carried out in male Wistar rats weighing 300—350 g, anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneally). Both KMUP-3- and sham-treated animals were anesthetized for the same length of time. Polyethylene tubing (PE50) was placed in the trachea to keep the airway patent, and rats continued to breathe spontaneously while hemodynamic and cardiovascular functions were recorded. Vascular catheters (PE50) were placed in the femoral arteries to measure mean arterial blood pressure (MABP) and heart rate (HR). Changes of MABP and HR caused by KMUP-3 were recorded from the femoral artery with a pressure transducer (Model P50; Gould, Oxnard, CA, USA) connected to a Pressure Processor Amplifier (Model 13-4615-52; Gould). A femoral vein was used for intravenous administration of KMUP-3. A specialized transducer tip catheter was placed in the right carotid artery and advanced into the left ventricle of the heart for measurement of LVSP or contractility. Maximal rate of pressure development (dp/dt) at 50 mm Hg, an index of cardiac contractility, was derived from the left ventricular pressure trace. Post-hoc vascular resistance was calculated as MABP/peripheral cardiac output [9]. Body temperature was monitored throughout the experiment and maintained between 37°C and 37.5°C using a heating pad.

Each dose of active drug was administered as a 20-minute infusion in a series of escalating doses dissolved in a 5% dextrose water vehicle. After termination of the high-dose infusion, animals were observed for 20 minutes. KMUP-3 HCl was infused at 0.03 μg/kg/20 minutes, 0.05 μg/kg/20 minutes and 0.075 μg/kg/20 minutes for the measurement of Free Acid-Binding Protein (FABP), intraperitoneally injected at 0.03 mg/kg, 0.05 mg/kg, and 0.1 mg/kg for the measurement of MABP and HR, and infused by intravenous injection for 2 minutes at 0.5 mg/kg, 1.0 mg/kg, and 3 mg/kg dissolved in 5% dextrose water vehicle (2 mL) for the measurement of LVSP, ABP, dp/dt, and HR. The primary hemodynamic variables were calculated using commercial software and a signal processing workstation. Animals were randomly divided into one of six treatment or vehicle groups. After the completion of the surgical protocol, animals were allowed to stabilize for 1 hour, and baseline data were collected at 5-minute intervals for 30 minutes before treatment. Three tissue samples from each group were used in the experiments.

Organ bath experiments and tissue procurement

Guinea pigs were killed by a blow on the head. Their hearts were quickly excised and trimmed of excess tissues. The atrium was carefully removed and placed at room temperature (20—25°C) in a Krebs solution composed of NaCl 113mM, KCl 4.8mM, $CaCl_2$ 2.2mM, KH_2PO_4 1.2mM, $MgCl_2$ 1.2mM, $NaHCO_3$ 25mM, and dextrose 11.0mM, which was bubbled with a 95% O_2 + 5% CO_2 mixture, followed by separation of the right and left atrium for subsequent experiments. Three tissue samples from each group were used in the experiments.

Spontaneously beating right atrium

The spontaneously beating right atrium was dissected from the heart and mounted in a 10-mL organ bath with one end fixed and the other end connected to a force displacement transducer (Model FT03; Grass, Quincy, MA, USA). The frequency of contraction was measured on a separate channel by a tachometer (Model S77-26; Coulbourn, Allentown, PA, USA) connected to a high-speed videograph (AT L19-69; Coulbourn). These experiments were carried out at 32.5°C in Krebs solution. The atrium strip was prestretched to a baseline tension of 0.5 g and equilibrated for 90 minutes in aerated (95% O_2 + 5% CO_2) Krebs solution before the experimental protocols were initiated. The atria were then allowed a 30—60-minute washout period to restabilize. KMUP-3 (0.1μM, 1.0μM, 10μM, and 100μM) was then cumulatively added to the organ bath. To examine the possible involvement of NO and cAMP/cGMP in the action of KMUP-3 (0.1μM, 1.0μM, and 10μM) on spontaneously beating atria, the beating atria were preincubated with atropine (1μM) and *N*-nitro-L-arginine methyl ester (L-NAME; 100μM), respectively. Three tissue samples from each group were used in the experiments.

Electrically stimulated beating left atrium

Quiescent left atria were dissected free of connective tissues and mounted in an organ bath under a resting tension of 0.5 g. Organs were bathed at 32.5°C in aerated Krebs solution and electrically stimulated with 1 Hz at 2-second intervals via two platinum electrodes placed at either side. The positive inotropic effect was measured by determining the cumulative concentration—response curves in the presence of KMUP-3 (0.1μM, 1.0μM, 10μM, and 100μM). To examine the possible involvement of NO and cAMP/cGMP in the action of KMUP-3 on electrically stimulated beating atria, preincubation with Y27632 (1μM and 10μM), propranolol (1μM), L-NAME (100μM), 7-nitroindazole (100μM), and ketanserin (1μM) was performed for 15 minutes prior to adding KMUP-3 to measure the development of beating force and heart rate for 10 minutes. Three tissue samples from each group were used in the experiments.

Protein extraction and Western blotting analysis

After incubation with test agents in an organ bath, isolated atria were frozen and pulverized to a fine powder at the temperature of liquid nitrogen. Atrium powders were then homogenized in protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA) by sonication. The samples were sonicated for 10 seconds three times and centrifuged at 20,000 g at 4°C for 30 minutes. The protein concentrations of supernatants were determined using bovine serum albumin as the standard. The cell extracts were then boiled in a ratio of 4:1 with sample buffer [Tris 100mM, pH 6.8; glycerol 20%, sodium dodecyl sulfate (SDS) 4%, and bromophenol blue 0.2%]. Electrophoresis was performed using 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). Membranes were blocked with Tris-buffered saline (TBS; 20mM Tris and 137mM NaCl, pH 7.6) containing 0.1% Tween 20 (TTBS) and 5% nonfat milk at room temperature for 1 hour, washed with TTBS, and then incubated overnight at 4°C in the appropriate primary antibody for PKA_{RI}, RhoA, ROCKII, p-CIP-17 (an endogenous 17-kDa inhibitory protein), CPI-17, and $PKG_{1\alpha1\beta}$. The membranes were washed in TTBS before being incubated with horseradish-peroxidase-conjugated antibody against mouse, goat, or rabbit immunoglobulin G for 1 hour. Membranes were then washed in TTBS and developed with enhanced chemiluminescence to detect the specific antigen. The intensity of the bands was measured by densitometry. Endothelial NO synthase (eNOS) or phosphorylated eNOS (p-eNOS) was extracted from the cell culture of rat neonatal myocytes, pulmonary artery ring and aortic ring (2—3 mm) and measured by western blotting [11]. Three tissue samples from each group were used in the experiments.

PK, ROCKII, and CIP-17 expression and pretreatment of beating left atrium

To examine whether KMUP-3 affects protein expression in beating atrium, PKA, ROCKII, PKG and phosphorylated CIP-17 were measured by Western blotting. To examine whether KMUP-3 affected crosstalk among ROCK and PKA, PKC and PKG, Y27632 (0.1μM, 1μM, 10μM, and 100μM) or L-NAME (10μM) were added to a beating atrium in the organ bath for 15 minutes, followed by protein extraction. Three tissue samples from each group were used in the experiments.

PKC translocation of beating left atrium

To determine the effect of KMUP-3 on PKCα translocation, isolated left beating atrium was incubated with KMUP-3 (0.1—10μM) for 30 minutes. Frozen left atrium was pulverized to a fine powder at the temperature of liquid nitrogen for preparation of cytosolic and membrane fractions. Isolated left atrial tissues were then homogenized by a sonicator (XL-2020; Heat Systems Ultrasonic, Plainview, NY, USA) at 4°C in 1 mL homogenization buffer containing 20mM Tris—HCl (pH 7.5), 1mM dithiothreitol, 5mM EGTA, 2mM EDTA, 0.5mM phenylmethylsulfonyl fluoride, 20μM leupeptin, and 20μM aprotinin. The cytosol fractions were obtained by collecting the supernatants after centrifuging the cells at 100,000 *g* for 45 minutes at 4°C. The pellets were resonicated in homogenizing buffer with 1% Triton X-100, centrifuged again at 100,000 *g* for 45 minutes at 4°C, and the supernatants collected as membrane fractions. The extracts for cytosol and membrane fractions were denatured by heating at 90°C for 10 minutes in sampling buffer, and an aliquot containing 20 μg total protein was subjected to 10% SDS-PAGE. After migration, proteins were transferred onto polyvinylidene difluoride transfer membranes (Millipore), and the membranes were successively incubated at room temperature with 5% (w/v) nonfat dry milk in TBS for 1 hour, and then incubated with primary antibody at 4°C overnight. Protein was detected with horseradish-peroxidase-conjugated secondary antibody (BD Transduction Laboratories, San Jose, CA, USA). At the end of the incubation, the membranes were extensively washed with TBS. The immunoreactive bands were detected by chemiluminescence (ECL) reagents (PerkinElmer Life Sciences). Three PKC expressions from each group were measured.

RhoA activation of neonatal myocytes

RhoA activation was determined using an affinity precipitation assay, which binds only the active GTP-bound form of Rho. Subconfluent neonatal rat myocytes were grown to 85—90% confluence, cells were exposed to KMUP-3 with 10% fetal bovine serum for 90 minutes at 37°C before addition of lysis buffer (25mM HEPES, pH 7.5, 150mM NaCl, 1% Igepal CA-630, 10mM $MgCl_2$, 1mM EDTA and 10% glycerol, 1 Ag/mL aprotinin, 10 Ag/mL leupeptin, and 1mM Na_3PO_4) for 15 minutes at 4°C. Cell lysates were clarified by centrifugation at 13, 000 *g* at 4°C for 10 minutes, and equal volumes of lysates were incubated with agarose-conjugated rhotekin-Rho binding domain (RBD) for 45 minutes at 4°C and then washed three times with lysis buffer. Agarose beads were boiled in SDS-PAGE sample buffer to release active RhoA and samples were resolved on a 12% polyacrylamide gel followed by immunoblotting with anti-Rho A (Clone 55). Six RhoA expressions from each group were measured.

Measurement of cellular cAMP and cGMP

Neonatal rat myocytes were incubated with KMUP-3 (0.1—10μM) in incubation plate wells for 24 hours and terminated by adding 10% trichloroacetic acid. Intracellular cAMP and cGMP concentrations were assayed as previously described [6]. The cAMP and cGMP levels were determined by a commercially available radioimmunoassay kit subsequently (GE Healthcare, Little Chalfont, Bucks, UK).

Compounds

The hydrochloride salt of KMUP-3, synthesized in this laboratory, was used in all experiments [1]. SQ 22536 [9-(terahydro- 2- furanyl)-9H-purin-6-amine], L-NAME, and KT5823 were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Y27632, atropine, ketanserin, and 7-imidazole were all obtained from Sigma—Aldrich, Inc. (St. Louis, MO, USA). Anti-PKCα, anti-eNOS and anti-phospho-eNOS (Ser1177) antibody were obtained from BD Transduction Laboratories and Cell Signaling Technology (Beverly, MA, USA), respectively. Anti-PKA_{RI} and anti-PKG_1 antibodies were purchased from Calbiochem (San Diego, CA, USA). Anti-RhoA antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-ROCKII, anti-phospho CPI-17 and anti-CPI-17 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY, USA). All drugs and reagents were dissolved in distilled water unless otherwise stated. KMUP-3 hydrochloride was dissolved in 5% glucose distilled solution. Serial dilutions were made with distilled water.

Statistical evaluation

All data are expressed as the mean ± standard error. Statistical differences were determined by independent and paired Student *t* test in unpaired and paired samples, respectively. Whenever a control group was compared with more than one treated group, one-way or two-way analysis of variance was used. When analysis of variance manifested a statistical difference, results were further analyzed with Dunnett's or Tukey's test. A p value < 0.05 or $p < 0.01$ were considered to be significant. Data analysis and figure plotting were done using SigmaPlot software version 8.0 (Chicago, IL, USA) and SigmaStat version 2.03 (Chicago, IL, USA) run on an IBM compatible computer.

Results

Cardiac Ca^{2+} entry blockade

In rat heart-derived H9c2 cells, KMUP-3 (1—100μM) concentration-dependently reduced inward currents (Figures 1A and 1B). The combination of KMUP-3 (10μM) with KT5823 (3μM) or Y27642 (50μM) restored the reduced currents (Figures 1C—1F).

LVSP, dP/dt, MABP, and HR

As shown by the trace recordings in Figures 2A—2D, intravenous infusion of KMUP-3 (0.5—3 mg/kg; intravenously) dose-dependently increased LVSP, dp/dt and MABP within 2 minutes in anesthetized rats. Pretreatment with intravenous Y27642 and ketanserin at 1 mg/kg respectively inhibited the effects induced by KMUP-3 (1 mg/kg, intravenously). Intravenous perfusion of KMUP-3 (0.03—0.075 μg/kg/min × 20 minutes) dose-dependently increased MABP and HR, due to agonist activity of GPCR, particularly β-adrenergic receptor (AR) activity (Figures 2E and 2F). Intraperitoneal injection of KMUP-3 (0.0 3—0.1 mg/kg) dose-dependently increased MABP and HR (Figures 2G and 2H).

Spontaneously beating right atria

As shown in Figures 3A and 3B, KMUP-3 (0.1—30 μM) concentration-dependently increased inotropic and decreased chronotropic effects in isolated spontaneously beating right atrium. In contrast, milrinone (0.1—30μM) increased both inotropic and chronotropic activities. The beating frequency decreased by KMUP-3, due to PKG-preconditioning or inhibited Ca^{2+} current in ventricle cells, was upregulated by pretreatment with atropine (1μM) and 7-nitroindazole (100μM; Figure 3C).

Electrically stimulated left atria

The inotropic effects of KMUP-3 and milrinone were studied under electrical stimulation (1 Hz) at a constant rate. As shown in Figure 3D, both KMUP-3 and milrinone (0.1—30μM) concentration-dependently increased left atrium contractility. KMUP-3 was more potent than milrinone for increasing contractility ($p < 0.05$).

Pretreatment with the adenylyl cyclase inhibitor SQ 22536 and the sGC inhibitor ODQ [1H-(1,2,4) oxadiazolo(4,3-a) quinoxalin-1-one] inhibited KMUP-3-induced contractility, suggesting the involvement of cAMP/cGMP (Figure 3E). KMUP-3 (10μM) increased left atrium contractility and the effect was attenuated by 7-nitroindazole (100μM; Figure 3F). Pretreatment with propranolol and Y27632 at 1μM, C3 exoenzyme (50 μg/mL), ketanserin (1μM), and L-NAME (100μM) all reduced KMUP-3-induced positive inotropic activity in the left atria (Figures 3G and 3H).

RhoA/ROCKII and PKA

RhoA, ROCKII, and PKA expression were significantly increased by KMUP-3 (0.1—10μM) in electrically stimulated left guinea pig atria (Figures 4A—4C).

PKC translocation and CPI-17 phosphorylation

The translocation of PKC from cytosol to membrane in guinea pig left atrial cells was significantly increased by KMUP-3 (1μM; Figure 4D). However, pretreatment with Y27632 (1μM and 10μM) nonsignificantly prevented KMUP-3-induced translocation of PKC. Different expression ratios of cytosol/cytosol + membrane or membrane/cytosol + membrane at each concentration are shown in Figure 4D. KMUP-3 concentration-dependently increased the phosphorylation of CPI-17 in electrically stimulated beating left guinea pig atria (Figure 4E).

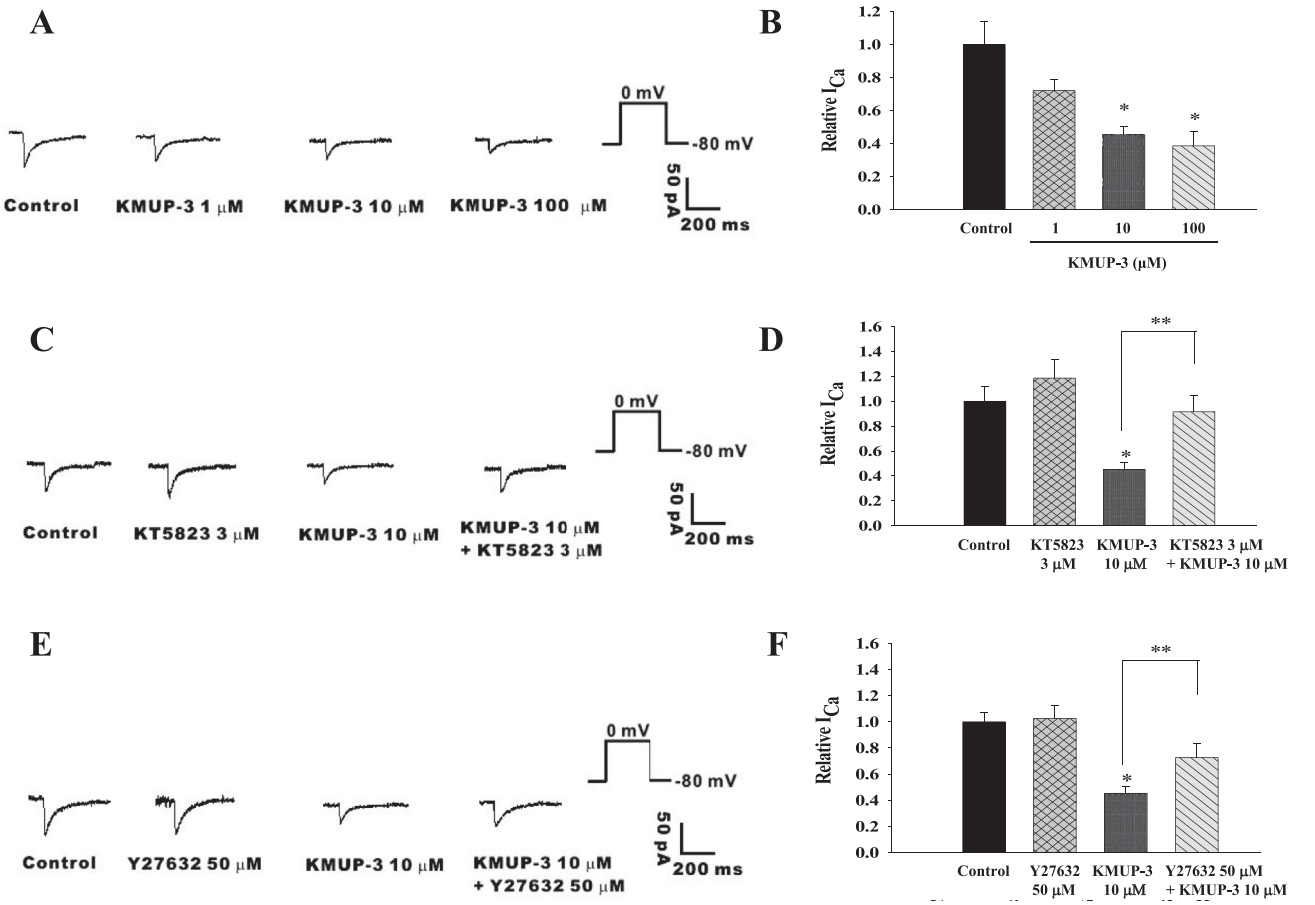

Figure 1. Effects of KMUP-3 on Ca^{2+}-entry currents in rat heart H9c2 cells. Cell patch-clamp electrophysiology was used to record the inward currents. Recording trace (left side) of (A), (C), and (E) indicated effects of KMUP-3 and combination with Y27632 or KT5823 on inward currents. (A, B) KMUP-3 (1μM, 10μM, and 100μM) concentration-dependently decreased the inward currents. (C, D) Combination with the protein kinase G antagonist KT5823 (3μM) restored the inward currents. (E, F) Combination with ROCK inhibitor Y27632 (50μM) restored the inward currents, compared to KMUP-3 alone. Values are expressed as means ± standard error ($n = 3$). * $p < 0.05$ versus control; ** $p < 0.05$ versus KMUP-3 (2 way repeated measures analysis of variance followed by Student—Newman—Keuls test).KMUP-3 = 7-{2-[4-(4-nitrobenzene) piperazinyl]ethyl}-1, 3-dimethylxanthine; KT5823 = 2,3,9,10,11,12-hexahydro-10R-methoxy-2,9-dimethyl-1-oxo-9S,12R-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl] pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester; ROCK = Rho kinase; Y27623 = (R)-(+)-trans-4-1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide.

Pretreatment with Y27632 and L-NAME

As shown in Figure 5, KMUP-3 significantly increased the expression of ROCKII (A) and PKA (B), but not PKG (C). KMUP-3-induced expression of PKA and ROCK II was inhibited by Y27632 (1—100μM) but not PKG. Pretreatment with L-NAME reduced the expression of PKG (Figure 5C).

eNOS/p-NOS, cAMP/cGMP, and GTP RhoA in neonatal myocytes

To determine the role of the NO/cGMP pathway in heart, neonatal rat myocyte protein was extracted followed by Western blotting to measure the expression of eNOS and p-eNOS (Figures 6A and 6B). Tissue extraction of eNOS in western blotting analysis usually appears as a vague banding that is difficult to separate. The illustrations are puzzling but actually accurate. KMUP-3 increased eNOS and p-eNOS in neonatal rat cardiomyocytes and the effect was reduced by L-NAME (100μM) pretreatment (Figures 6A and 6B). KMUP-3 increased both cAMP and cGMP. The latter was increased more than the former (Figures 6D and 6E).

RhoA activation of neonatal myocytes

As shown in Figure 6C, RhoA activation, determined using an affinity precipitation assay which binds only the active GTP-bound form of Rho, was increased by KMUP-3.

ROCKII expression and vascular resistance

To determine whether the ROCKII activity of KMUP-3 increased vascular resistance, aorta and pulmonary artery ring protein was extracted followed by Western blotting to measure the expression of ROCKII and eNOS. We found that KMUP-3 did not increase vascular ROCKII, but did increase eNOS (Figure 7).

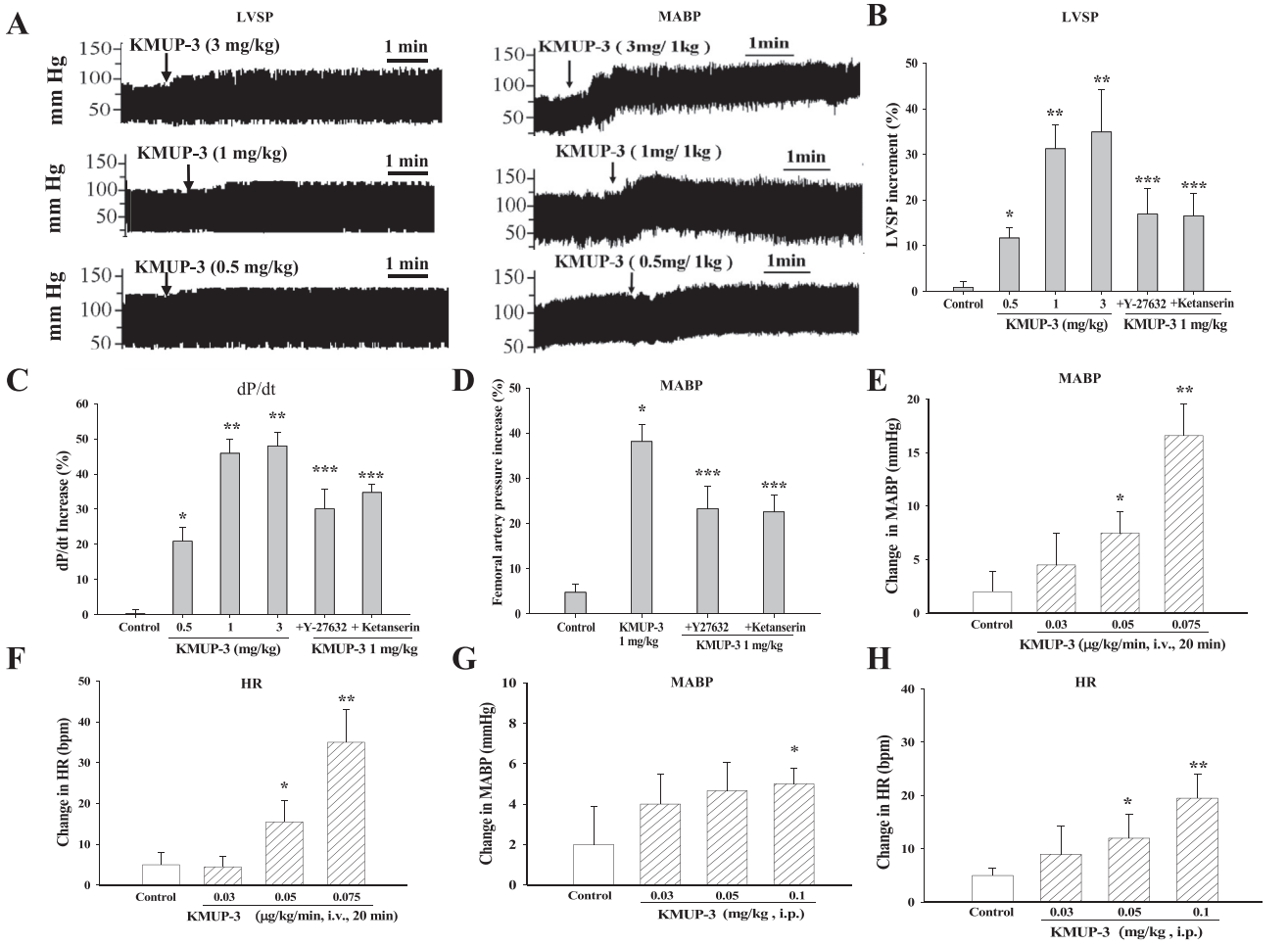

Figure 2. Hemodynamic effects of KMUP-3. (A) Recording traces of LVSP and MABP indicated that KMUP-3 (0.5 mg/kg, 1 mg/kg, and 3 mg/kg, i.v.) increased cardiac output, as shown in: (B) LVSP; (C) dp/dt; (A, D, E, G) MABP; and (F, H), HR. KMUP-3-induced effects were inhibited by pretreatment with Y27632 or ketanserin. Rats were treated with KMUP-3 (0.03—0.075 mg/kg, i.v., 20 minutes or 0.03—0.1 mg/kg, i.p.). + Y27632: KMUP-3 (1 mg/kg) + Y27632 (1 mg/kg); + ketanserin: KMUP-3 (1 mg/kg) + ketanserin (1 mg/kg). Values are expressed as means ± standard error ($n = 3$). * $p < 0.05$; ** $p < 0.01$ versus control; *** $p < 0.05$ versus KMUP-1 (1 mg/kg) alone (2-way repeated-measures analysis of variance followed by Student—Newman—Keuls test). dp/dt = maximal rate of pressure development; HR = heart rate; i.v. = intravenously; KMUP-3 = 7-{2-[4-(4-nitrobenzene) piperazinyl]ethyl}-1, 3-dimethylxanthine; LVSP = left ventricular systolic pressure; MABP = mean arterial blood pressure; Y27623 = (R)-(+)-trans-4-1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide.

Discussion

Hemodynamic examination is crucial for assessing cardiac performance in drug experiments for improving hypoperfused myocardium. KMUP-3, a PDE-3/PDE-4/PDE-5A inhibitor, has been used successfully to inhibit rat myocardial infarction [1]. Healthy animal tissues are also required to re-examine the benefits of KMUP-3 for improving outcomes in hypoperfused myocardium using an experimental model of electrically evoked muscle contraction and Ca^{2+}-current influx. H9c2, a clonal myogenic cell line derived from embryonic rat ventricles, was used to evaluate cardioprotection by an electrophysiologic technique, avoiding noise from tissue-derived motion in experiments. In this study, KMUP-3 displayed cardioprotective Ca^{2+} entry blockade and increased LVSP, dp/dt, inotropy, and PK expression, particularly RhoA/ROCKII, to correlate Ca^{2+}-sensitization and cardiac motion. The crosslink between β-AR/5-hydroxytryptamine (HT) receptor activation/inactivation determines the effect of KMUP-3 on inotropy and chronotropy [8,27—29]. Based on its Ca^{2+} sensitization, KMUP-3 is a potentially valuable new cardiac GPCR agonist that can therapeutically target cardiogenic shock or myocardial hypoperfusion by increasing blood supply, tissue oxygenation, and cardioprotection. Future experiments will evaluate sarco/endoplasmic reticulum calcium-ATPase as another effective method to determine the inotropic effect of KMUP-3.

ROCK enhanced cardiac contractility via Ca^{2+} sensitization, as it does in the vasculature. KMUP-3 activation of RhoA/ROCK leads to Ca^{2+} sensitization and cardiac muscle contractility even in the limited presence of Ca^{2+} [30].

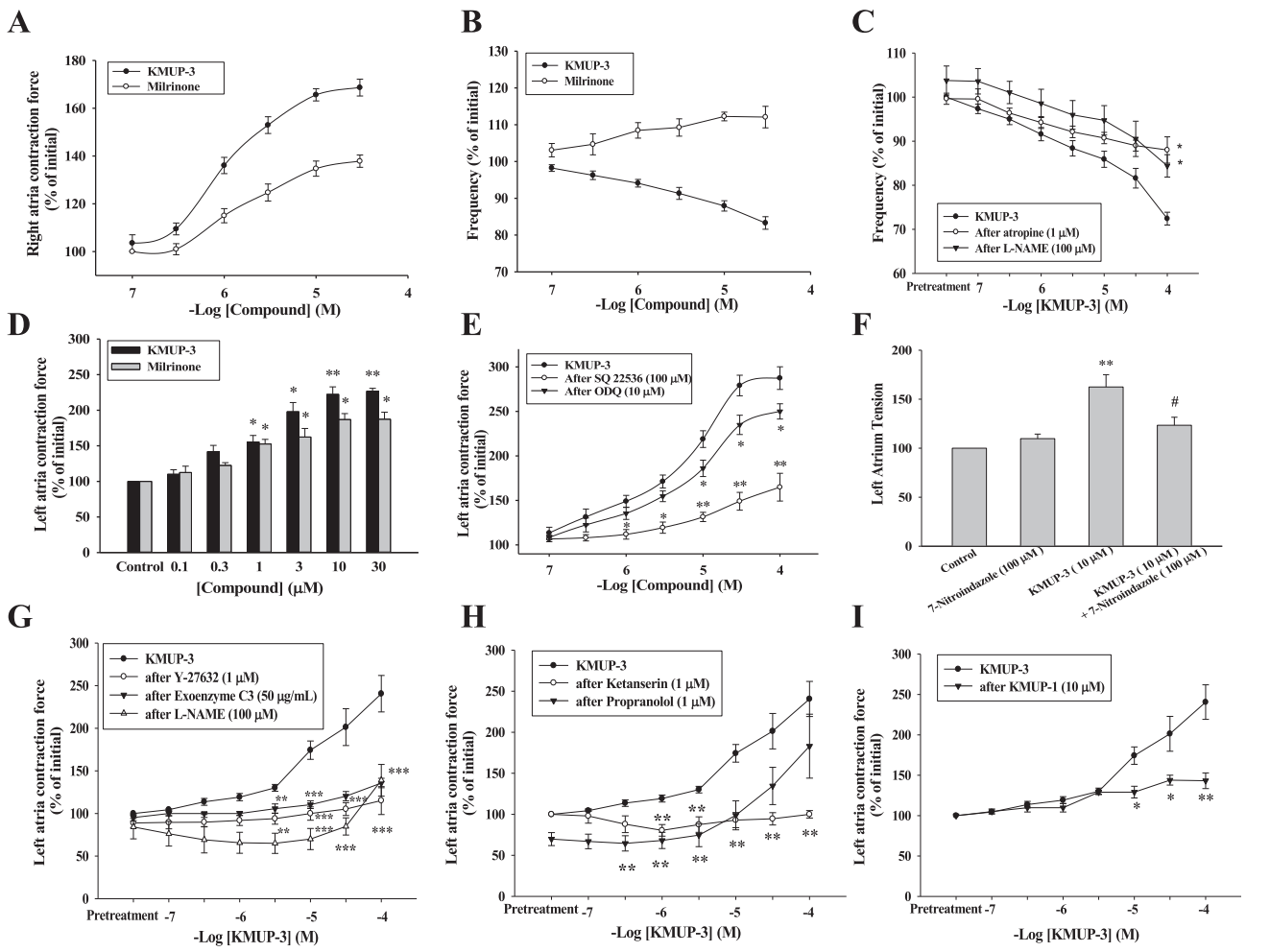

Figure 3. Inotropic and chronotropic activities of KMUP-3 in spontaneously beating right and electrically stimulated beating left atrium. (A) Positive inotropic activities of KMUP-3 and milrinone on beating right atrium. (B) Effects of KMUP-3 and milrinone on frequency of beating right atrium. (C) Effects of KMUP-3 on frequency of beating right atrium and pretreatment with atropine (1μM) or L-NAME (100μM). (D) Positive inotropic activities of KMUP-3 and milrinone on electronically stimulated left atrial contraction. (E) Effects of pretreatment with SQ22536 (100μM) or ODQ (10μM) on KMUP-3-induced electronically stimulated left atrial contraction. (F) Effects of pretreatment with 7-nitroindazole on KMUP-3-induced left atrial contraction. (G) Effects of pretreatment with Y27632 (1.0μM), exoenzyme C3 (50 μg/mL) or L-NAME (100μM) on KMUP-3-induced left atrial contraction. (H) Effects of pretreatment with ketanserin (1μM) or propranolol (1μM) on KMUP-3-induced left atrial contraction. Values are expressed as means ± standard error ($n = 3$). (A—E) and (G—I) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus KMUP-3; (G—I) share the same KMUP-3 data. (F) ** $p < 0.01$ versus control; # $p < 0.05$ versus KMUP-3 (2-way repeated-measures analysis of variance followed by Student—Newman—Keuls test). KMUP-3 = 7-[2-[4-(4-nitrobenzene) piperazinyl]ethyl]-1, 3-dimethylxanthine; L- NAME, *N*-nitro-L-arginine methyl ester; ODQ = 1*H*-[1,2,4] oxadiazolo[4,3-*a*] quinoxalin-1-one; SQ22536 = 9-(terahydro- 2- furanyl)-9*H*-purin-6-amine; Y27623 = (R)-(+)-trans-4-1-aminoethyl)-N- (4-pyridyl) cyclohexane carboxamide.

Expression of PKA, ROCKII, and phosphorylation of CPI-17 by KMUP-3 is suggested to increase atrial Ca^{2+} sensitization; increased CPI-17 phosphorylation indicates upstream activation via PKC and elevated Ca^{2+} [31]. The PKC-potentiated inhibitor protein of 17 kDa, called CPI-17, specifically inhibits MLCP. We suggest that KMUP-3 activates ROCKII and also enhances CPI-17 (*P*-CPI-17) production through PKC translocation to inhibit MLCP, leading to aortic and cardiac Ca^{2+} sensitization.

The GPCRs are a large protein family of transmembrane receptors. There are four pathways affecting the GPCR activity of KMUP-3: (1) Y27632-sensitive activation of GPCRs by RhoA/ROCK; (2) inactivation of β-AR type GPCRs via PKG-dependent PDE-5A; (3) propranolol-sensitive inactivation of β-AR type GPCRs by PDE3/PDE4; and (4) ketanserin-sensitive inactivation of 5-HT type GPCRs. Taken together, pathways (1), (3), and (4) display cardiac GPCR agonist activity of KMUP-3. We suggest that KMUP-3 at higher doses activates β-AR-type GPCRs through inhibition of PDE-3/PDE-4 (2), and at lower doses KMUP-3 enhances β-AR type inhibition of GPCRs via PDE-5A inhibition/eNOS activation [8,9]. KMUP-3 can be regarded as a cardiac GPCR agonist, while it increases LVSP, dp/dt, inotropy, PKs, and particularly activation of RhoA/ROCKII by inhibiting PDE-3/PDE-4/PDE-5. KMUP-1 inhibits KMUP-3-induced atrial contractility, therefore, KMUP-1 is suggested to be an antagonist of KMUP-3 via PDE and GPCRs.

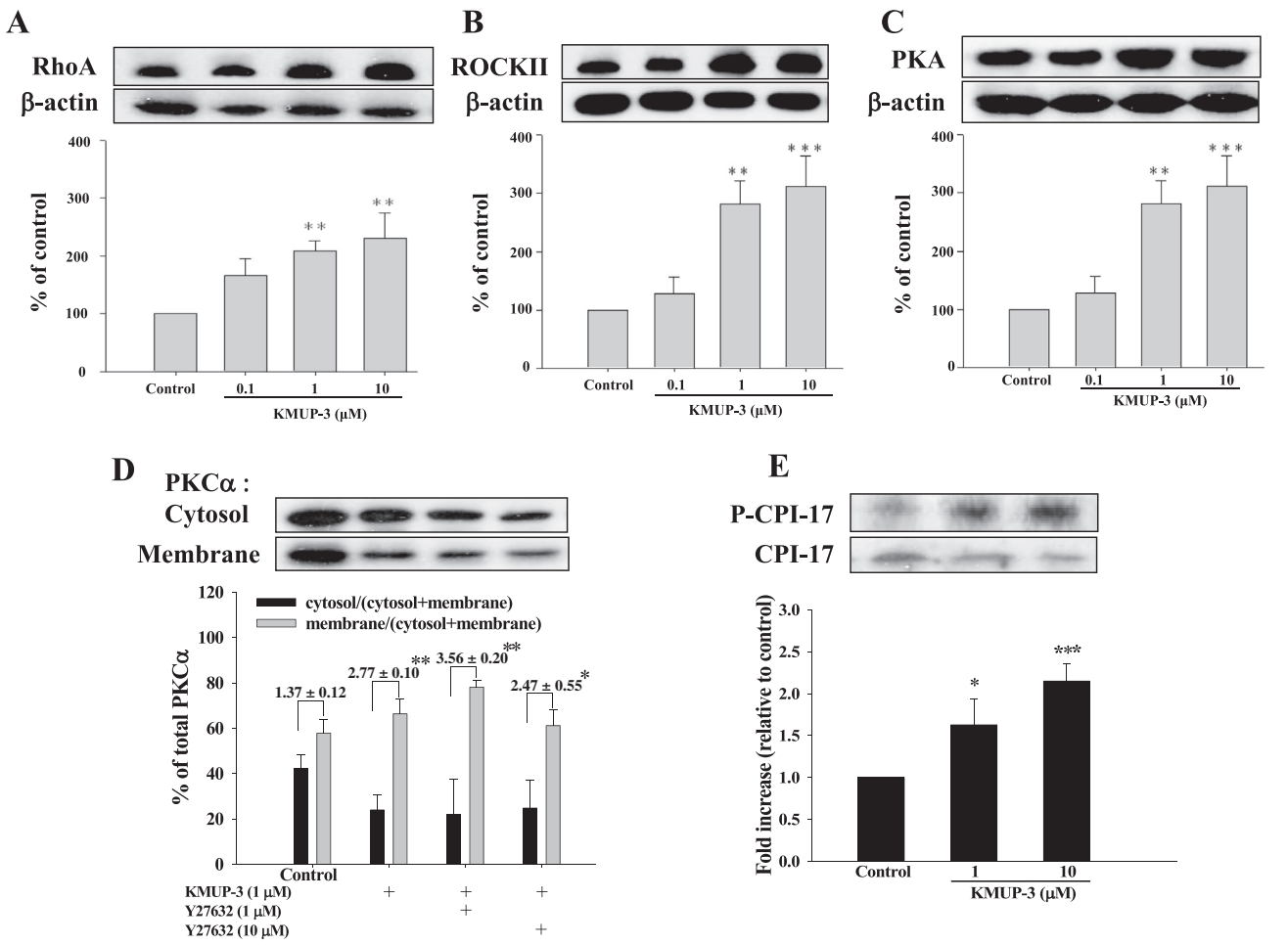

Figure 4. Protein expression of (A) RhoA, (B) ROCKII, (C) PKA, (D) PKC, and (E) phosphorylated CPI-17, induced by KMUP-3 in electrically stimulated guinea pig left atrium. In (D), the ratio of membrane to cytosol PKCα expression, compared to control, indicates the extent of translocation; each peak indicates the percentage of total PKCα expression. Values are expressed as means ± standard error ($n = 3$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus control (2-way repeated-measures analysis of variance followed by Student—Newman—Keuls test). CPI-17 = an endogenous 17-kDa inhibitory protein; KMUP-3 = 7-{2-[4-(4-nitrobenzene) piperazinyl]ethyl}-1, 3-dimethylxanthine; L- NAME, *N*-nitro-L-arginine methyl ester; PK = protein kinase; ROCK = Rho kinase.

PDE inhibitors are reported to increase cAMP/cGMP, and expression of RhoA/ROCK can be cAMP-dependent or cAMP-independent. Both cAMP and cGMP lead to the phosphorylation of RhoA, resulting in RhoA inhibition [32]. In this study, KMUP-3 activated cardiac RhoA/ROCK in the presence of increased cGMP and increased atrial contraction. By enhancing cGMP/PKG, KMUP-1 may selectively inhibit KMUP-3 GPCR agonist activity beyond PDE inhibition via PKA.

There are three pathways contributing to the inotropic and electrophysiological activity of KMUP-3: (1) RhoA/ROCK-mediated sensitization; (2) cAMP/PKA-mediated Ca^{2+}entry; and (3) cGMP/PKG-mediated blockade of Ca^{2+} entry. KMUP-3 predominately displays cGMP-dependent Ca^{2+} blockade and ROCK-mediated Ca^{2+} sensitization activity. The Ca^{2+} current inhibited by KMUP-3 were reversed by ROCK inhibitor Y27632 and PKG inhibitor KT5823, indicating that KMUP-3 is a ROCK activator and also a cGMP/PKG-enhancer [8,28,31]. Increased eNOS/p-eNOS in cultured neonatal rat myocytes and inhibited Ca^{2+} entry in cardiac H9c2 cells indicate that KMUP-3 has L-type Ca^{2+} blocking properties [28,30].

PKA increases open the L-type calcium channel, leading to calcium influx and Ca^{2+} release from the sarcoplasmic reticulum in cardiomyocytes via an excitation—contraction coupling pathway. Continuous stimulation of cAMP/PKA has calcium overloading potential and the resultant cardiac arrhythmia and myocardial cell injury has been considered the primary defect of PDE-3 inhibitors [33,34]. The electrically stimulated right atrial contractility shown by KMUP-3 is inhibited by the sGC inhibitor ODQ, indicating the involvement of cGMP. KMUP-3-induced positive inotropy can be inhibited by SQ22536, indicating the involvement of cAMP. The positive inotropy of KMUP-3, inhibited by propranolol, is due to increased activation of β-AR. Therefore, we suggest that the positive inotropy of KMUP-3 is partly associated with the activation of β-AR type GPCRs.

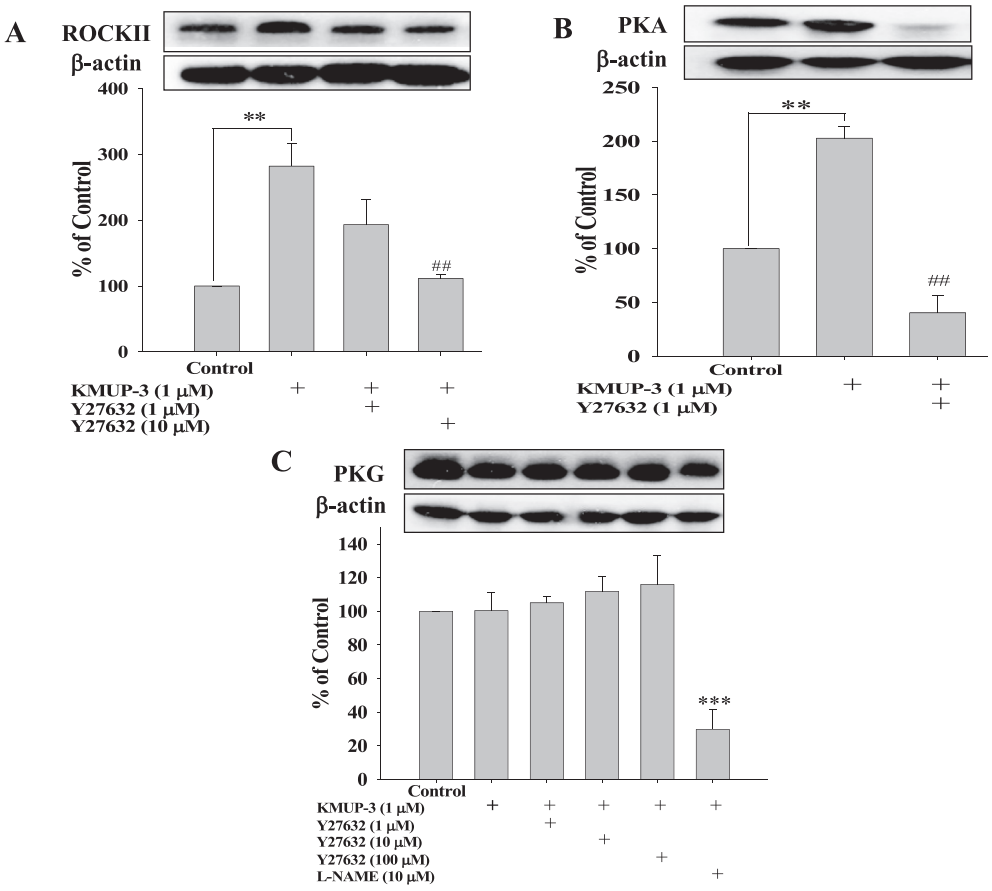

Figure 5. Effects of KMUP-3 on expression of (A) ROCKII, (B) PKA, and (C) PKG pretreatment with Y27632 and L-NAME in electrically stimulated guinea pig left atrium. Values are expressed as means ± standard error ($n = 3$). ** $p < 0.01$; *** $p < 0.001$ versus control; ## $p < 0.01$ versus KMUP-3 (2-way repeated-measures analysis of variance followed by Student—Newman—Keuls test). KMUP-3 = 7-[2-[4-(4-nitrobenzene); piperazinyl]ethyl]-1, 3-dimethylxanthine; PK = protein kinase; ROCK = Rho kinase; Y27623 = (R)-(+)-trans-4-1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide.

The KMUP-3-induced increase in cardiac output and electrically stimulated inotropy was inhibited by the ROCK inhibitor Y27632, indicating ROCK involvement. Specifically, pretreatments with C3 exoenzyme, a RhoA inhibitor, inhibited KMUP-3 inotropy, indicating the selective activation of RhoA. Also, ketanserin, a 5-HT2A antagonist, inhibited KMUP-3 inotropy, indicating 5-HT type GPCR involvement. Therefore, we suggest that the positive inotropy of KMUP-3 is partly associated with activation of 5-HT-type GPCRs and therefore RhoA/ROCKII [29,35,36]. In particular, C3 exoenzyme inhibited KMUP-3-induced contractility, indicating that 5-HT type GPCR is selectively activated to increase cardiac muscle contractility [34,35]. However, Y27632 is a nonspecific ROCK inhibitor displaying inhibition activity on PKs and thus able to reduce PKA and PKCα translocation in electrically stimulated left atrium [35].

Activation of NO/cGMP can stimulate the hyperpolarization-activated pacemaking current in the sinoatrial node of myocytes. NO acts presynaptically, displaying vagally mediated bradycardia [36]. KMUP-3 concentration-dependently decreased the spontaneous beating frequency of isolated guinea pig atria, indicating negative chronotropic activity possibly involving NO/cGMP. To elucidate whether parasympathetic efferent activity is involved in KMUP-3 negative chronotropy via the NO/cGMP pathway, electrically stimulated left atria pretreated with atropine and the NOS inhibitor 7-nitroindazole were enhanced, and the results suggested NO/cGMP-dependent acetylcholine release from the parasympathetic efferent nervous system.

The positive inotropy of KMUP-3 in beating atria is accompanied by increased PKA, PKC, and ROCKII expression, but not PKG. It is interesting that KMUP-3 more effectively increased ROCKII than PKA in the presence of PKG. However, increased PKC translocation by KMUP-3 was inhibited by Y27632, indicating that Ca^{2+} sensitization does not occur through ROCKII alone. In particular, crystal structure investigation indicated that PKA is in a complex with ROCK inhibitors; Y-27632, a relatively selective inhibitor of ROCK, inhibits PKC and cAMP-dependent PKA [35]. Accordingly, KMUP-3 causes crosstalk between PKA/PKC and ROCKII in the beating atrium.

RhoA activation might protect cardiomyocytes from ischemic damage [24]. Here, KMUP-3 exhibits cardioprotective effects via the expression of eNOS/cGMP/

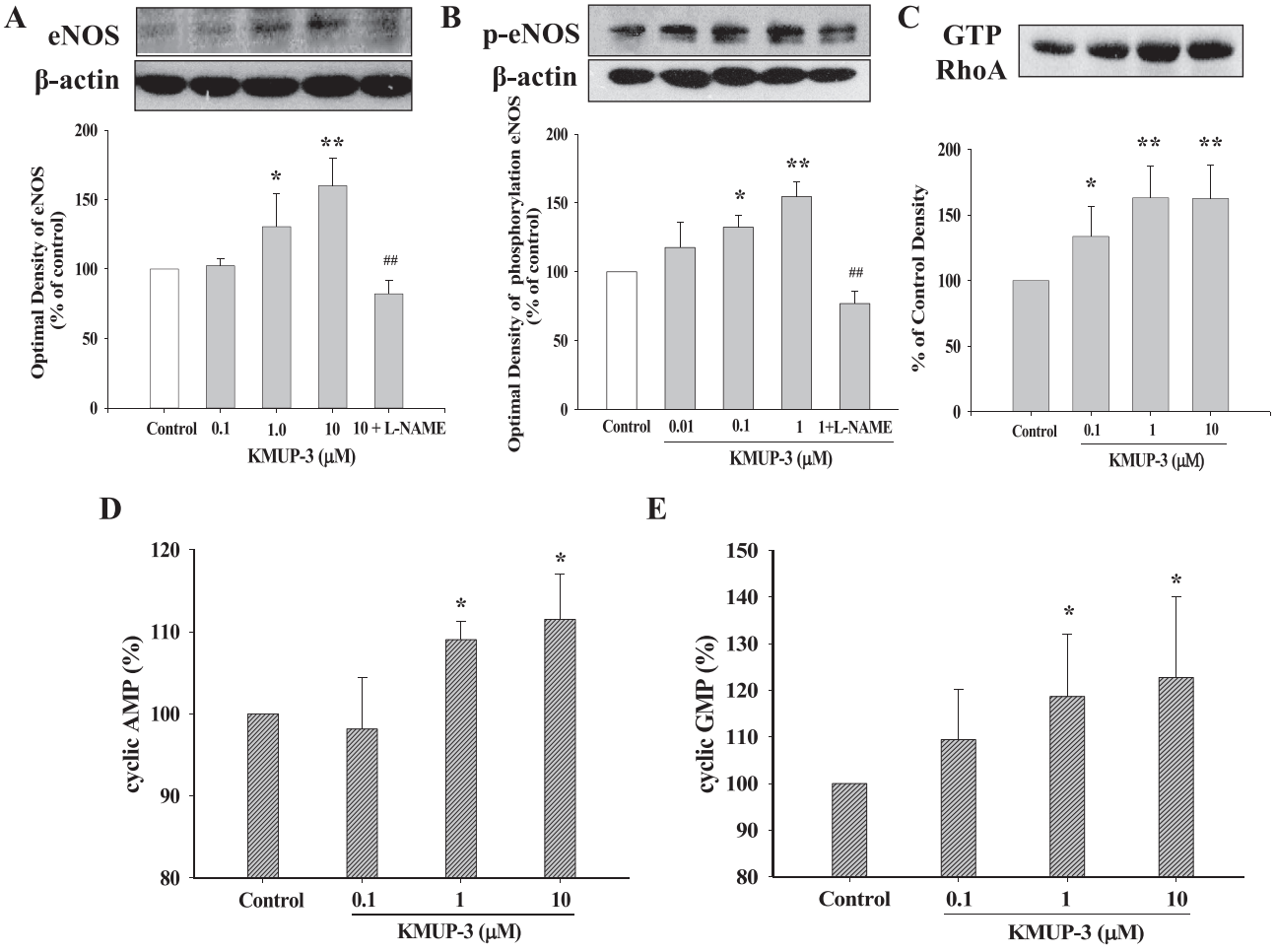

Figure 6. Effects of KMUP-3 on expression of (A, B) eNOS/p-eNOS and (C) GTP RhoA, and levels of (D) cAMP and (E) cGMP in cultured neonatal rat myocytes. In (A, B), KMUP-3-induced effects were inhibited by pretreatment with L-NAME. KMUP-3 increased (A) eNOS, (B) p-eNOS, (C) GTP RhoA, (D) cyclic AMP, and (E) cyclic GMP. Values are expressed as means ± standard error ($n = 3$). * $p < 0.05$; ** $p < 0.01$ versus control; ## $p < 0.01$ versus KMUP-3 (2-way repeated-measures analysis of variance followed by Student–Newman–Keuls test). eNOS = endothelial NO synthase; KMUP-3 = 7-{2-[4-(4-nitrobenzene) piperazinyl]ethyl}-1, 3-dimethylxanthine; L- NAME, *N*-nitro-L-arginine methyl ester; p-eNOS = phosphorylated eNOS.

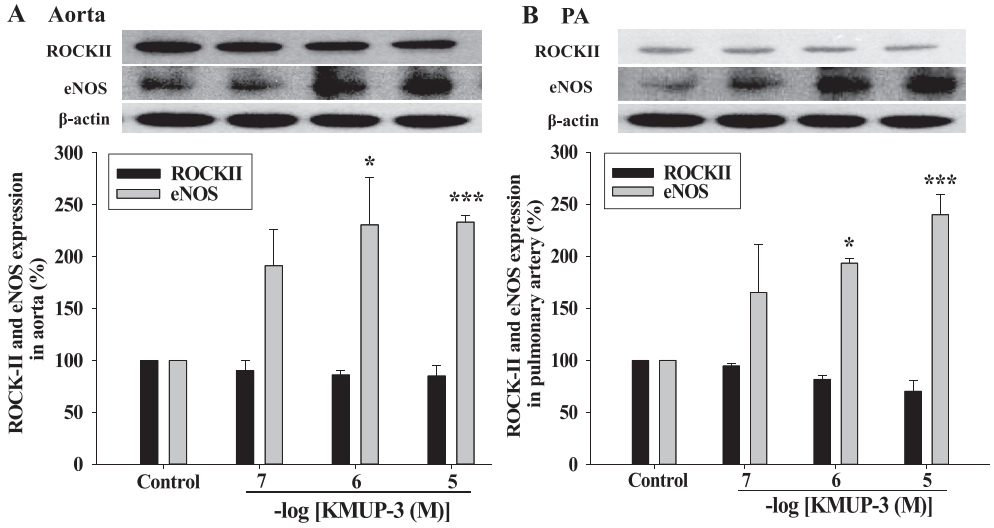

Figure 7. Expression of ROCK and eNOS in the vascular system. Isolated (A) rat aorta and (B) pulmonary artery were incubated with KMUP-3 (0.1μM, 1μM, and 10μM) for 90 minutes. eNOS expression in the aorta and pulmonary artery was increased by KMUP-3. ROCK was nonsignificantly altered by KMUP-3. Values are expressed as means ± standard error ($n = 3$). * $p < 0.05$; *** $p < 0.001$ versus control (2-way repeated-measures analysis of variance followed by Student–Newman–Keuls test). eNOS = endothelial NO synthase; KMUP-3 = 7-{2-[4-(4-nitrobenzene) piperazinyl]ethyl}-1, 3-dimethylxanthine; ROCK = Rho kinase.

PKG, although able to be regulated by PKA and PKC. KMUP-3 did not increase aortic and pulmonary vascular ROCKII expression, nor did it decrease ROCKII, indicating lack of peripheral vascular resistance. However, caution is required to prevent hypertensive crises by acute overdose. The divide between Ca^{2+}sensitization and Ca^{2+} entry blockade in the heart requires careful dosage adjustment.

Milrinone decreased vascular resistance and caused vascular hypotension beyond treating CHF, besides actions resulting from NO release [36,37]. By contrast, KMUP-3 activates ROCKII to increase cardiac output without inducing hypotension, while vasodilatory eNOS is increased or activated. Hypotension in response to KMUP-3 might be masked by increased LVSP due to PDE inhibition via PKA and/or GPCRs agonist activity related to Ca^{2+} sensitization.

Vascular GPCR agonist activity, characterized by Ca^{2+}-entry properties, has been shown by thrombin and lysophosphatidic acid in vascular smooth muscle cells but not in cardiac myocytes [25,26]. However, KMUP-3 did not increase vascular contraction, nor activate vascular ROCK, but did reduce cardiac Ca^{2+}entry via cGMP/PKG in H9c2 cells. We suggest that the agonist activity of KMUP-3 is inhibited by the GPCR antagonist KMUP-1.

In conclusion, KMUP-3 increases cardiac cGMP/PKG and displays cardioprotective activity at lower doses and activates GPCRs followed by Ca^{2+}sensitization at higher doses, along with PDE inhibition. The ability of KMUP-3 to increase cardiac output via Ca^{2+} sensitization/PDE inhibition at higher doses and protect the ventricular cells by Ca^{2+}-entry blockade via cGMP/PKG enhancement at lower doses would be useful for the treatment of myocardial hypoperfusion.

Acknowledgments

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References

[1] Liu CP, Yeh JL, Wu BN, Chai CY, Chen IJ, Lai WT. KMUP-3 attenuates ventricular remodelling after myocardial infarction through eNOS enhancement and restoration of MMP-9/TIMP-1 balance. Br J Pharmacol 2011;162:126—35.

[2] Zhang S, Rodriguez R, Scholz PM, Weiss HR. Functional interaction of a beta-adrenergic agonist and cyclic GMP phosphodiesterase inhibitor in control and hypertrophic cardiomyocytes. Pharmacology 2006;76:53—60.

[3] Stehlik J, Movsesian MA. Inhibitors of cyclic nucleotide phosphodiesterase 3 and 5 as therapeutic agents in heart failure. Expert Opin Investig Drugs 2006;15:733—42.

[4] Castro LR, Verde I, Cooper DM, Fischmeister R. Cyclic guanosine monophosphate compartmentation in rat cardiac myocytes. Circulation 2006;113:2221—8.

[5] Yeh JL, Liu CP, Hsu JH, Tseng CJ, Wu PJ, Wang YY, et al. KMUP-1 inhibits hypertension-induced left ventricular hypertrophy through regulation of nitric oxide synthases, ERK1/2, and calcineurin. Kaohsiung J Med Sci 2012;28:567—76.

[6] Endoh M. Could Ca^{2+}sensitizers rescue patients from chronic congestive heart failure? Br J Pharmacol 2007;150:826—8.

[7] Lin RJ, Wu BN, Lo YC, An LM, Dai ZK, Lin YT, et al. 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-alpha-induced tracheal contraction, involving nitric oxide release and expression of cGMP and protein kinase G. J Pharmacol Exp Ther 2006;316:709—17.

[8] Christ T, Galindo-Tovar A, Thoms M, Ravens U, Kaumann AJ. Inotropy and L-type Ca^{2+}current, activated by β1- and β2-adrenoceptors, are differently controlled by phosphodiesterases 3 and 4 in rat heart. Br J Pharmacol 2009;156:62—83.

[9] Lee DI, Vahebi S, Tocchetti CG. PDE5A suppression of acute beta-adrenergic activation requires modulation of myocyte beta-3 signaling coupled to PKG-mediated troponin I phosphorylation. Basic Res Cardiol 2010;105:337—47.

[10] Yeh JL, Hsu JH, Wu PJ, Liou SF, Liu CP, Chen IJ, et al. KMUP-1 attenuates isoprenaline induced cardiac hypertrophy in rats through NO/cGMP/PKG and ERK1/2/calcineurin A pathways. Br J Pharmacol 2010;159:1151—60.

[11] Liu CP, Dai ZK, Huang CH, Yeh JL, Wu BN, Wu JR, et al. Endothelial nitric oxide synthase-enhancing G-protein coupled receptor antagonist inhibits pulmonary artery hypertension by endothelin-1-dependent and endothelin-1-independent pathways in a monocrotaline model. Kaohsiung J Med Sci 2014;30:267—78.

[12] Chung HH, Dai ZK, Wu BN, Yeh JL, Chai CY, Chu KS, et al. The xanthine derivative KMUP-1 inhibits models of pulmonary artery hypertension via increased NO and cGMP-dependent inhibition of RhoA/Rho kinase. Br J Pharmacol 2010;160:971—86.

[13] Chung HH, Dai ZK, Wu BN, Yeh JL, Chai CY, Chu KS, et al. KMUP-1 inhibits pulmonary artery proliferation by targeting serotonin receptors/transporter and NO synthase, inactivating RhoA and suppressing AKT/ERK phosphorylation. Vascul Pharmacol 2010;53:239—49.

[14] Dai ZK, Lin TC, Liou JC, Cheng KI, Chen JY, Chu LW, et al. Xanthine derivative KMUP-1 reduces inflammation and hyperalgesia in a bilateral chronic constriction injury model by suppressing MAPK and NFκB activation. Mol Pharmacol 2014;11:1621—31.

[15] Kuo KK, Wu BN, Liu CP, Yang TY, Yang LP, Wu JR, et al. Xanthine-based KMUP-1 improves HDL via PPARγ/SR-B1, LDL via LDLRs and HSL via PKA/PKG for hepatic fat loss. J Lipid Res 2015;56:2070—84.

[16] Wu BN, Tu HF, Welsh DG, Chen IJ. KMUP-1 activates BKCa channels in basilar artery myocytes via cyclic nucleotide-dependent protein kinases. Br J Pharmacol 2005;146:862—71.

[17] Hoshijima M, Sah VP, Wang Y, Chien KR, Brown JH. The low molecular weight GTPase Rho regulates myofibril formation and organization in neonatal rat ventricular myocytes. Involvement of Rho kinase. J Biol Chem 1998;273:7725—30.

[18] Mizuno Y, Isotani E, Huang J, Ding H, Stull JT, Kamm KE. Myosin light chain kinase activation and calcium sensitization in smooth muscle *in vivo*. Am J Physiol Cell Physiol 2008;295:C358—64.

[19] Segreti JA, Marsh KC, Polakowski JS, Fryer RM. Evoked changes in cardiovascular function in rats by infusion of levosimendan, OR-1896 [(R)-N-(4-(4- methyl-6-oxo-1,4,5,6- tetrahydropyridazin-3-yl) phenyl) acetamide], OR-1855 [(R)-6-(4-aminophenyl)-5-methyl-4,5-dihydro pyridazin-3(2H)-one], dobutamine, and milrinone: comparative effects on peripheral resistance, cardiac output, dP/dt, pulse rate, and blood pressure. J Pharmacol Exp Ther 2008;325:331—40.

[20] Takimoto E, Champion HC, Li M, Belardi D, Ren S, Rodriguez ER, et al. Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. Nat Med 2005;11:214—22.

[21] Peters SL, Michel MC. The RhoA/Rho kinase pathway in the myocardium. Cardiovasc Res 2007;75:3—4.
[22] Phrommintikul A, Tran L, Kompa A, Wang B, Adrahtas A, Cantwell D, et al. Effects of a Rho kinase inhibitor on pressure overload induced cardiac hypertrophy and associated diastolic dysfunction. Am J Physiol Heart Circ Physiol 2008;294: H1804—14.
[23] Vlasblom R, Muller A, Beckers CM, van Nieuw Amerongen GP, Zuidwijk MJ, van Hardeveld C, et al. RhoA-ROCK signaling is involved in contraction-mediated inhibition of SERCA2a expression in cardiomyocytes. Pflugers Arch 2009;458:785—93.
[24] Miyamoto S, Del Re DP, Xiang SY, Zhao X, Florholmen G, Brown JH. Revisited, revised: Is RhoA always a villain in cardiac pathophysiology? J Cardiovasc Transl Res 2010;3: 330—43.
[25] Liu K, Southall N, Titus SA, Inglese J, Eskay RL, Shinn P, et al. A multiplex calcium assay for identification of GPCR agonists and antagonists. Assay Drug Dev Technol 2010;8:367—79.
[26] Seasholtz TM, Majumdar M, Kaplan DD, Brown JH. Rho, Rho kinase mediate thrombin-stimulated vascular smooth muscle cell DNA synthesis and migration. Circ Res 1999;84:1186—93.
[27] Walsh CT, Stupack D, Brown JH. G protein-coupled receptors go extracellular: RhoA integrates the integrins. Mol Interv 2008;8:165—73.
[28] Chen JY, Cheng KI, Tsai YL, Hong YR, Howng SL, Kwan AL, et al. Potassium-channel openers KMUP-1 and pinacidil prevent subarachnoid hemorrhage-induced vasospasm by restoring the BK_{Ca}-channel activity. Shock 2012;38:203—12.
[29] Wu BN, Chen IC, Lin RJ, Chiu CC, An LM, Chen IJ. Aortic smooth muscle relaxants, KMUP-3 and KMUP-4, two nitrophenylpiperazine derivatives of xanthine, display cGMP-enhancing activity: roles of endothelium, phosphodiesterase, and K+ channel. J Cardiovasc Pharmacol 2005;46:600—8.
[30] Seguchi O, Takashima S, Yamazaki S, Asakura M, Asano Y, Shintani Y, et al. Cardiac myosin light chain kinase regulates sarcomere assembly in the vertebrate heart. J Clin Invest 2007;117:2812—24.
[31] Shimokawa H, Takeshita A. Rho-Kinase is an important therapeutic target in cardiovascular medicine. Arterioscler Thromb Vasc Biol 2005;25:1767—75.
[32] Guilluy C, Rolli-Derkinderen M, Loufrani L, Bourgé A, Henrion D, Sabourin L, et al. Ste20-related kinase SLK phosphorylates Ser188 of RhoA to induce vasodilation in response to angiotensin II Type 2 receptor activation. Circ Res 2008; 102:1265—74.
[33] Haddad GE, Coleman BR, Zhao A, iBlackwell KN. Modulation of atria contraction by PKA and PKC during the compensated phase of eccentric cardiac hypertrophy. Basic Res Cardiol 2004;99:317—27.
[34] Somlyo AP, Somlyo AV. Ca^{2+}sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol Rev 2003;83:1325—58.
[35] Liao JK, Seto M, Noma K. Rho Kinase (ROCK) Inhibitors. J Cardiovasc Pharmacol 2007;50:17—24.
[36] Herring N, Paterson DJ. Nitric oxide-cGMP pathway facilitates acetylcholine release and bradycardia during vagal nerve stimulation in the guinea-pig *in vitro*. J Physiol 2001;535: 507—18.
[37] Albrecht CA, Giesler GM, Kar B, Hariharan R, Delgado 3rd RM. Intravenous milrinone infusion improves congestive heart failure caused by diastolic dysfunction: a brief case series. Tex Heart Inst J 2005;32:220—3.