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KMUP-1 activates BK_{Ca} channels in basilar artery myocytes *via* cyclic nucleotide-dependent protein kinases

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1 This study investigated whether KMUP-1, a synthetic xanthine-based derivative, augments the delayed-rectifier potassium (K_{DR})- or large-conductance Ca^{2+}-activated potassium (BK_{Ca}) channel activity in rat basilar arteries through protein kinase-dependent and -independent mechanisms.

2 Cerebral smooth muscle cells were enzymatically dissociated from rat basilar arteries. Conventional whole cell, perforated and inside-out patch-clamp electrophysiology was used to monitor K^+- and Ca^{2+} channel activities.

3 KMUP-1 (1 μM) had no effect on the K_{DR} current but dramatically enhanced BK_{Ca} channel activity. This increased BK_{Ca} current activity was abolished by charybdotoxin (100 nM) and iberiotoxin (100 nM). Like KMUP-1, the membrane-permeable analogs of cGMP (8-Br-cGMP) and cAMP (8-Br-cAMP) enhanced the BK_{Ca} current.

4 BK_{Ca} current activation by KMUP-1 was markedly inhibited by a soluble guanylate cyclase inhibitor (ODQ 10 μM), an adenylate cyclase inhibitor (SQ 22536 10 μM), competitive antagonists of cGMP and cAMP (Rp-cGMP, 100 μM and Rp-cAMP, 100 μM), and cGMP- and cAMP-dependent protein kinase inhibitors (KT5823, 300 nM and KT5720, 300 nM).

5 Voltage-dependent L-type Ca^{2+} current was significantly suppressed by KMUP-1 (1 μM), and nearly abolished by a calcium channel blocker (nifedipine, 1 μM).

6 In conclusion, KMUP-1 stimulates BK_{Ca} currents by enhancing the activity of cGMP-dependent protein kinase, and in part this is due to increasing cAMP-dependent protein kinase. Physiologically, this activation would result in the closure of voltage-dependent calcium channels and the relaxation of cerebral arteries.
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Abbreviations: AC, adenylate cyclase; 8-Br-cAMP, 8-bromo-adenosine 3′,5′-cyclic monophosphate; 8-Br-cGMP, 8-bromo-guanosine 3′,5′-cyclic monophosphate; BK_{Ca} channels, large-conductance Ca^{2+}-activated potassium channels; ChTX, charybdotoxin; IbTX, iberiotoxin; K_{DR} channels, delay-rectifying potassium channels; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PKA, protein kinase A; PKG, protein kinase G; sGC, soluble guanylate cyclase; SQ 22536, 9-(terahydro-2-furanyl)-9*H*-purin-6-amine

Introduction

Relaxation of blood vessels can be mediated by several mechanisms. One major mechanism of vasodilatation is activation of guanylate cyclase and increased production of cGMP (Rembold, 1992). A second major mechanism that mediates vasodilatation is activation of adenylate cyclase (AC) and production of cAMP. Previous evidence (Faraci & Heistad, 1998) suggests that several types of K^+ channels are present in cerebral blood vessels, and that activation of these channels may constitute a key mechanism of relaxation in cerebral blood vessels. Activation of K^+ channels in arterial smooth muscle hyperpolarizes the cell membrane, and subsequently closes voltage-dependent calcium channels, resulting in a decrease in intracellular calcium, and vascular relaxation (Kitazono *et al*., 1995). The membrane potential of cerebral arterial muscle measured *in vitro* has ranged widely from approximately −40 to −70 mV, and changes in this potential of only a few millivolts are associated with significant changes in vascular tone (Nelson & Quayle, 1995).

Large-conductance calcium-activated potassium (BK_{Ca}) channels were first described in skeletal muscle (Latorre *et al*., 1982), chromaffin cells (Marty, 1981), and vascular smooth muscle, including cerebral vessels (Brayden & Nelson, 1992). Pharmacological agents commonly used to inhibit BK_{Ca} channels include tetraethylammonium (TEA; $\leqslant 1\,mmol\,l^{-1}$), charybdotoxin (ChTX), and iberiotoxin (IbTX) (Giangiacomo *et al*., 1995). ChTX blocks BK_{Ca} channels in arterial smooth muscle, although it may inhibit some other types of K^+ channels in other tissues (Galvez *et al*., 1990). IbTX is a highly selective blocker of BK_{Ca} channels.

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BK_{Ca} channels are activated by increases in intracellular Ca^{2+} and membrane depolarization (Nelson & Quayle, 1995). High levels of Ca^{2+}, of the order of 3–10 μmol l^{-1}, are required for K^+ channel activity in the physiological range of membrane potentials (−60 to −30 mV) in relaxed cells (Jackson & Blair, 1998). On the other hand, the functional role of BK_{Ca} channels is enhanced in arterial smooth muscle during chronic hypertension. This phenomenon occurs similarly throughout the vasculature, including the aorta (Rusch *et al*., 1992) and carotid artery, and the mesenteric, femoral (Asano *et al*., 1993), and cerebral vascular beds (Paterno *et al*., 1997). Therefore, increased BK_{Ca} channel function in arterial smooth muscle cells may provide a protective mechanism against progressive increases in blood pressure. This negative-feedback mechanism would modulate increased pressure and vascular tone, and subsequently limit pressure-induced vasoconstriction and preserve local blood flow.

In arterial smooth muscle patch-clamp experiments, cGMP and cAMP activate protein kinase G (PKG) and protein kinase A (PKA), respectively, and this leads to BK_{Ca} activation (Robertson *et al*., 1993; Schubert *et al*., 1996; White *et al*., 2000). BK_{Ca} channels play an important role in regulating the smooth muscle contractility and in controlling the diameter of small myogenic cerebral arteries (Brayden & Nelson, 1992; Nelson *et al*., 1995). Thus any agent (e.g., NO) that activates BK_{Ca} channels would not only tend to hyperpolarize and relax arteries but it would also alter how arteries respond to changes in pressure.

Delayed rectifier potassium (K_{DR}) channels have been described in nearly all excitable membranes including vascular muscle. These channels are activated by membrane depolarization with threshold potentials for substantial activation of ~−30 mV. When cells are depolarized, these potassium channels are activated, resulting in an outward current that returns the membrane potential toward the resting level (Nelson & Quayle, 1995). Thus, the K_{DR} channel appears to be a negative feedback system to regulate vascular tone. K_{DR} channels are inhibited by 4-aminopyridine, cesium, and high concentrations of TEA (Hirst & Edwards, 1989). Relatively little is known about the physiological importance of these potassium channels in the cerebral circulation.

KMUP-1, a chemically synthetic xanthine-based derivative, has been demonstrated to raise cyclic nucleotides, inhibit phosphodiesterases (PDEs), and activate K^+ channels resulting in relaxations in aortic (Wu *et al*., 2001), corporeal carvenosa (Lin *et al*., 2002), and tracheal smooth muscles (Wu *et al*., 2004). Recently, we proposed that tracheal relaxations of KMUP-1 could be mediated *via* two major pathways, either through (1) activation of K^+ channels that are independent of cellular cyclic nucleotides; or (2) increases in both cAMP and cGMP, followed by stimulation of PKA and PKG cascades. Increased PKA and PKG appear to activate K^+ channels, thus resulting in the lowering of cellular Ca^{2+} levels (Wu *et al*., 2004). The main objective of this study was to investigate further the mechanism by which KMUP-1 could modulate BK_{Ca} channels in rat basilar artery myocytes. Both conventional and perforated patch-clamp techniques were used to determine whether KMUP-1 enhanced the BK_{Ca} channel activity through cGMP/cAMP-dependent and -independent signaling pathways.

Methods

Animal procedures and tissue preparations

All procedures and protocols were approved by the Animal Care and Use Committee at Kaohsiung Medical University. Briefly, female Sprague–Dawley rats (10–12 weeks of age) were killed 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). Basilar arteries were dissected free of the surrounding tissue and cut into 2 mm segments.

Preparation of isolated arterial smooth muscle cells

Smooth muscle cells from rat basilar arteries were enzymatically isolated as previously described (Welsh *et al*., 2000). 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 glucose, and 10 HEPES with 1 mg ml^{-1} albumin (pH 7.2) for 10 min. After this equilibration step, arterial segments were initially incubated (37°C) in 1 mg ml^{-1} papain and 3 mg ml^{-1} dithioerythritol for 20 min. This was followed by a second incubation (37°C) in isolation medium containing 100 μM Ca^{2+}, 0.7 mg ml^{-1} type F collagenase, and 0.4 mg ml^{-1} type H collagenase for 10 min. 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. Cells were stored in ice-cold isolation medium for use on the same day.

Patch-clamp electrophysiology

Conventional whole cell patch-clamp electrophysiology was used to measure the K_{DR} currents in basilar artery myocytes. In brief, basilar artery myoctyes were placed in a recording dish and perfused with a solution containing (in mM) 120 NaCl, 3 $NaHCO_3$, 4.2 KCl, 1.2 KH_2PO_4, 2 $MgCl_2$, 0.1 $CaCl_2$, 10 glucose, and 10 HEPES. A recording electrode was pulled from borosilicate glass (resistance 4–7 MΩ), the tip was covered with sticky wax and backfilled with pipette solution containing (in mM): 110 K-gluconate, 30 KCl, 0.5 $MgCl_2$, 5 HEPES, 5 EGTA, 5 Na_2ATP, and 1 GTP (pH 7.2, KOH) and was 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 (−60 mV). Membrane currents were recorded on an Axopatch 700A amplifier (Axon Instruments, Union City, CA, U.S.A.), 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. A 1 M NaCl–agar salt bridge between the bath and the Ag-AgCl reference electrode was used to minimize offset potentials. All electrical recordings were performed at room temperature and cell capacitance averaged 16.5 ± 0.6 pF.

Whole cell BK_{Ca} currents were measured using the conventional or perforated patch-clamp configuration. Under both recording conditions, the bathing solution contained (in mM): 140 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose (pH 7.4, NaOH). In comparison, the pipette solution contained (in mM) 110 K-gluconate, 30 KCl, 0.5 $MgCl_2$,

5 HEPES, 5 EGTA, 5 Na_2ATP, and 1 GTP (pH 7.2, KOH). Amphotericin B (200 $\mu g\,ml^{-1}$) was included in the pipette solution for the perforated patch-clamp recordings. With respect to employing the inside-out patch-clamp configuration to monitor single-channel BK_{Ca} activity, recording pipettes were backfilled with a solution containing (in mM) 140 NaCl, 6 KCl, 0.5 $MgCl_2$, and 10 HEPES. The bath solution contained (in mM): 140 KCl, 1 $MgCl_2$, 10 HEPES, 1.8 $CaCl_2$, 5 EGTA, and 10 glucose. Note, the free Ca^{2+} concentration of these solutions was first calculated using Max Chelator Sliders software (C. Patton, Standford University) and secondarily measured with a Ca^{2+}-selective electrode (Corning, Acton, MA, U.S.A.). Single-channel activity in excised patches was recorded at 0 mV, filtered at 2.5 kHz, and digitized at 10 kHz. To measure voltage-dependent Ca^{2+} currents, KCl inside the pipette solution was replaced with equimolar CsCl, and the pH was adjusted to 7.2 with CsOH, whereas the bathing solution contained 1 μM tetrodotoxin and 10 mM tetraethylammonium chloride.

Experimental procedures

Voltage-clamped cells were equilibrated for 15 min prior to experimentation. Following equilibration, whole cell K_{DR} and BK_{Ca} currents were monitored in the presence and absence of KMUP-1 (1 μM), ChTX (100 nM), or IbTX (100 nM). To ascertain whether PKG or PKA signaling was involved in the KMUP-1-induced increases in BK_{Ca}, cerebral smooth muscle cells were preincubated for 15 min with ODQ (10 μM), SQ 22536 (10 μM), KT5823 (300 nM), KT5720 (300 nM), Rp-cGMP (100 μM), or Rp-cAMP (100 μM) prior to the addition of KMUP-1. ODQ, SQ 22536, KT5823 and KT5720 were continuously superfused in the bath whereas Rp-cGMP and Rp-cAMP were added to the pipette solution. In general, the net current–voltage (*I-V*) relationship was determined at 5 min intervals by measuring the peak current at the end of a 300 ms pulse to voltages between -70 and $+40$ mV for K_{DR} currents, and 0 and $+40$ mV for BK_{Ca} currents. To evoke whole-cell Ca^{2+} currents, cells were clamped at -40 mV with step depolarizations (200 ms) from -40 to 10 mV and the currents were recorded in the presence and absence of KMUP-1 or nifedipine.

Chemicals

Buffer reagents, 4-aminopyridine (4-AP), 8-bromo-adenosine 3′,5′-cyclic monophosphate (8-br-cAMP), 8-bromo-guanosine 3′,5′-cyclic monophosphate (8-br-cGMP), ChTX, collagenase (type F and H), dithioerythritol, IbTX, isoproterenol, KT 5720, KT 5823, ODQ, papain, and SQ 22536 were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, U.S.A.). All drugs and reagents were dissolved in distilled water unless otherwise stated. ChTX and ODQ were dissolved in DMSO at 10 mM; KMUP-1 was dissolved in 10% absolute alcohol, 10% propylene glycol, and 2% 1 N HCl at 10 mM. Serial dilutions were made in phosphate buffer solution, with the final solvent concentration $\leqslant$0.01%.

Data analysis and statistics

For single-channel analysis, BK_{Ca} channel activity (NP_0) was determined from continuous gap-free data by using Clampfit 9.0. The NP_0 was calculated from the following equation:

$$NP_0 = (\Sigma t_i i)/T,$$

where i is the number of channels open, t_i is the open time for each level i and T is the total time of analysis.

For BK_{Ca} currents analysis, $I-I_{min}/I_{max}-I_{min}$ was used to normalize the currents. I_{max} and I_{min} indicate the values of control BK_{Ca} current at 40 and 0 mV, respectively, where I represents the values of BK_{Ca} current for test agent measured between 0 and 40 mV.

Data are expressed as means $\pm$ s.e. and n indicates the number of cells. Repeated measure ANOVAs compared values at a given voltage. When appropriate, a Tukey-Kramer pairwise comparison was used for *post hoc* analysis. *P*-values $\leqslant$0.05 were considered statistically significant.

Results

Lack of modulation of K_{DR} currents by KMUP-1

Using conventional whole-cell patch-clamp and pipette solutions that minimize BK_{Ca} channel activity, the K_{DR} current was isolated in rat basilar artery smooth muscle cells. In

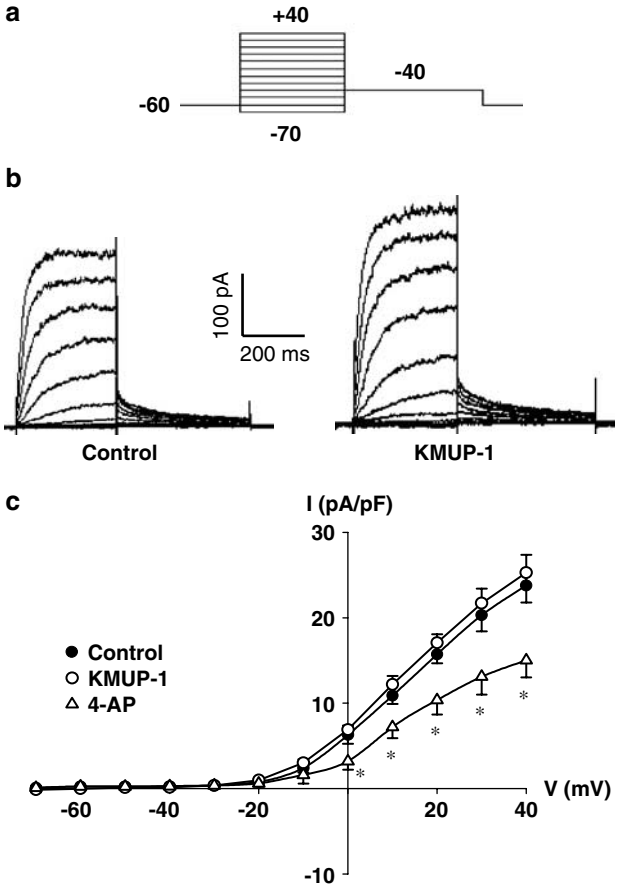

Figure 1 Effects of KMUP-1 on delayed rectifier K^+ (K_{DR}) current in myocytes isolated from rat basilar arteries. (a) Voltage protocol was designed to measure steady-state activation of the K_{DR} current. (b) Representative recordings of K_{DR} current before and after the addition of KMUP-1 (1 μM) or 4-AP (5 mM). (c) Average current–voltage (*I–V*) relationships under control conditions and in the presence of 1 μM KMUP-1 ($n=6$).

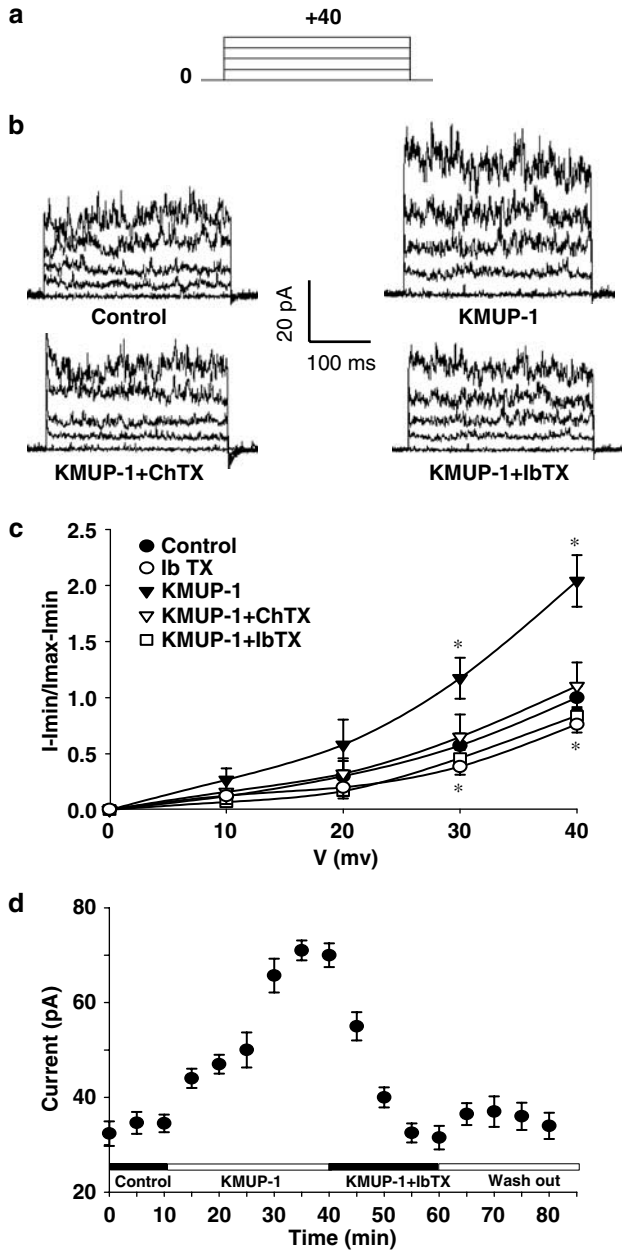

Figure 2 Effects of KMUP-1 (1 μM) on large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) currents. Cells were bathed in high Ca^{2+} solution containing 1.8 mM $CaCl_2$. (a) Voltage protocol. (b) Representative recordings of BK_{Ca} currents under control conditions and in the presence of KMUP-1, KMUP-1 + ChTX (100 nM) or KMUP-1 + IbTX (100 nM). (c) Average *I–V* relationships under control conditions and in the presence of KMUP-1, KMUP-1 + ChTX, or KMUP-1 + IbTX. (d) Time course of KMUP-1 on BK_{Ca} currents. The horizontal bars of the diagram indicate the periods of drug perfusion ($n = 7$). *Significant difference from control.

general, brief voltage steps positive to −30 mV activated K_{DR} (Figure 1b). In the basilar artery myocytes, the K_{DR} current was recognized by the addition of 4-AP (5 mM) as previously described (Luykenaar *et al*., 2004). Superfused KMUP-1 (1 μM) had no significant effect on the K_{DR} current. In contrast, perfusion with 4-AP did significantly inhibit this current (38.6 ± 4.1% at + 40 mV) (Figure 1c).

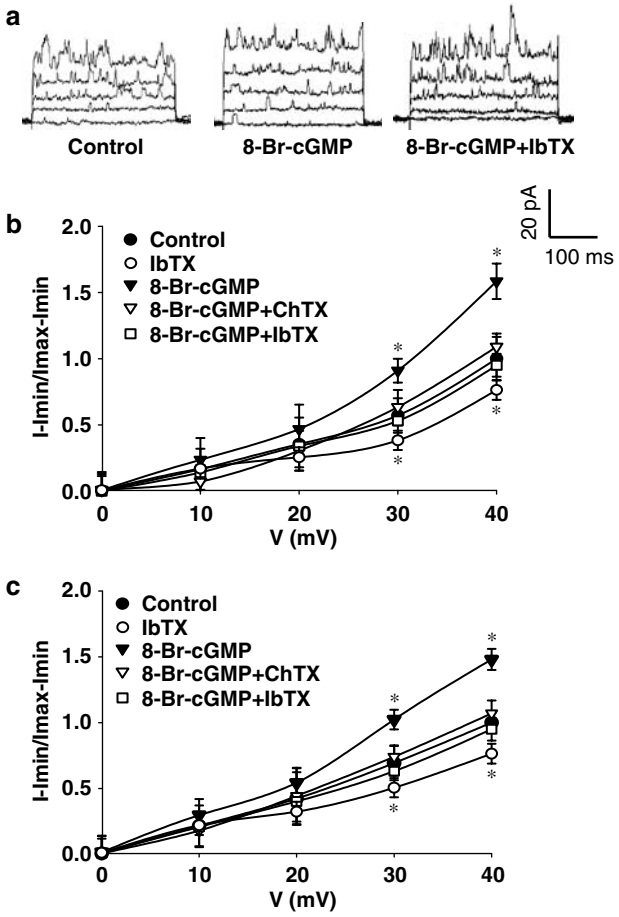

Figure 3 Enhanced BK_{Ca} currents elicited by extracellular application of membrane-permeable cGMP and cAMP analogs 8-Br-cGMP (1 μM) and 8-Br-cAMP (1 μM). (a) Representative recordings of BK_{Ca} currents under control conditions and in the presence of 8-Br-cGMP or 8-Br-cGMP + ChTX. (b) Average *I–V* relationships under control conditions and in the presence of 8-Br-cGMP, 8-Br-cGMP + ChTX (100 nM) or 8-Br-cGMP + IbTX (100 nM) ($n = 6$). (c) Average *I–V* relationships under control conditions and in the presence of 8-Br-cAMP, 8-Br-cAMP + ChTX or 8-Br-cAMP + IbTX ($n = 6$). *Significant difference from control.

Activation of BK_{Ca} currents by KMUP-1

Conventional and perforated patch-clamp was used to assess the effect of KMUP-1 on the regulation of outward BK_{Ca} conductance. Openings of BK_{Ca} channels were identified based on the characteristic single-channel conductance and blocked by ChTX or IbTX as previously described (Jaggar *et al*., 2002; Xi *et al*., 2004). The BK_{Ca} channels were also recognized by their conductance over the voltage range of −40 to + 40 mV (249.5 ± 3.2 pS, $n = 6$) in excised inside-out patches bathed in symmetrical 140 mM KCl (unpublished data). In brief, rat basilar artery myocytes were voltage clamped at 0 mV to inactivate voltage-dependent K^+ currents (Wu *et al*., 1999), and continuously superfused with an isotonic physiological solution containing 1.8 mM Ca^{2+} ± 1 μM KMUP-1 (Figure 2b). When KMUP-1 was < 1 μM in the perfusate, there was little or no effect on BK_{Ca} channels. As noted in Figure 2c, the KMUP-1-induced increase in BK_{Ca} current (+ 30 mV: 1.1 ± 0.1 to 2.4 ± 0.3 pA pF^{-1}; + 40 mV: 2.0 ± 0.2 to 4.1 ± 0.7 pA pF^{-1}, $n = 7$, $P < 0.05$) was inhibited by ChTX

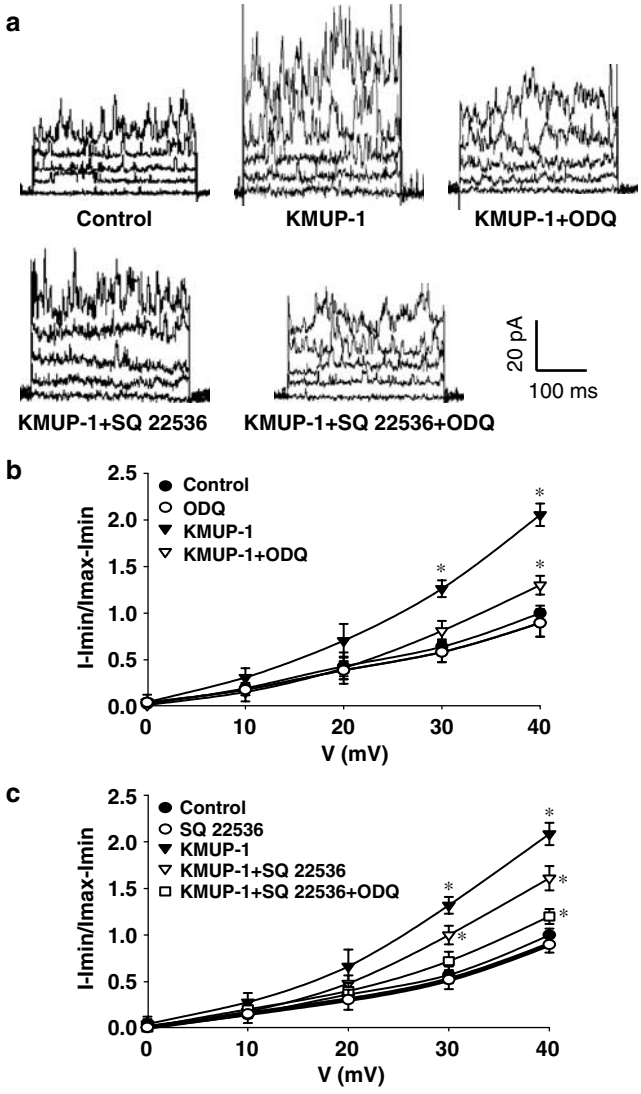

Figure 4 BK_{Ca} current activation by KMUP-1 (1 μM) is dependent on soluble guanylate cyclase and adenylate cyclase. (a) Representative recordings of BK_{Ca} currents under control conditions and in the presence of KMUP-1, KMUP-1 + ODQ (10 μM), KMUP-1 + SQ 22536 (10 μM) or KMUP-1 + SQ 22536 + ODQ. (b) Average *I–V* relationships under control conditions and in the presence of KMUP-1 or KMUP-1 + ODQ ($n=6$). (c) Average *I–V* relationships under control conditions and in the presence of KMUP-1, KMUP-1 + SQ 22536, or KMUP-1 + SQ 22536 + ODQ ($n=7$). *Significant difference from control.

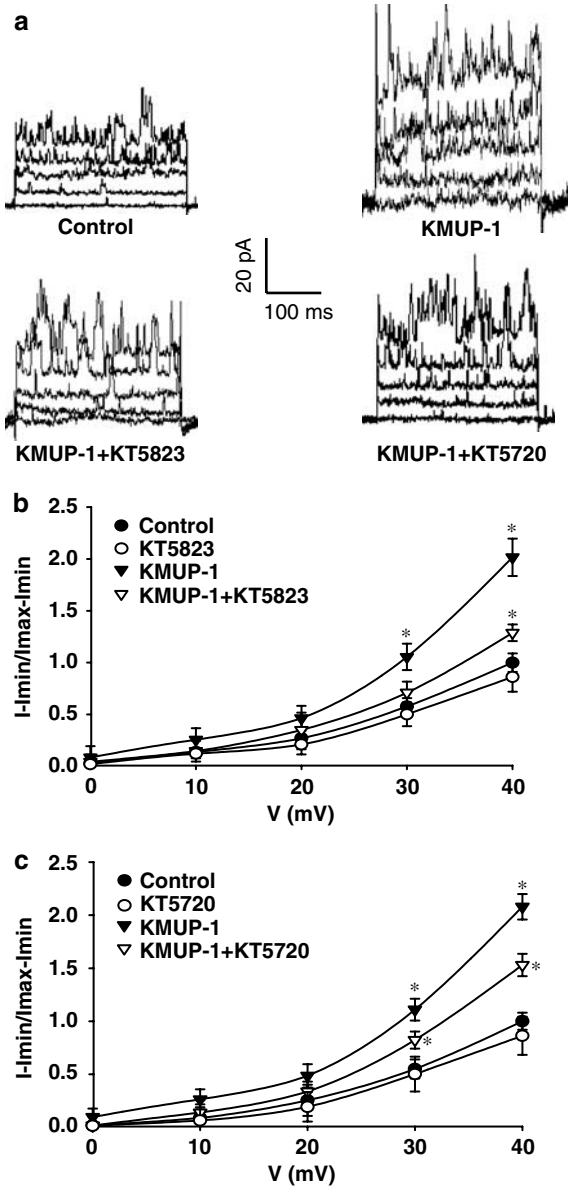

Figure 5 BK_{Ca} current activation by KMUP-1 (1 μM) is dependent on PKG and PKA. (a) Representative recordings of BK_{Ca} currents under control conditions and in the presence of KMUP-1, KMUP-1 + KT5823 (300 nM) or KMUP-1 + KT5720 (300 nM). (b) Average *I–V* relationships under control conditions and in the presence of KMUP-1 or KMUP-1 + KT5823 ($n=6$). (c) Average *I–V* relationships under control conditions and in the presence of KMUP-1 or KMUP-1 + KT5720 ($n=6$). *Significant difference from control.

(100 nM) or IbTX (100 nM; +30 mV: 1.1 ± 0.1 to 0.8 ± 0.1 pA pF^{-1}; +40 mV: 2.0 ± 0.2 to 1.5 ± 0.1 pA pF^{-1}, $n=7$, $P<0.05$). The increases of BK_{Ca} current were consistently observed 5–10 min after the addition of KMUP-1, with the peak steady-state level occurring by 30 min (Figure 2d). Similarly, the membrane-permeable analogs of cGMP (8-Br-cGMP, 1 μM; +30 mV: 1.1 ± 0.1 to 1.8 ± 0.2 pA pF^{-1}; +40 mV: 2.1 ± 0.2 to 3.2 ± 0.5 pA pF^{-1}, $n=6$, $P<0.05$) and cAMP (8-Br-cAMP, 1 μM; +30 mV: 1.4 ± 0.2 to 2.0 ± 0.2 pA pF^{-1}; +40 mV: 2.0 ± 0.2 to 3.0 ± 0.3 pA pF^{-1}, $n=6$, $P<0.05$) increased BK_{Ca} activity, a response that was completely abolished in the presence of ChTX (100 nM) and IbTX (100 nM; +30 mV: 1.2 ± 0.1 to 0.8 ± 0.1 pA pF^{-1}; +40 mV: 2.1 ± 0.2 to 1.5 ± 0.2 pA pF^{-1}, $n=6$, $P<0.05$; Figure 3).

KMUP-1 activates BK_{Ca} currents via *sGC/cGMP- and AC/cAMP-dependent mechanisms*

To investigate further the signaling mechanisms that lead to BK_{Ca} channel activation, KMUP-1 was applied to voltage-clamped cells in the presence of ODQ, a soluble guanylate cyclase (sGC) inhibitor (+40 mV: 2.6 ± 0.2 to 2.1 ± 0.1 pA pF^{-1}, $n=6$, $P<0.05$). ODQ (10 μM) markedly blocked the KMUP-1-induced increases in BK_{Ca} activity, indicating that the modulatory effect of this compound involved the sGC/cGMP pathway (Figure 4). The KMUP-1-induced increase in BK_{Ca} activity was also partially attenuated (40 ± 5.3% at +40 mV) by the AC inhibitor SQ 22536 (10 μM;

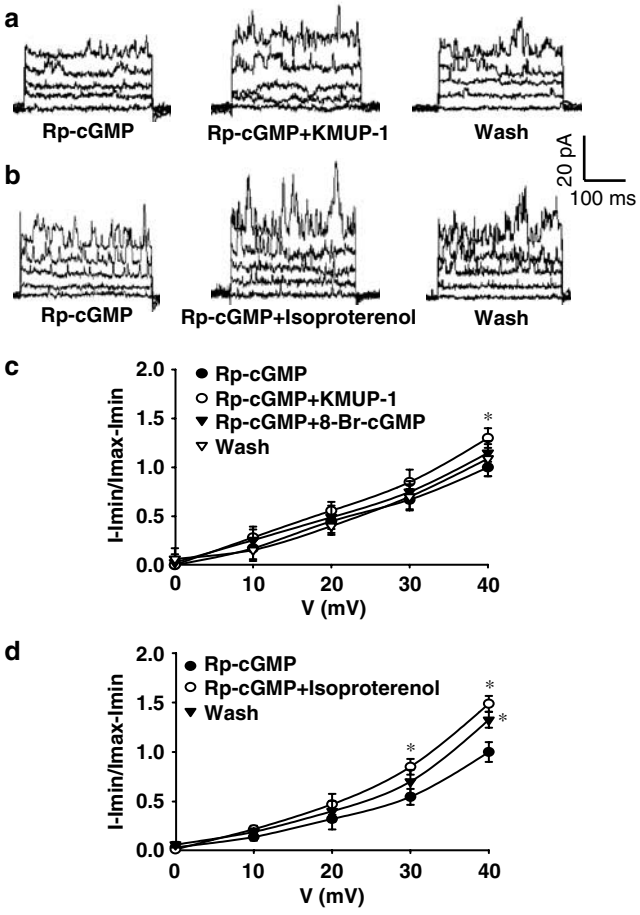

Figure 6 BK_{Ca} current activation by KMUP-1 (1 μM) is dependent on cGMP/PKG activity. (a) Representative recordings demonstrating the effect of KMUP-1 (1 μM) on BK_{Ca} currents with Rp-cGMP (100 μM) in the pipette solution. (b) Representative recordings demonstrating the effect of isoproterenol (1 μM) on BK_{Ca} currents with Rp-cGMP (100 μM) in the pipette solution. (c) Average *I–V* relationships in the presence of Rp-cGMP, Rp-cGMP + KMUP-1 or Rp-cGMP + 8-Br-cGMP, and wash out ($n=6$). (d) Average *I–V* relationships in the presence of Rp-cGMP or Rp-cGMP + isoproterenol, and wash out ($n=6$). *Significant difference from control.

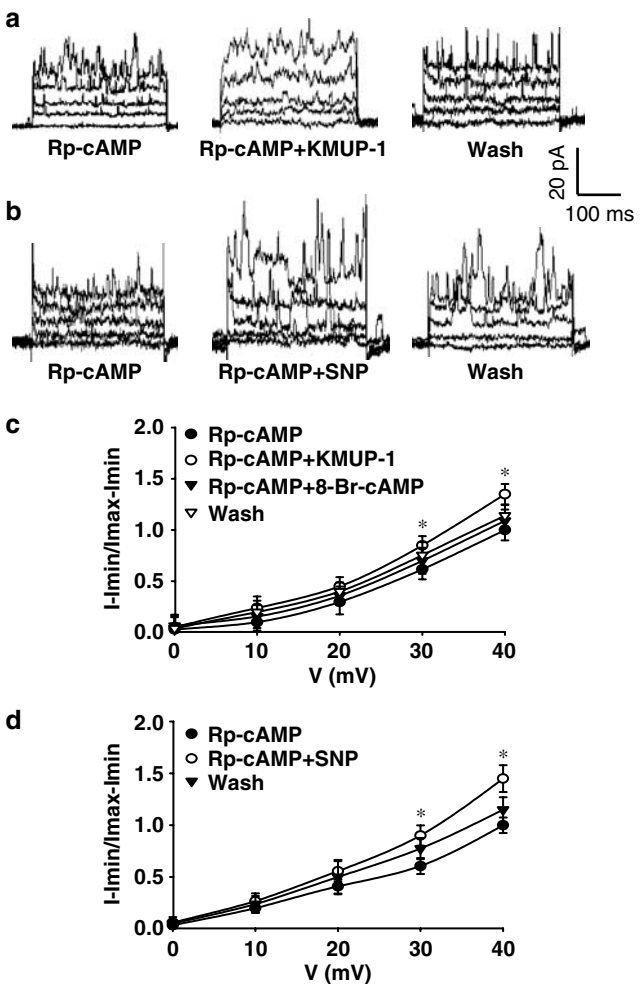

Figure 7 BK_{Ca} current activation by KMUP-1 (1 μM) is dependent on cAMP/PKA activity. (a) Representative recordings demonstrating the effect of KMUP-1 (1 μM) on BK_{Ca} currents with Rp-cAMP (100 μM) in the pipette solution. (b) Representative recordings demonstrating the effect of SNP (1 μM) on BK_{Ca} currents with Rp-cAMP (100 μM) in the pipette solution. (c) Average *I–V* relationships in the presence of Rp-cAMP, Rp-cAMP + KMUP-1 or Rp-cAMP + 8-Br-cAMP, and wash out ($n=6$). (d) Average *I–V* relationships in the presence of Rp-cAMP or Rp-cAMP + SNP, and wash out ($n=6$). *Significant difference from control.

+30 mV: 2.0±0.2 to 1.1±0.2 pA pF^{-1}; +40 mV: 3.2±0.3 to 2.0±0.1 pA pF^{-1}, $n=7$, $P<0.05$), while the cells superfused with ODQ (10 μM) and SQ 22536 (10 μM) inhibited but did not abolish the channel activity (+40 mV: 2.4±0.2 to 2.0±0.1 pA pF^{-1}, $n=7$, $P<0.05$) (Figure 4). This result suggests that KMUP-1 appears to have a direct action on BK_{Ca} channels in addition to activation of the sGC/cGMP and AC/cAMP pathways.

KMUP-1 activates BK_{Ca} currents via *PKG- and PKA-dependent pathways*

The KMUP-1-induced increase in BK_{Ca} channel activity was inhibited in the presence of cGMP- and cAMP-dependent protein kinase inhibitors KT5823 (300 nM; +40 mV: 2.6±0.2 to 2.0±0.2 pA pF^{-1}, $n=6$, $P<0.05$) and KT5720 (300 nM; +30 mV: 1.7±0.2 to 1.1±0.2 pA pF^{-1}; +40 mV: 3.1±0.2 to 1.9±0.2 pA pF^{-1}, $n=6$, $P<0.05$), respectively (Figure 5). The competitive antagonist of cGMP, Rp-cGMP (100 μM), prevented the stimulatory effect of KMUP-1 on BK_{Ca} when dialyzed into the cell *via* the patch pipette, but not at 40 mV (Figure 6a and c; from 2.6±0.2 to 2.0±0.2 pA pF^{-1}, $n=6$, $P<0.05$). As in the KMUP-1 experiments, Rp-cGMP (100 μM) fully prevented 8-Br-cGMP (1 μM) from enhancing BK_{Ca} activity (Figure 6c). Interestingly, the inclusion of Rp-cGMP in the pipette solution did not prevent the β-adrenoceptor agonist isoproterenol (1 μM) from stimulating the BK_{Ca} current (+30 mV: 1.7±0.2 to 1.1±0.2 pA pF^{-1}; +40 mV: 3.0±0.2 to 1.9±0.2 pA pF^{-1}, $n=6$, $P<0.05$). The stimulatory effects of isoproterenol were not fully reversible during washout (+40 mV: 2.7±0.1 to 1.9±0.2 pA pF^{-1}, $n=6$, $P<0.05$), indicating that this agonist might have a high binding affinity to its own receptors in basilar artery myocytes (Figure 6b and d).

Further experiments revealed that Rp-cAMP (100 μM in the pipette), a competitive antagonist of cAMP, attenuated the effect of KMUP-1-induced increases in BK_{Ca} activity, but did not prevent the effect of KMUP-1 at $\geqslant$30 mV (Figure 7a and

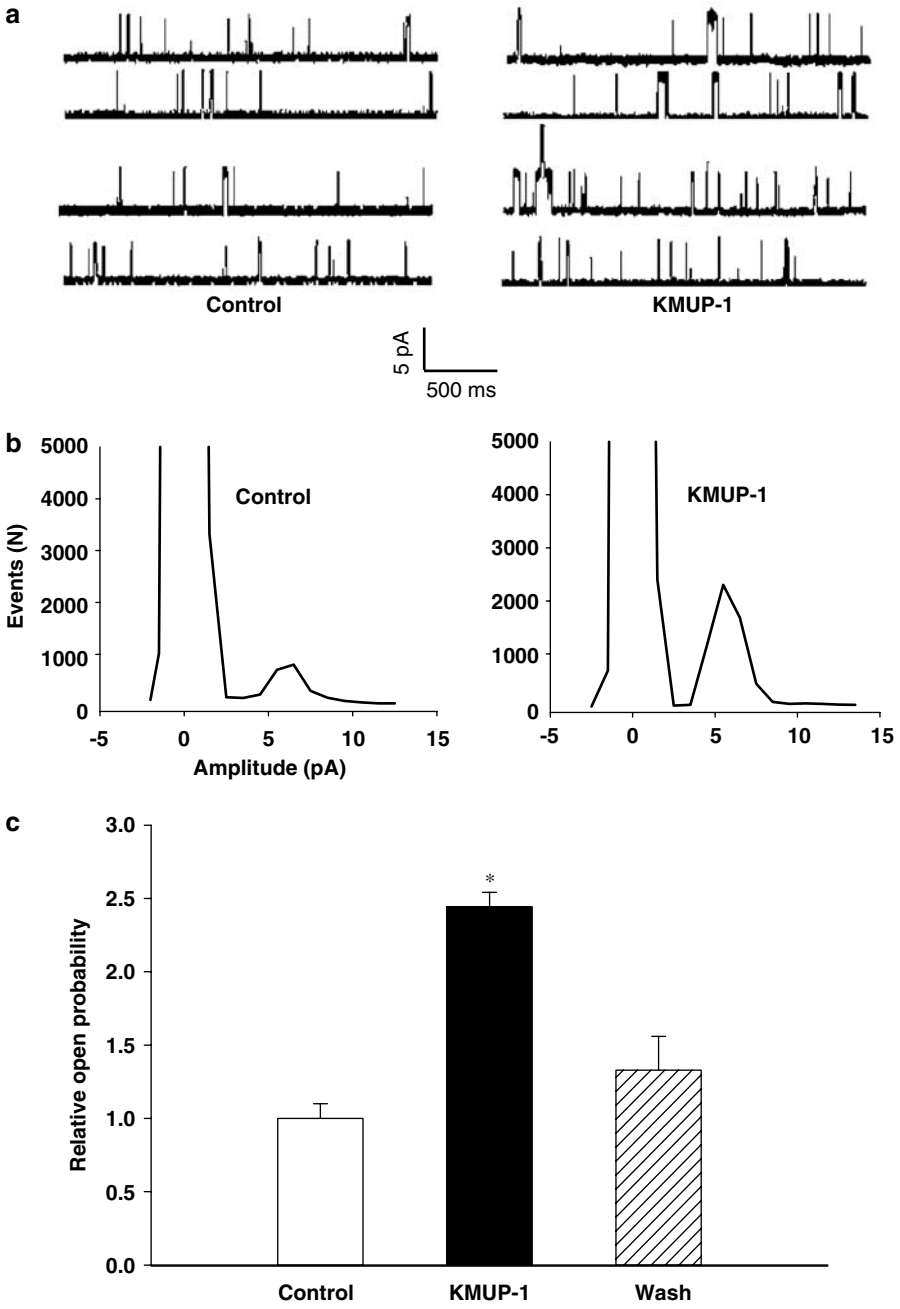

Figure 8 KMUP-1 activates BK_{Ca} channels in excised inside-out membrane patches. (a) Original current recording illustrating BK_{Ca} channels activation by KMUP-1 (1 μM), voltage-clamped at 0 mV. (b) Current amplitude histograms constructed from the traces shown in a. (c) Bar graph showing the control and in the presence of KMUP-1 on the relative open probability of BK_{Ca} channels ($n=7$). *Significant difference from control.

c; +30 mV: 1.7 ± 0.2 to 1.2 ± 0.2 pA pF^{-1}; +40 mV: 2.7 ± 0.2 to 2.0 ± 0.2 pA pF^{-1}, $n=6$, $P<0.05$). The addition of Rp-cAMP to the pipette solution prevented 8-Br-cAMP (1 μM) from activating the BK_{Ca} current (Figure 7c). In contrast, in the presence of Rp-cAMP, the cGMP-dependent NO donor, SNP (100 μM), stimulated BK_{Ca} (Figure 7b and d; +30 mV: 1.8 ± 0.2 to 1.2 ± 0.1 pA pF^{-1}; +40 mV: 2.9 ± 0.3 to 2.0 ± 0.1 pA pF^{-1}, $n=6$, $P<0.05$). From these results, this study cannot exclude the involvement of a PKA-dependent signaling pathway in the activation of BK_{Ca} channels by KMUP-1.

KMUP-1 activates BK_{Ca} channels in excised membrane patches

Since BK_{Ca} channel activation by KMUP-1 was caused by stimulation of the sGC/PKG and AC/PKA pathways, we sought to investigate whether KMUP-1 could activate BK_{Ca} channels in the complete absence of intracellular signaling factors. KMUP-1 regulation of BK_{Ca} channel activity was measured in excised inside-out membrane patches with 300 nM free Ca^{2+} present in the bathing solution. At 0 mV, KMUP-1 (1 μM) increased the mean BK_{Ca} channel open probability

~2.5-fold (NP_0 from 0.024 ± 0.007 to 0.061 ± 0.015, $n=7$; Figure 8). These data suggest that KMUP-1 could directly activate BK_{Ca} channels located on the cerebral artery myocyte membrane, and is not required for stimulating the enzyme systems contained in cytosol.

Inhibition of L-type Ca^{2+} channels by KMUP-1

The experiment was conducted with a Cs^+-containing solution. Perfusion with KMUP-1 (1 μM) was found to suppress significantly the voltage-dependent L-type Ca^{2+} currents ($I_{Ca,L}$), and the perfusate with a calcium channel blocker nifedipine (1 μM) nearly abolished the currents (Figure 9). KMUP-1 reduced the amplitude of $I_{Ca,L}$ to 60 ± 7 pA from a control value of 100 ± 9 pA ($P<0.05$, $n=7$) when cells were depolarized from −40 to 10 mV, but it did not modify the I–V relationship of $I_{Ca,L}$ in these cells (unpublished data).

Discussion

KMUP-1 has been shown to relax aortic, corporeal carvenosa, and tracheal smooth muscles, and elevate cAMP and cGMP levels through inhibition of PDEs. We suggested that the smooth muscle relaxant effects of KMUP-1 are mediated *via* cyclic nucleotide elevation and K^+ channel activation (Wu *et al*., 2001; 2004; Lin *et al*., 2002). In this study, we first investigated the ability of KMUP-1 to activate BK_{Ca} channels through cyclic nucleotide-dependent and -independent pathways using patch-clamp electrophysiology.

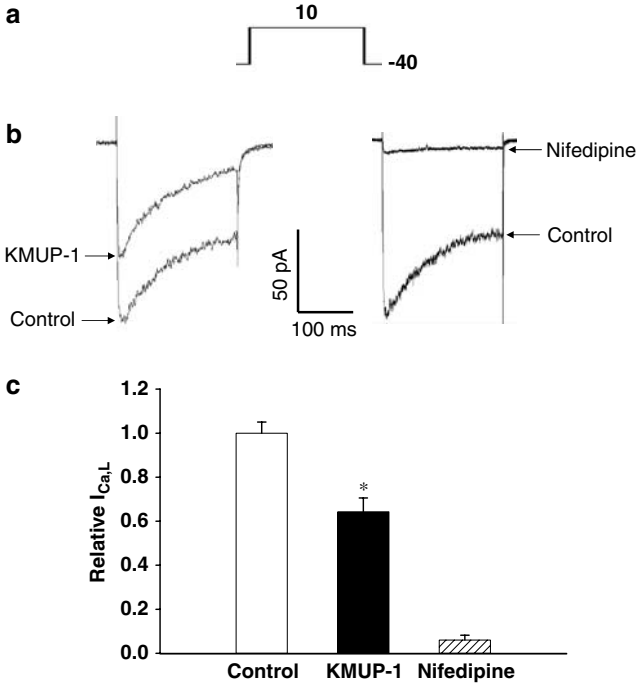

Figure 9 Effects of KMUP-1 on voltage-dependent L-type Ca^{2+} currents ($I_{Ca,L}$) in rat basilar artery myocytes. (a) Voltage protocol. (b) Representative recordings of $I_{Ca,L}$ before and after the addition of KMUP-1 or nifedipine (1 μM). (c) Bar graph showing the control and in the presence of KMUP-1 or nifedipine ($n=6$). *Significant difference from control.

Potassium channels are the main determinant of resting membrane potential, and their activation causes hyperpolarization, the inhibition of voltage-gated calcium channels, and vascular relaxation. Although multiple classes of K^+ channels are expressed in a variety of vascular beds, the BK_{Ca} channel is a particularly important target for physiological regulation (Faraci & Heistad, 1998). The BK_{Ca} channel is a large conducting channel with conductance values measured at physiological levels of K^+, ranging from 100 to 250 pS (Faraci & Heistad, 1998; Barman *et al*., 2003). Similar values were also found in this study in the rat basilar artery. The BK_{Ca} channel is voltage- and calcium-dependent, and does not display voltage-dependent inactivation (Latorre *et al*., 1989; Holland *et al*., 1996). To study the BK_{Ca} in basilar artery myocytes, we first inactivated K_{DR} channels through a step depolarization to 0 mV. Under these conditions, BK_{Ca} is the dominant outward current and KMUP-1 significantly increases the magnitude of this ChTX/IbTX-sensitive conductance (Figure 2). In contrast, KMUP-1 only slightly affected the voltage-sensitive K_{DR} channels.

To explore whether KMUP-1 could directly affect the BK_{Ca} channel or a closely associated site, inside-out patch-clamp electrophysiology was used in a number of experiments. In general, we observed that KMUP-1 applied to the cytoplasmic face of inside-out patches could directly activate these channels. These findings are consistent with our previous reports that KMUP-1 rapidly relaxes smooth muscle (Wu *et al*., 2001; 2004; Lin *et al*., 2002) presumably through a mechanism that involves the modulation of a K^+ channel.

Under whole-cell recording conditions, there was a substantial delay (~5 min) between the addition of KMUP-1 and the activation of BK_{Ca} currents. This delay contrasts sharply with the very rapid activation of BK_{Ca} channels in excised patches. A possible explanation for the discrepancy is that the primary site of action of KMUP-1 is intracellular. Thus, the delayed activation of BK_{Ca} channels by KMUP-1 in whole-cell preparations might be rationally explained by the additional time required to penetrate the cell membrane and to activate the second messenger cascades leading to the stimulation of K^+ effluxes.

Alterations in BK_{Ca} channel activity play a central role in mediating vasoconstriction and vasodilatation. For example, studies have noted the involvement of BK_{Ca} channels in mediating vascular relaxation to agents that elevate cGMP (Zhao *et al*., 1997). In addition, the relaxation induced by 8-Br-cGMP has been demonstrated to be attenuated by IbTX (Tanaka *et al*., 1998), a finding consistent with BK_{Ca} channels being a target of cGMP signaling. This study confirmed that 8-Br-cGMP does indeed increase the magnitude of the BK_{Ca} current in basilar artery myocytes. Agents that elevate cAMP have also been shown to modulate this current and initiate vascular relaxation (Paterno *et al*., 1996). In basilar artery myocytes, superfused 8-Br-cAMP did enhance the BK_{Ca} current; this result is consistent with the work of White *et al*. (2000) in coronary artery smooth muscle cells. Taken together, PKA and PKG pathways were shown to participate in the regulation of BK_{Ca} activity in the rat basilar artery because 8-Br-cAMP and 8-Br-cGMP caused a marked augmentation in BK_{Ca} currents, which was reversed by the competitive antagonists of cAMP (Rp-cAMP) and cGMP (Rp-cGMP). These findings were consistent with previous studies in rabbit cerebral artery (Robertson *et al*., 1993) and rat tail artery (Schubert *et al*., 1996). Torphy (1994) also documented that increases in cAMP

and cGMP simultaneously activated the PKA and PKG pathways resulting in the opening of BK_{Ca} channels.

Recently, we have demonstrated that KMUP-1, like the representative sGC activator YC-1, possesses multiple pharmacological activities including sGC activation, inhibition of PDEs, elevation of cyclic nucleotide levels, enhancement of the expression of PKA and PKG, and increase in associated K^+ channels opening (Wu *et al*., 2001; 2004). In this study, KMUP-1-induced increases in BK_{Ca} currents are extremely inhibited, but not abolished, by ODQ together with SQ 22536. This finding indicates that activation of the BK_{Ca} channel by KMUP-1 is predominantly dependent on both cGMP- and cAMP-mediated signaling mechanisms. However, at least part of the response to KMUP-1 is cGMP- and cAMP-independent mechanisms, and may be due to the direct BK_{Ca} channel opening. Additionally, enhanced BK_{Ca} currents by KMUP-1 are reduced by competitive antagonists of cGMP (Rp-cGMP) and cAMP (Rp-cAMP), and by inhibitors of PKG (KT5823) and PKA (KT5720). Compared with KT5823, KT5720 attenuated the KMUP-1-induced increases in BK_{Ca} currents to a lesser extent. These results suggest that BK_{Ca} currents activation by KMUP-1 is principally *via* PKG and partially *via* PKA.

In summary, we provide the first direct evidence that KMUP-1 activates BK_{Ca} currents in rat basilar artery. We suggest 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. Functionally, both these effects could contribute to its vasorelaxant property, and the hypothesis is confirmed in rat aorta (Wu *et al*., 2001). Finally, the stimulatory effect of KMUP-1 on BK_{Ca} channels is mediated by PKA and PKG, which phosphorylate the channels or associated regulatory proteins, enhancing the K^+ effluxes, and could lead to membrane hyperpolarization and closure of voltage-dependent L-type Ca^{2+} channels.

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