Kaohsiung Journal of Medical Sciences (2011) 27, 538–543

Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: http://www.kjms-online.com

ORIGINAL ARTICLE

KMUP-1 inhibits L-type Ca^{2+} channels involved the protein kinase C in rat basilar artery myocytes

Jun-Yih Chen [a,b], Min-Chi Jiang [c], Li-Wen Chu [d], Su-Ling Hsieh [e], Ing-Jun Chen [c], Bin-Nan Wu [c,*]

[a] *Department of Neurosurgery, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan*
[b] *Institute of Biological Sciences, National Sun Yat-Sen University, Kaohsiung, Taiwan*
[c] *Department of Pharmacology, School of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[d] *School of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan*
[e] *Department of Pharmacy, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan*

Received 20 May 2011; accepted 10 June 2011
Available online 25 November 2011

KEYWORDS
Basilar artery;
Ca^{2+} channels;
KMUP-1;
Patch-clamp technique;
Protein kinase C

Abstract This study investigated whether KMUP-1, a xanthine-based derivative, inhibits L-type Ca^{2+} currents ($I_{Ca,L}$) in rat basilar artery smooth muscle cells (RBASMCs). We used whole cell patch-clamp recording to monitor Ba^{2+} currents (I_{Ba}) through L-type Ca^{2+} channels (LTCCs). Under voltage–clamp conditions, holding at −40 mV, KMUP-1 (1, 3, 10 μM) inhibited I_{Ba} in a concentration-dependent manner and its IC_{50} value was 2.27 ± 0.45 μM. A high concentration of KMUP-1 (10 μM) showed without modifying the I_{Ba} current–voltage relationship. On the other hand, the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA, 1 μM) increase I_{Ba} was inhibited by KMUP-1. Pretreatment with the PKC inhibitor chelerythrine (5 μM) intensified KMUP-1-inhibited I_{Ba}. However, the Rho kinase inhibitor Y-27632 (30 μM) failed to affect the I_{Ba} inhibition by KMUP-1. In light of these results, we suggest that KMUP-1 inhibition of LTCCs in concentration- and voltage-dependent manners in RBASMCs may be due, at least in part, to its modulation of the PKC pathway.

Introduction

KMUP-1 (7-{2-[4-(2-chlorobenzene)piperazinyl]ethyl}-1,3-dimethylxanthine) has been demonstrated to raise cyclic nucleotides, inhibit phosphodiesterases and stimulate K^+ channels resulting in relaxations in aortic [1], corporeal cavernosa [2], and tracheal smooth muscles [3]. Its smooth

* Corresponding author. Department of Pharmacology, School of Medicine, College of Medicine, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung 807, Taiwan.
E-mail address: binnan@kmu.edu.tw (B.-N. Wu).

1607-551X/$36
doi:10.1016/j.kjms.2011.10.026

muscles relaxations could be mediated via two major pathways, either via activation of K^+ channels that are independent of cellular cyclic nucleotides, or through increases in both cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), followed by stimulation of protein kinase A (PKA) and G (PKG) cascades. Elevated PKA and PKG appear to activate K^+ channels, thus resulting in the lowering of cellular Ca^{2+} levels [3]. Furthermore, we have provided the evidence that KMUP-1 activates large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) currents in basilar artery myocytes [4]. The findings suggested that KMUP-1 could reduce the activity of Ca^{2+} channels by two routes; directly, by its Ca^{2+} channel blocking action, and indirectly by activating BK_{Ca} channels producing hyperpolarization, thus decreasing the open probability of Ca^{2+} channels through their voltage-dependence. However, the direct ionic mechanism by which KMUP-1 inhibits basilar artery Ca^{2+} currents remains undetermined. Therefore, this study aimed to look into the effects of KMUP-1 on voltage-dependent Ba^{2+} currents ($I_{Ba,}$) through L-type Ca^{2+} channels (LTCCs), targeting the applicability of KMUP-1 for cerebral artery dilatation.

LTCCs are activated by membrane depolarization, and their function is subject to modulation by signaling molecules such as protein kinase C (PKC) [5,6] or RhoA/Rho kinase [7,8]. These channels play an indispensable role in many signaling pathways requiring Ca^{2+} influx for activation of intracellular Ca^{2+}-dependent molecules [9]. LTCCs and intracellular Ca^{2+} [$(Ca^{2+})_i$] have a critical role in regulating many cellular functions in vascular smooth muscle cells (VSMCs), including contraction and relaxation. This may be of importance because alterations in the expression of LTCCs and $[Ca^{2+}]_i$ levels have been linked to several pathologic processes that involve VSMCs, including hypertension, coronary vasospasm, and atherosclerosis [7].

The main objective of this study was to address the mechanism by which KMUP-1 could modulate LTCCs in rat basilar artery smooth muscles (RBASMCs). Accordingly, whole cell patch-clamp electrophysiology was used to study whether KMUP-1 inhibited the LTCCs activity through PKC-dependent and/or -independent signaling pathways.

Methods

Animal procedures and tissue preparations

All procedures and protocols were approved by the Animal Care and Use Committee at the Kaohsiung Medical University. Briefly, female Sprague Dawley rats (10—12 weeks of age) were euthanized by carbon dioxide asphyxiation. The brain was carefully removed and placed in cold phosphate-buffered saline containing (in mM): 138 NaCl, 3 KCl, 10 Na_2HPO_4, 2 NaH_2PO_4, 5 glucose, 0.1 $CaCl_2$, and 0.1 $MgSO_4$ (pH 7.4). The basilar arteries were dissected free of the surrounding tissue and cut into 2-mm segments.

Preparation of isolated arterial smooth muscle cells

RBASMCs from rat basilar arteries were enzymatically isolated as previously described [4,10]. In brief, arterial segments were placed in warm (37°C) cell isolation medium containing (in mM): 60 NaCl, 80 Na-glutamate, 5 KCl, 2 $MgCl_2$, 10 N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), and 10 glucose with 1 mg/ml albumin (pH 7.2) for 10 minutes. After this equilibration step, arterial segments were initially incubated (37°C) in 1 mg/ml papain and 3 mg/ml dithioerythritol for 20 minutes. This was followed by a second incubation (37°C) in isolation medium containing 100 μM Ca^{2+}, 0.7 mg/ml type F collagenase, and 0.4 mg/ml type H collagenase for 10 minutes. After enzyme treatment, the tissue was washed three times in ice-cold isolation medium and triturated with a fire-polished pipette to release the myocytes. RBASMCs were stored in ice-cold isolation medium for use on the same day.

Patch-clamp electrophysiology

To measure the I_{Ba} through LTCCs, whole cell patch-clamp recording was performed [10]. In brief, RBASMCs were put in a recording dish and perfused with a bath solution containing (in mM): 120 NaCl, 10 tetraethylammonium (TEA)-Cl, 5 $BaCl_2$, 0.5 $MgCl_2$, 5.5 glucose, 10 HEPES, 5 CsCl, and 0.001 tetrodotoxin (pH 7.4, CsOH). To minimize outward K^+ currents, Cs^+ rather than K^+ was used in the pipette solution. A recording electrode was pulled from borosilicate glass (resistance: 4—7 megaohm), the pipette was coated with sticky wax close to the tip to reduce capacitance, and it was backfilled with pipette solution containing (in mM): 140 CsCl, 5 ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetra-acetic acid (EGTA), 1 $MgCl_2$, 5 Na_2ATP, 1 guanosine triphosphate, 5 HEPES, and 5 CsOH (pH 7.2, CsOH). It was then gently lowered onto a smooth muscle cell. Negative pressure was briefly applied to rupture the membrane and a gigaohm seal was obtained. Cells were subsequently voltage clamped (—40 mV). Membrane currents were recorded on an Axopatch 700 A amplifier (Axon Instruments, Union City, CA), 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 (Axon Instruments, Union City, CA). A 1-M NaCl-agar salt bridge between the bath and the Ag—AgCl reference electrode was used to minimize offset potentials. All electrophysiologic recordings were performed at room temperature.

Experimental procedures

To evoke whole-cell I_{Ba} (or I_{Ca}), RBASMCs were clamped at —40 mV with step depolarizations (200 milliseconds) from —40 to 50 mV. Voltage clamped cells were equilibrated for 15 minutes prior to experimentation. Following equilibration, inward I_{Ba} was monitored in the presence and absence of KMUP-1 (1 μM). To ascertain whether PKC or Rho kinase signaling were involved in the KMUP-1-mediated I_{Ba} inhibition, RBASMCs were preincubated for 15 minutes with phorbol 12-myristate 13-acetate (PMA, 1 μM), chelerythrine (5 μM), or Y-27632 (30 mM) prior to the addition of KMUP-1. PMA, chelerythrine, or Y-27632 was continuously superfused in the bath solution.

Data analysis and statistics

Data are expressed as means ± SE and *n* indicates the number of cells. Repeated measures of analyses of variance

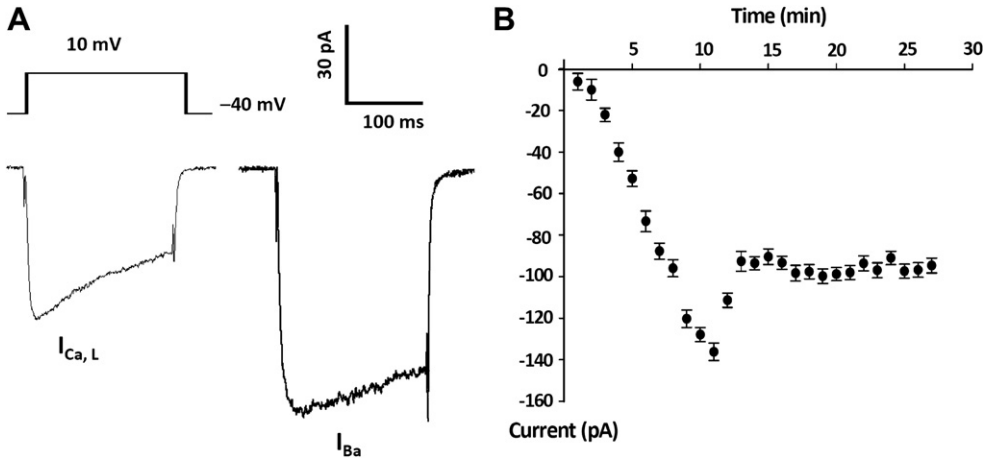

Figure 1. External Ca^{2+} solution was substituted by $BaCl_2$ (Ba^{2+}). (A) Replacement of 1.8 mM Ca^{2+} with 5 mM Ba^{2+} resulted in increases of the inward current evoked by a step from −40 to 10 mV; (B) time course of Ba^{2+} current (I_{Ba}) under control conditions. The peak I_{Ba} amplitude was plotted against time. About 12 minutes later, the current amplitude reached the steady state ($n = 6$).

compared values at a given voltage. When appropriate, a Tukey-Kramer pairwise comparison was used for *post hoc* analysis. The p values ≤ 0.05 were considered statistically significant.

Results

Steady-state of inward I_{Ba}

Using a whole-cell patch clamp and pipette solution containing Cs^+ in this experiment, RBASMC was clamped at −40 mV and continuously superfused with an isotonic physiologic solution containing 1.8 mM Ca^{2+} or 5 mM Ba^{2+}. RBASMC was depolarized from −40 to 10 mV to produce an inward I_{Ba} through LTCCs. Rapid inactivation of LTCCs observed in perfusing 1.8 mM Ca^{2+} solution while replaced with 5 mM Ba^{2+} retarded the inactivation and enhanced this inward current. The I_{Ba} was constantly observed 30 minutes after cell disruption, with the peak, steady-state level occurring ~12 minutes (Fig. 1).

Inhibition of inward I_{Ba} by KMUP-1

Whole-cell patch clamp recording was used to measure the effect of KMUP-1 (1, 3, 10 μM) on the regulation of LTCCs (Fig. 2). At the holding potential of −40 mV, KMUP-1 exhibited a concentration-dependent inhibition of inward I_{Ba} and its IC_{50} value was 2.27 ± 0.45 μM. KMUP-1 (10 μM) inhibited I_{Ba} in ~25% of control cells, but the dihydropyridine-sensitive LTCC blocker nifedipine (1 μM) nearly abolished the current (data not shown). Additionally, depolarizing step pulses (10 mV increments from −40 to 50 mV for 200 milliseconds) were applied from a holding potential of −40 mV. Fig. 3 shows the current-voltage (I–V) relationships of 10 μM KMUP-1-mediated I_{Ba} obtained at −40 mV. We observed that at potentials more positive than −30 mV, an inward I_{Ba} was evoked; this reached a peak and then gradually decayed. The amplitude of this current was voltage-dependent; the maximum peak amplitude was observed at approximately 10 mV and then was reduced at more positive potentials. After 20 minutes of drug washout, the recovery of peak current of KMUP-1-mediated I_{Ba} was ~85%.

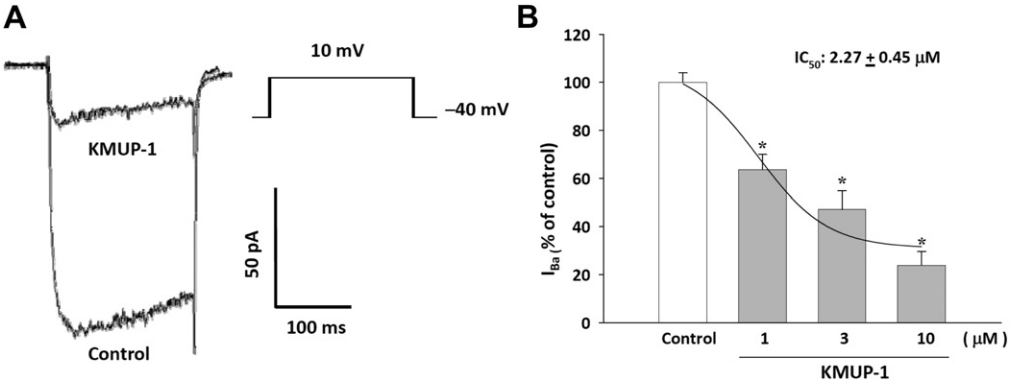

Figure 2. Effects of KMUP-1 (1, 3, 10 μM) on inward I_{Ba} in RBASMCs. (A) Representative recordings of I_{Ba} evoked by a test pulse to 10 mV from a holding potential of −40 mV, before and after perfusion with KMUP-1. (B) Bar graph showing the effects in the absence and presence of different concentrations of KMUP-1 ($n = 6$). * Denotes significant difference from control.

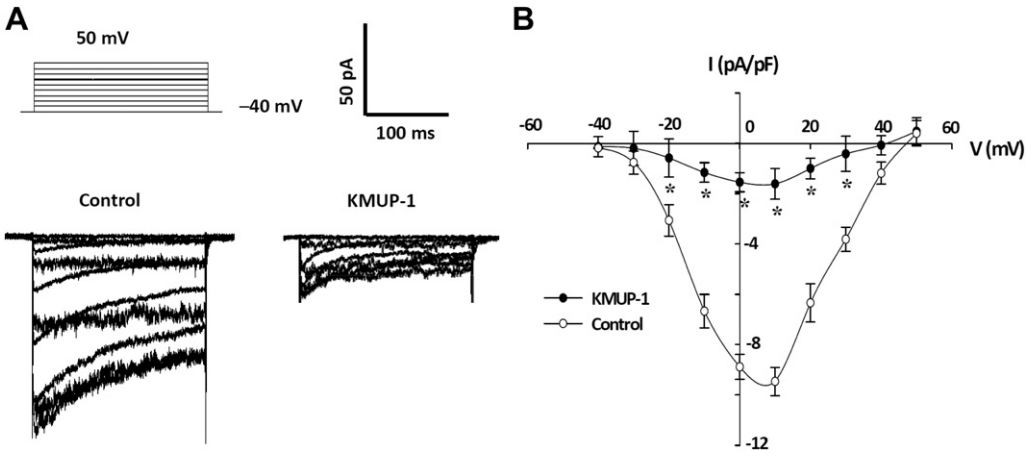

Figure 3. The *I*−*V* relationships of KMUP-1 (10 μM) on inward I_{Ba} in RBASMCs. (A) Voltage protocol was designed to measure the I_{Ba}. Representative recordings of I_{Ba} evoked by a series of depolarizing pulse (200 ms, range from −40 to 50 mV) from a holding potential of −40 mV in the absence and presence of KMUP-1; (B) average *I*−*V* relationship of the peak I_{Ba} in the absence (○) and presence (●) of KMUP-1 ($n = 6$). * Denotes significant difference from control.

PKC and/or Rho kinase involved the KMUP-1-mediated I_{Ba} inhibition

A perfused PKC activator PMA (1 μM) enhanced the inward I_{Ba} in RBASMCs. KMUP-1 (10 μM) fully eliminated the PMA-enhanced inward current and further reduced the basal I_{Ba} (Fig. 4). Perfusate with a PKC inhibitor chelerythrine (5 μM, Fig. 5) or a Rho kinase inhibitor Y-27632 (30 mM, Figure 6) inhibited the I_{Ba} in RBASMCs. Chelerythrine reinforced KMUP-1 (1 μM)-mediated I_{Ba} inhibition (Fig. 5), but Y-27632 did not affect KMUP-1-mediated current inhibition (Fig. 6). From these results, we suggest that KMUP-1-mediated I_{Ba} inhibition could be due, at least in part, to its modulation of the PKC pathway.

Discussion

The pivotal findings of this study are that KMUP-1 inhibits the LTCCs in RBASMCs in a concentration-dependent manner. We observed that the PKC activator, PMA-induced inward I_{Ba} increment is reversed by adding KMUP-1 in perfusate. The PKC inhibitor chelerythrine augments KMUP-1-mediated I_{Ba} inhibition, but it was not found in Rho kinase inhibitor Y-27632. Taken together, we suggest that KMUP-1-mediated LTCCs inhibition results at least partly from modulation of the PKC pathway.

Voltage-dependent LTCCs play a crucial role in the regulation of vascular tone by membrane potential: hyperpolarization closes these channels and leads to

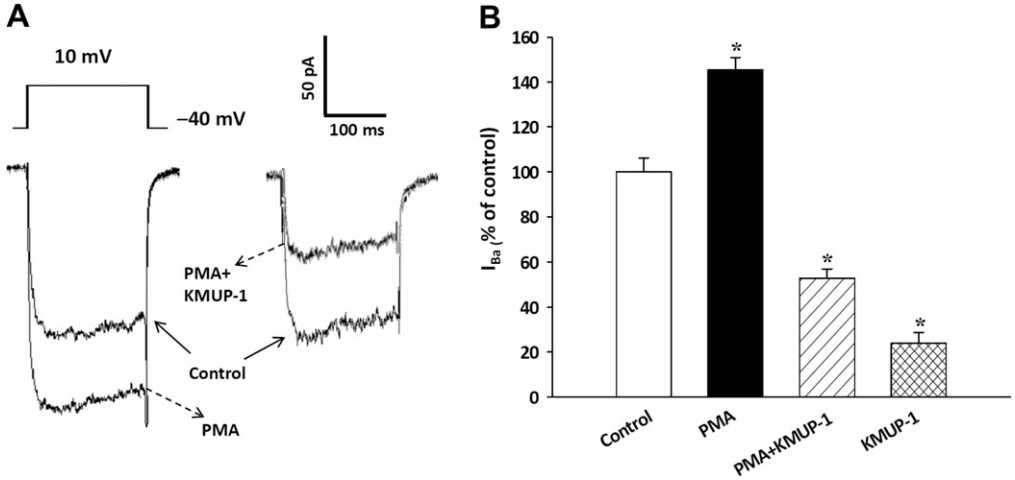

Figure 4. Effects of KMUP-1 (10 μM) on PMA (1 μM)-induced activation of inward I_{Ba} in RBASMCs. (A) Representative recordings of I_{Ba} evoked by a test pulse to 10 mV from a holding potential of −40 mV before and after perfusion with PMA or PMA + KMUP-1; (B) bar graph showing the relative I_{Ba} under control conditions and in the presence of PMA, KMUP-1 or PMA + KMUP-1 ($n = 6$). * Denotes significant difference from control.

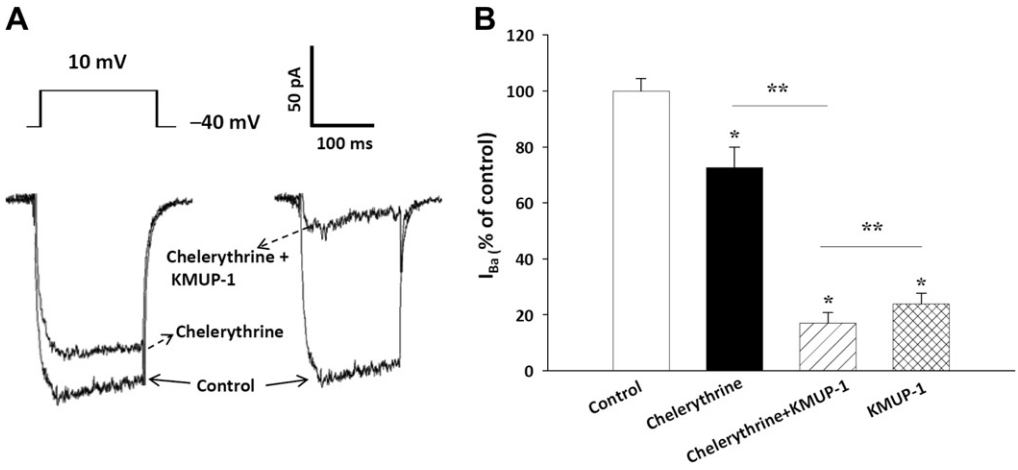

Figure 5. Effects of the PKC inhibitor chelerythrine (5 μM) on KMUP-1 (10 μM)-inhibited inward I_{Ba} in RBASMCs. (A) Representative recordings of I_{Ba} evoked by a test pulse to 10 mV from a holding potential of −40 mV before and after perfusion with chelerythrine or chelerythrine + KMUP-1; (B) bar graph showing the relative I_{Ba} under control conditions and in the presence of chelerythrine, KMUP-1, or chelerythrine + KMUP-1 ($n = 6$). * Denotes significant difference from control. ** Denotes significant difference between the two groups.

vasodilation, whereas depolarization opens them, resulting in vasoconstriction [11]. Dihydropyridine-sensitive LTCCs seem to be dominant in most VSMCs. In the microcirculation, LTCCs appear to play a particularly important role in myogenic reactivity and vasomotion [12]. Voltage-dependent LTCCs are modulated by several signaling systems. They appear to be activated by vasoconstrictors that activate the PKC pathway [12]. In this study, KMUP-1-mediated I_{Ba} inhibition through LTCCs is attributed to its voltage-dependence in RBASMCs, also found in dihydropyridine-sensitive LTCC blocker nifedipine [13]. In addition, KMUP-1-sensitive I_{Ba} is not mediated by a Na^+ or K^+ channel because the extracellular and/or intracellular solutions used contain the voltage-sensitive Na^+ channel blocker tetrodotoxin and the K^+ channel blockers Cs^+ and TEA. From the findings, we suggest that KMUP-1-mediated voltage-dependent LTCCs inhibition would modulate the cerebral artery tone and circulation.

It is widely accepted that the activation of PKC promotes the opening of voltage-dependent LTCCs [5,6,12]. In this study, we further confirmed that the PKC activator PMA enhanced the inward I_{Ba} through LTCCs in RBASMCs [13]. Notably, KMUP-1 not only abolished the PMA-increased inward current, but it also attenuated the basal I_{Ba}. The PKC inhibitor chelerythrine—but not the Rho kinase inhibitor Y-27632—enhanced KMUP-1-mediated I_{Ba} inhibition. These results strongly indicated that KMUP-1-mediated I_{Ba} inhibition through LTCCs could be attributed to PKC—but not Rho kinase—modulation. In addition to PKC modulation by KMUP-1, however, we cannot exclude the possibility that

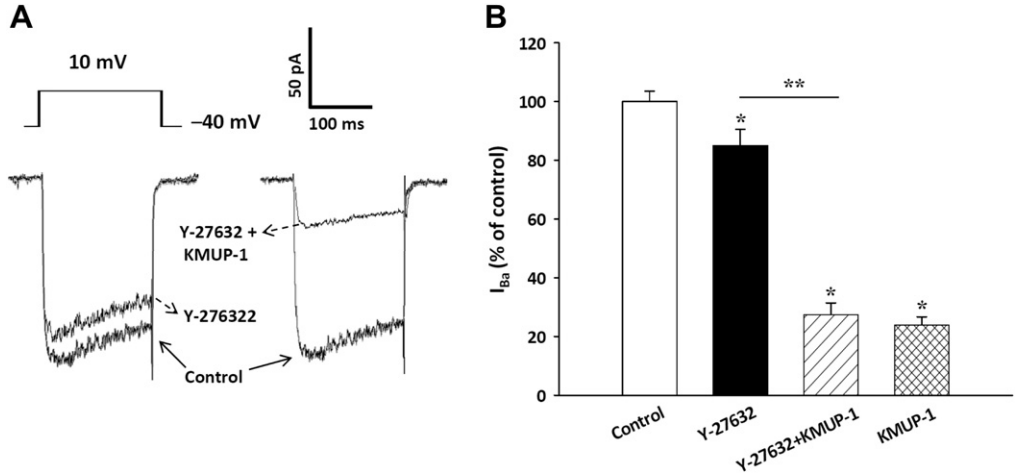

Figure 6. Effects of the Rho kinase inhibitor Y-27632 (30 μM) on KMUP-1 (10 μM)-inhibited inward I_{Ba} in RBASMCs. (A) Representative recordings of I_{Ba} evoked by a test pulse to 10 mV from a holding potential of −40 mV before and after perfusion with Y-27632 or Y-27632 + KMUP-1; (B) bar graph showing the relative I_{Ba} under control conditions and in the presence of Y-27632, KMUP-1, or Y-27632 + KMUP-1 ($n = 6$). * Denotes significant difference from control. ** Denotes significant difference between the two groups.

PKC-independent and other unidentified mechanisms, e.g., store-operated Ca^{2+} channels, might be also involved.

In conclusion, this study provides the direct evidence that a xanthines derivative KMUP-1 inhibits Ca^{2+}/Ba^{2+} entry via voltage-dependent LTCCs in RBASMCs, which could be attributed to its modulation of the PKC pathway. From one of our published reports [4], we also suggest that KMUP-1 could have an indirect action on Ca^{2+} entry due to its activation of BK_{Ca} channels producing hyperpolarization, thus decreasing the open probability of Ca^{2+} channels. In light of our results, we suggest that KMUP-1's Ca^{2+} entry inhibitory mechanisms could be primarily responsible for its vasorelaxant effect on RBASMCs during PKC inhibition. Thus, we suggest that the inhibitory action of KMUP-1 on voltage-dependent LTCCs may be of pharmacologic advantage to attenuate subarachnoid hemorrhage-induced cerebral vasospasm.

Acknowledgments

We thank Ms. Li-Mei An for her excellent technical assistance and Dr. Susan Olmstead-Wang at Johns Hopkins University for her editorial assistance with the manuscript. This study was supported by grants NSC-97-2320-B-037-006-MY3 from the National Science Council, Taiwan, and VGHKS96-033 and VGHKS97-044 from Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan.

References

[1] Wu BN, Lin RJ, Lin CY, Shen KP, Chiang LC, Chen IJ. A xanthine-based KMUP-1 with cyclic GMP enhancing and K^+ channels opening activities in rat aortic smooth muscle. Br J Pharmacol 2001;134:265—74.

[2] Lin RJ, Wu BN, Lo YC, Shen KP, Lin YT, Huang CH, et al. KMUP-1 relaxes rabbit corpus cavernosum smooth muscle in vitro and in vivo: involvement of cyclic GMP and K^+ channels. Br J Pharmacol 2002;135:1159—66.

[3] Wu BN, Lin RJ, Lo YC, Shen KP, Wang CC, Lin YT, et al. KMUP-1, a xanthine derivative, induces relaxation of guinea-pig isolated trachea: the role of the epithelium, cyclic nucleotides and K^+ channels. Br J Pharmacol 2004;142:1105—14.

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

[5] Slish DF, Welsh DG, Brayden JE. Diacylglycerol and protein kinase C activate cation channels involved in myogenic tone. Am J Physiol Heart Circ Physiol 2002;283:H2196—201.

[6] Kimura M, Obara K, Sasase T, Ishikawa T, Tanabe Y, Nakayama K. Specific inhibition of stretch-induced increase in L-type calcium channel currents by herbimycin A in canine basilar arterial myocytes. Br J Pharmacol 2000;130:923—31.

[7] Wamhoff BR, Bowles DK, McDonald OG, Sinha S, Somlyo AP, Somlyo AV, et al. L-type voltage-gated Ca^{2+} channels modulate expression of smooth muscle differentiation marker genes via a rho kinase/myocardin/SRF-dependent mechanism. Circ Res 2004;95:406—14.

[8] Wilson DP, Susnjar M, Kiss E, Sutherland C, Walsh MP. Thromboxane A2-induced contraction of rat caudal arterial smooth muscle involves activation of Ca^{2+} entry and Ca^{2+} sensitization: Rho-associated kinase-mediated phosphorylation of MYPT1 at Thr-855, but not Thr-697. Biochem J 2005; 389:763—74.

[9] Navedo MF, Amberg GC, Votaw VS, Santana LF. Constitutively active L-type Ca^{2+} channels. Proc Natl Acad Sci USA 2005;102: 11112—7.

[10] Wu BN, Luykenaar KD, Brayden JE, Giles WR, Corteling RL, Wiehler WB, et al. Hyposmotic challenge inhibits inward rectifying K^+ channels in cerebral arterial smooth muscle cells. Am J Physiol Heart Circ Physiol 2007;292:H1085—94.

[11] McFadzean I, Gibson A. The developing relationship between receptor-operated and store-operated calcium channels in smooth muscle. Br J Pharmacol 2002;135:1—13.

[12] Jackson WF. Ion channels and vascular tone. Hypertension 2000;35:173—8.

[13] Wu BN, Chen ML, Dai ZK, Lin YL, Yeh JL, Wu JR, et al. Inhibition of voltage-gated L-type calcium channels by labedipinedilol-A involves protein kinase C in rat cerebrovascular smooth muscle cells. Vascul Pharmacol 2009;51:65—71.