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RESEARCH PAPER

Activation of BK_{Ca} channels via cyclic AMP- and cyclic GMP-dependent protein kinases by eugenosedin-A in rat basilar artery myocytes

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Background and purpose: The study investigated whether eugenosedin-A, a 5-hydroxytryptamine and α/β adrenoceptor antagonist, enhanced delayed-rectifier potassium (K_{DR})- or large-conductance Ca^{2+}-activated potassium (BK_{Ca})-channel activity in basilar artery myocytes through cyclic AMP/GMP-dependent and -independent protein kinases.
Experimental approach: Cerebral smooth muscle cells (SMCs) 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.
Key results: Eugenosedin-A (1 μM) did not affect the K_{DR} current but dramatically augmented BK_{Ca} channel activity in a concentration-dependent manner. Increased BK_{Ca} current was abolished by charybdotoxin (ChTX, 0.1 μM) or iberiotoxin (IbTX, 0.1 μM), but not affected by a small-conductance K_{Ca} blocker (apamin, 100 μM). BK_{Ca} current activation by eugenosedin-A was significantly inhibited by an adenylate cyclase inhibitor (SQ 22536, 10 μM), a soluble guanylate cyclase inhibitor (ODQ, 10 μM), competitive antagonists of cAMP and cGMP (Rp-cAMP, 100 μM and Rp-cGMP, 100 μM), and cAMP- and cGMP-dependent protein kinase inhibitors (KT5720, 0.3 μM and KT5823, 0.3 μM). Eugenosedin-A reversed the inhibition of BK_{Ca} current induced by the protein kinase C activator, phorbol myristyl acetate (PMA, 0.1 μM). Eugenosedin-A also prevented BK_{Ca} current inhibition induced by adding PMA, KT5720 and KT5823. Moreover, eugenosedin-A reduced the amplitude of voltage-dependent L-type Ca^{2+} current ($I_{Ca,L}$), but without modifying the voltage-dependence of the current.
Conclusions and implications: Eugenosedin-A enhanced BK_{Ca} currents by stimulating the activity of cyclic nucleotide-dependent protein kinases. Physiologically, this activation would result in the closure of voltage-dependent calcium channels and thereby relax cerebral SMCs.
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Keywords: eugenosedin-A; basilar artery myocytes; BK_{Ca} channels; K_{DR} channels; patch clamp electrophysiology; protein kinases

Abbreviations: AC, adenylate cyclase; 8-Br-cGMP, 8-bromo-guanosine 3′,5′-cyclic monophosphate; BK_{Ca} channels, large-conductance Ca^{2+}-activated potassium channels; ChTX, charybdotoxin; IBMX, 3-isobutyl-1-methylxanthine; IbTX, iberiotoxin; $I_{Ca,L}$, voltage-dependent L-type Ca^{2+} currents; K_{DR} channels, delay-rectifying potassium channels; KT5720, (9R,10S,12S)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-l][1,6]benzodiazocine-10-carboxylic acid hexyl ester; KT5823, (9S,10R,12R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1*H*-diindolo-[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-l][1,6]benzodiazocine-10-carboxylic acid methyl ester; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PMA, phorbol 12-myristate 13-acetate; Rp-cAMP, Rp-adenosine 3′,5′-cyclic monophosphorothioate; Rp-cGMP, Rp-guanosine 3′,5′-cyclic monophosphorothioate; sGC, soluble guanylate cyclase; SK_{Ca} channels, small-conductance Ca^{2+}-activated potassium channels; SQ 22536, 9-(terahydro-2-furanyl)-9H-purin-6-amine

Introduction

Eugenosedin-A (4-{2-hydroxy-3-[1-(2-chlorophenyl-piperazinyl)]-propoxy}-3-methoxy-1-propylenyl-benzene) was synthesized by combining isoeugenol-based aryloxypropanolamine and chlorophenylpiperazine (CPP) (Shen *et al.*, 2004).

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Isoeugenol-based aryloxypropanolamine had β-adrenoceptor blocking actions, and the CPP moiety had α_2-adrenoceptor and 5-hydroxytryptamine (5-HT) receptor antagonist activities (Malomvolgyi *et al.*, 1991; Shen *et al.*, 2004). In isolated aortae, eugenosedin-A was shown to inhibit the cumulative 5-HT-, norepinephrine- and clonidine-induced contractile responses concentration-dependently (Shen *et al.*, 2004). Previous evidence (Alioua *et al.*, 2002) suggests that agonist-induced vasoconstriction by 5-HT, angiotensin II and phenylephrine involves inhibition of large-conductance Ca^{2+}-activated potassium (BK_{Ca}) channels by c-Src via direct phosphorylation of the channel protein. This c-Src-BK_{Ca} signalling pathway plays an important role in regulating rat and human vasoconstriction (Alioua *et al.*, 2002), providing a link between electromechanical and pharmacomechanical coupling which is primarily found in cyclic nucleotide-modulated vasorelaxations (Somlyo and Somlyo, 1968). In addition, activation of BK_{Ca} channels increases K^+ efflux, counteracting the depolarization and constriction caused by pressure and vasoconstrictors (for review see Nelson and Quayle, 1995). While eugenosedin-A has been shown to counteract constrictions, the possibility that the observed reduction in constriction might also involve the activation of BK_{Ca} channels remains to be addressed. To clarify the contribution of BK_{Ca} channels to eugenosedin-A-inhibited vasoconstriction, we used patch clamp techniques to measure its K^+-channel activity in rat basilar artery.

BK_{Ca} channels are activated by increases in intracellular Ca^{2+} and membrane depolarization. In arterial smooth muscle patch clamp experiments, cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) activate protein kinase G (PKG) and protein kinase A (PKA), respectively, which leads to activation of BK_{Ca} (Schubert *et al.*, 1996; White *et al.*, 2000). BK_{Ca} channels play an important role in regulating smooth muscle contractility and in controlling the diameter of small cerebral arteries (Brayden and Nelson, 1992; Nelson *et al.*, 1995). Previous reports have shown that protein kinase C (PKC) inhibits the BK_{Ca} channel activity (Bayguinov *et al.*, 2001; Barman *et al.*, 2004). Therefore, any activation of PKA or PKG would be likely to reduce activation of PKC and increase activity of BK_{Ca} by means of cross-talk between signalling cascades (for review see Jaggar *et al.*, 2000).

The main objectives of this study were to investigate (1) whether eugenosedin-A activates a BK_{Ca} current and (2) the signalling mechanisms that underlie this BK_{Ca} activation. Both conventional and perforated patch clamp techniques were used to determine whether eugenosedin-A enhanced the BK_{Ca} channel activity through cyclic nucleotide-dependent and -independent signalling 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 killed by carbon dioxide asphyxiation. Brains were 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 (SMCs) from rat basilar arteries were enzymatically isolated as described previously (Wu *et al.*, 2005, 2007). In brief, arterial segments were placed in a warm (37°C) cell isolation medium containing (in mM) 60 NaCl, 80 Na-glutamate, 5 KCl, 2 $MgCl_2$, 10 HEPES and 10 glucose 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 delayed rectifier potassium (K_{DR}) currents in basilar artery myocytes. In brief, basilar artery myoctyes were placed in a recording dish and perfused with a bath 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 HEPES and 10 glucose (pH 7.4, NaOH). A recording electrode was pulled from borosilicate glass (resistance: 4–7 MΩ). Its tip was covered with sticky wax and backfilled with pipette solution containing (in mM) 110 K-gluconate, 30 KCl, 0.5 $MgCl_2$, 10 EGTA, 5 HEPES, 5 Na_2ATP, and 1 GTP (pH 7.2, KOH) and then gently lowered onto an SMC. 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, 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. 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.

To study the BK_{Ca} current, we inactivated K_{DR} channels by step depolarization to 0 mV. Under the conditions, BK_{Ca} becomes the dominant outward current. Whole-cell BK_{Ca} and voltage-dependent Ca^{2+} currents were measured using the conventional or perforated patch clamp configuration. Under both recording conditions, the bath solution contained (in mM) 140 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose (pH 7.4, NaOH). The pipette solution contained (in mM) 110 K-gluconate, 30 KCl, 0.5 $MgCl_2$, 1 EGTA, 5 HEPES, 5 Na_2ATP and 1 GTP (pH 7.2, KOH). Amphotericin B (200 μg ml^{-1}) was included in the pipette solution for the perforated patch clamp recordings. When

using 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 $MgCl_2$, 1.8 $CaCl_2$, 5 EGTA, 10 HEPES and 10 glucose. The free Ca^{2+} concentration of these solutions was first calculated (0.1 μM) using Max Chelator Sliders software (C Patton, Stanford University) and then measured again (0.3 μM) using a Ca^{2+}-selective electrode (Corning, Acton, MA, USA). Single channel activity in excised patches were recorded at 0 mV, filtered at 2.5 kHz and digitized at 10 kHz. To measure voltage-dependent Ca^{2+} currents, 140 mM K^+ inside the pipette solution was replaced with equimolar CsCl and pH was adjusted to 7.2 with CsOH, whereas the bathing solution contained 1 μM tetrodotoxin and 10 mM tetraethylammonium chloride.

Experimental procedures

To ascertain whether PKG or PKA signalling was involved in the eugenosedin-A-induced increases in BK_{Ca}, the perforated patch clamp configuration was used. Voltage-clamped cells were equilibrated for 15 min before experimentation. Following equilibration, whole-cell K_{DR} and BK_{Ca} currents were monitored in the presence and absence of eugenosedin-A (1 μM), charybdotoxin (ChTX, 0.1 μM) or iberiotoxin (IbTX, 0.1 μM). Cerebral SMCs were preincubated for 15 min with 9-(terahydro-2-furanyl)-9H-purin-6-amine (SQ 22536, 10 μM), 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM), (9R,10S,12S)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-l][1,6]benzodiazocine-10-carboxylic acid hexyl ester (KT5720, 0.3 μM), (9S,10R,12R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1*H*-diindolo- [1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-l][1,6]benzodiazocine-10-carboxylic acid methyl ester (KT5823, 0.3 μM), Rp-adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMP, 100 μM), Rp-guanosine 3′,5′-cyclic monophosphorothioate (Rp-cGMP, 100 μM) or phorbol 12-myristate 13-acetate (PMA, 0.1 μM) before eugenosedin-A was added. SQ 22536, ODQ, KT5720, KT5823 and PMA were continuously superfused in the bath, while Rp-cAMP and Rp-cGMP were added to the pipette solution. The effects of eugenosedin-A and various drug treatments were measured in the same cell. In general, the net current–voltage (*I-V*) relationship was determined at 5 min intervals by measuring the peak current at the end of 300 ms pulse to voltages between −70 and +40 mV for K_{DR} currents, 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 50 mV and the currents were recorded in the presence and absence of eugenosedin-A.

Cyclic nucleotide radioimmunoassay

cAMP and cGMP levels in endothelium-denuded cerebral arteries were measured as described previously (Wu *et al*., 2001, 2006). In brief, cerebral artery segments were preincubated for 10 min at 37°C in 10 ml phosphate-buffered saline, which were bubbled with 95% O_2/5% CO_2. Endothelial cells were removed by passing air bubbles through the vessel lumen (~2 min) and confirmed as described previously (Wu *et al*., 2007). Subsequently, the vessel preparation was incubated with eugenosedin-A (1, 10 μM) and 3-isobutyl-1-methylxanthine (IBMX, 100 μM) for 30 min, preventing the breakdown of cAMP/cGMP. The vessel was then transferred to a 1.5 ml vial, and the reaction was terminated by adding 1 ml ice-cold 1N hydrochloric acid. The samples were homogenized and then centrifuged at 2500 *g* for 15 min at 4°C. Then, the supernatants were lyophilized, and the concentrations of cAMP and cGMP of each sample were measured using cGMP-[^{125}I] and cAMP-[^{125}I] radioimmunoassay kits (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA).

Data analysis and statistics

For single channel analysis, BK_{Ca} channel activity (NP_o) was determined from continuous gap-free data using Clampfit 9.0. The NP_o was calculated from the following equation: $NP_o = (\Sigma t_i i)/T$, where i is the number of channels open, t_i the open time for each level i and T the total time of analysis.

Data are expressed as means ± s.e., n indicating the number of cells. Repeated measures analysis of variance (ANOVA) compared values at a given voltage. When appropriate, a Tukey–Kramer pairwise comparison was used for *post hoc* analysis. ANOVA followed by Dunnett's test was performed to statistically compare the open probability of BK_{Ca} channels. $P \leqslant 0.05$ was considered statistically significant.

Chemicals

Buffer reagents, 4-aminopyridine (4-AP), apamin, ChTX, chelerythrine, collagenase (type F and H), dithioerythritol, IBMX, IbTX, KT5720, KT5823, ODQ, papain, PMA, Rp-cAMP, Rp-cGMP and SQ 22536 were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). All drugs and reagents were dissolved in distilled water unless otherwise noted. IBMX, chelerythrine, ChTX, eugenosedin-A, ODQ and PMA were dissolved in dimethylsulphoxide at 10 mM. Serial dilutions were made in phosphate-buffered solution to a final solvent concentration of ⩽0.01%.

Results

Lack of modulation of K_{DR} currents by eugenosedin-A

Using conventional whole-cell patch clamp and pipette solutions (containing 10 mM EGTA) that minimize BK_{Ca} channel activity, the K_{DR} current was isolated in rat basilar artery SMCs. Brief voltage steps positive to −30 mV generally activated K_{DR} without an induction of inactivation (Figure 1b). This K_{DR} current was recognized by the addition of 4-AP (5 mM) as described previously (Luykenaar *et al*., 2004; Wu *et al*., 2005). Superfused eugenosedin-A (1 μM) had no significant effect on the K_{DR} current (Figure 1c). In addition, tail currents (at −40 mV) were recorded after a set of 300 ms voltage pulses (range −70 to +40 mV) (Figure 1b, see inset). Eugenosedin-A also did not change the *I–V* relationships of the tail currents (Figure 1d).

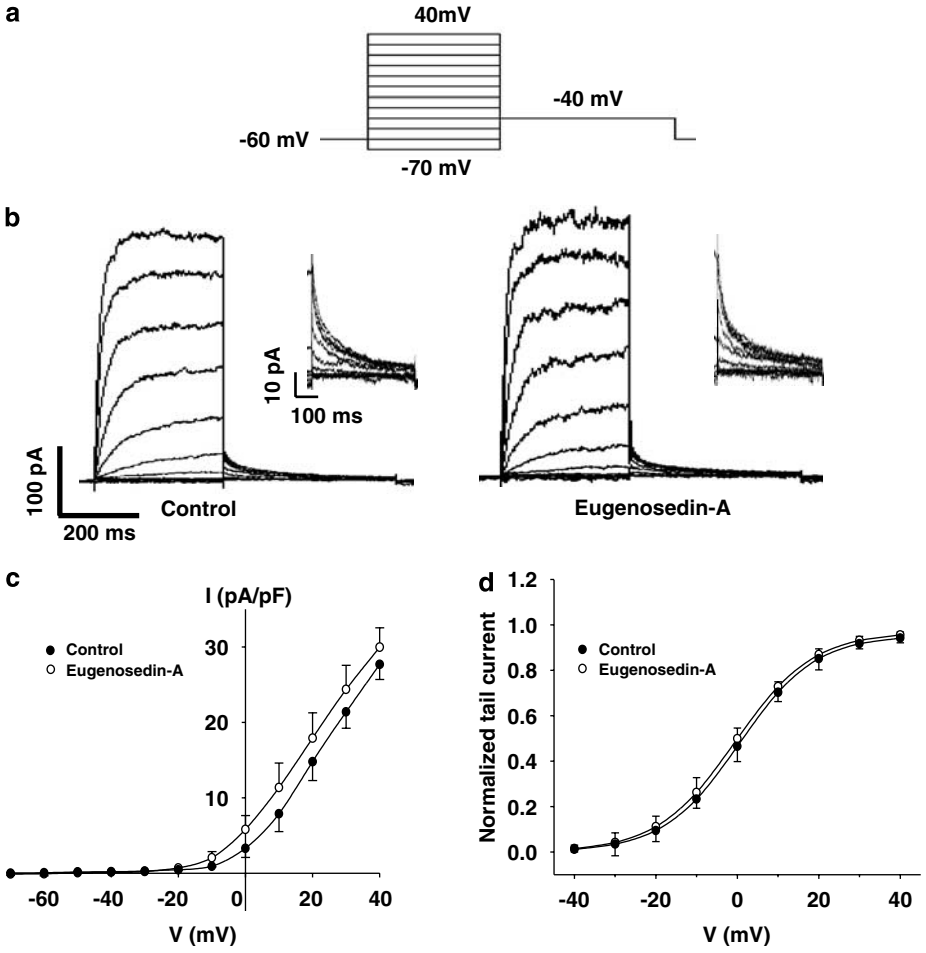

Figure 1 Effects of eugenosedin-A on delayed rectifier K^+ (K_{DR}) current in myocytes isolated from rat basilar arteries. (**a**) The voltage protocol designed to measure steady-state activation of the K_{DR} current. (**b**) Representative recordings of K_{DR} current before and after the addition of eugenosedin-A (1 μM). (**c**) Average current-voltage (*I–V*) relationships under control conditions and in the presence of 1 μM eugenosedin-A ($n=6$). (**d**) Average *I–V* relationships obtained by plotting the normalized tail current peak amplitude against the depolarization potentials.

Activation of BK_{Ca} currents by eugenosedin-A

Conventional and perforated patch clamp was used to assess the effect of eugenosedin-A on the regulation of outward BK_{Ca} conductance. BK_{Ca} channels were identified based on the characteristic single channel conductance and were blocked by ChTX or IbTX as described previously (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 in excised inside-out patch bathed in symmetrical 140 mM KCl (Figure 2b). The effects of eugenosedin-A on theses channels was studied in rat basilar artery myocytes, voltage-clamped at 0 mV to inactivate voltage-dependent K^+ currents (Wu *et al*., 2005) and continuously superfused with an isotonic physiological bath solution containing 1.8 mM Ca^{2+}. Eugenosedin-A (0.1, 1, 10 μM) produced concentration-dependent increases in BK_{Ca} channels (Figures 3b and c). Notably, the increase in BK_{Ca} current induced by eugenosedin-A (1 μM) was inhibited by IbTX (0.1 μM) or ChTX (0.1 μM), but hardly affected by a small-conductance K_{Ca} blocker apamin (100 μM) (Figures 4a and b). The increases of BK_{Ca} current were consistently observable $\sim$10 min after the addition of eugenosedin-A, with the peak steady-state level occurring by 30 min (Figure 4c).

Eugenosedin-A activates BK_{Ca} currents via AC/cAMP- and sGC/cGMP-dependent mechanisms

To further investigate the signalling mechanisms that lead to activation of BK_{Ca} channels, eugenosedin-A (1 μM) was applied to voltage-clamped cells in the presence of SQ 22536, an adenylate cyclase (AC) inhibitor. SQ 22536 (10 μM) blocked the eugenosedin-A-induced increases in BK_{Ca} activity, which indicated that this compound's modulatory effect involved the AC/cAMP pathway (Figure 5). The eugenosedin-A-induced increase in BK_{Ca} activity was also attenuated by the soluble guanylate cyclase (sGC) inhibitor ODQ (10 μM); cell superfusion with ODQ (10 μM) and SQ 22536 (10 μM) strongly inhibited, but did not abolish channel activity (Figure 5). Eugenosedin-A may have had another effect on the regulation of BK_{Ca} channels besides its activation of the AC/cAMP and sGC/cGMP pathways.

Eugenosedin-A activates BK_{Ca} currents via PKA- and PKG-dependent pathways

Eugenosedin-A (1 μM)-induced increases in BK_{Ca} channel activity were reduced in the presence of cAMP- and cGMP-dependent protein kinase inhibitors KT5720 (0.3 μM;

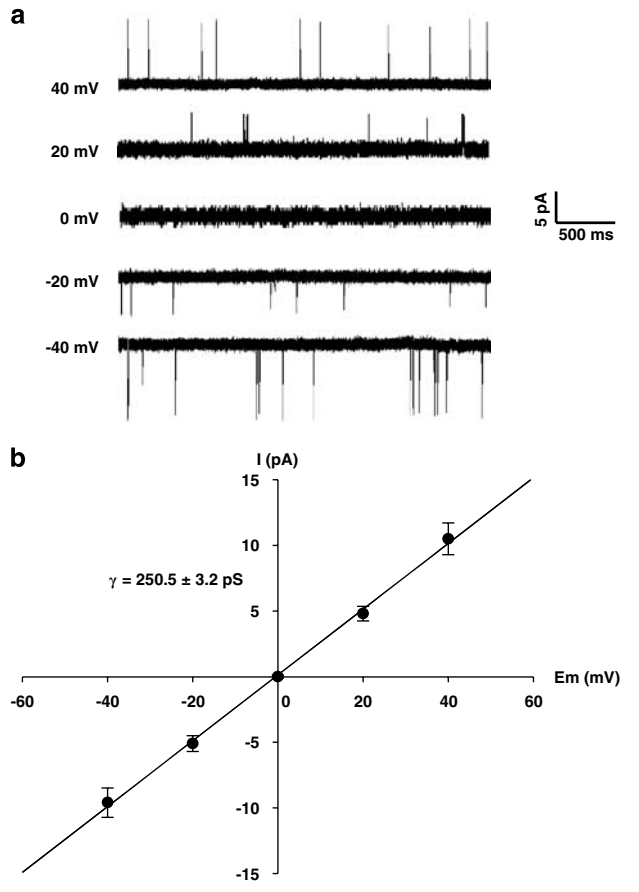

Figure 2 Large-conductance Ca^{2+}-activated potassium channels (BK_{Ca}) channels are the prominent ion channels in membranes of rat basilar artery myocytes. (**a**) In symmetrical gradients of K^+ (140 mM), channel activity recorded from the same inside-out patch varies as a function of membrane potential ($n=8$). Channel openings are upward or downward deflections from baseline (closed) state. (**b**) Plot of BK_{Ca} current amplitude (I) as a function of membrane potential (Em). A linear fit revealed an average single-channel conductance (γ) of 250.5 ± 3.2 pS ($n=7$).

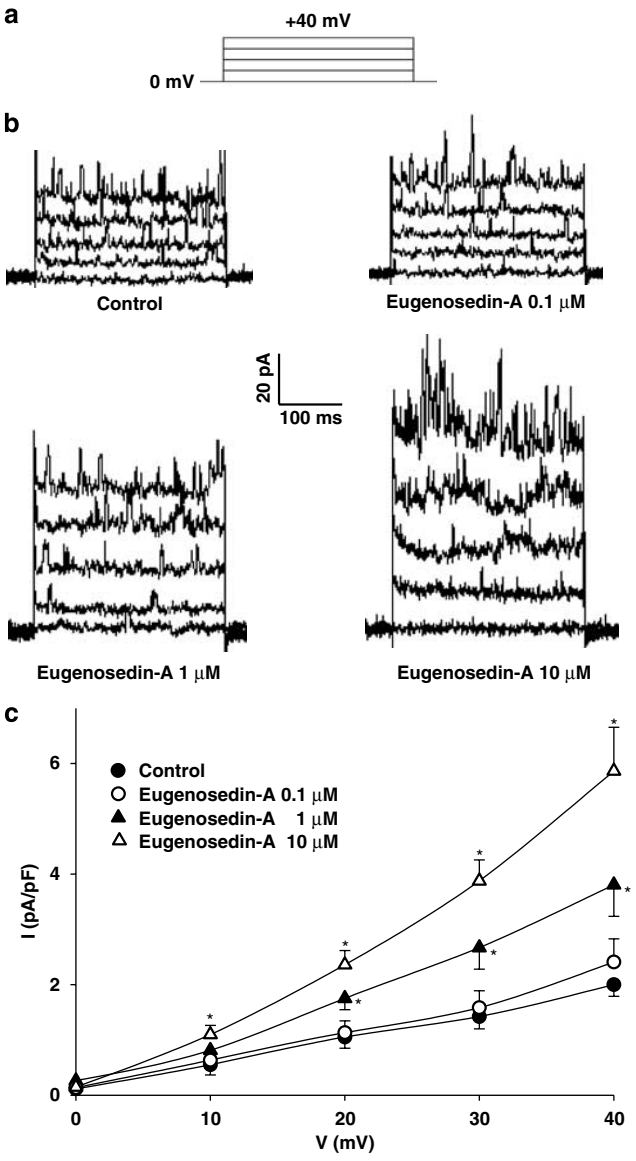

Figure 3 Concentration-dependent increases in BK_{Ca} currents by eugenosedin-A. Cells were bathed in high-Ca^{2+} solution containing 1.8 mM $CaCl_2$. (**a**) Voltage protocol. (**b**) Representative recordings of BK_{Ca} currents under control conditions and in the presence of eugenosedin-A (0.1, 1, 10 μM). (**c**) Average *I–V* relationships under control conditions and in the presence of eugenosedin-A (0.1, 1, 10 μM, $n=7$). *denotes significant difference from control. BK_{Ca}, large-conductance Ca^{2+}-activated potassium.

Figure 6a) and KT5823 (0.3 μM; Figure 6b), respectively; cell superfusion with KT5720 and KT5823 greatly inhibited, but did not abolish channel activity (Figure 6b). The competitive antagonist of cAMP, Rp-cAMP (100 μM), prevented the stimulatory effect of eugenosedin-A on BK_{Ca} when dialysed into the cell through the patch pipette, though not at 40 mV (Figure 7a). Further experiments revealed that Rp-cGMP (100 μM in the pipette), a competitive antagonist of cGMP, attenuated the effect of eugenosedin-A-induced increases in BK_{Ca} activity, but could not prevent eugenosedin-A's effect $\geqslant$30 mV (Figure 7b). Taken together, these results indicate that PKA- and PKG-dependent signalling pathways are involved in the activation of BK_{Ca} channels by eugenosedin-A.

Eugenosedin-A reverses the inhibition of BK_{Ca} current induced by a PKC activator

As shown in Figure 8, the PKC activator PMA (0.1 μM) significantly inhibited BK_{Ca} currents $\geqslant$20 mV. Perfusion with eugenosedin-A (1 μM) completely reversed the activity of PMA-inhibited BK_{Ca} channels (Figure 8a). Likewise, the PKC inhibitor chelerythrine (5 μM) prevented PMA-induced BK_{Ca} channel inhibition (Figure 8b). Eugenosedin-A, as well as chelerythrine, also prevented the BK_{Ca} channel inhibitory effects induced by PMA, KT5720 and KT5823 (Figures 8a and b). These results suggest that BK_{Ca} channels are modulated by eugenosedin-A not only via cyclic nucleotide-dependent protein kinases but also via a transduction pathway involving PKC.

Eugenosedin-A activates BK_{Ca} channels in excised membrane patches

Because BK_{Ca} channel activation by eugenosedin-A was caused by stimulation of the AC/PKA and sGC/PKG

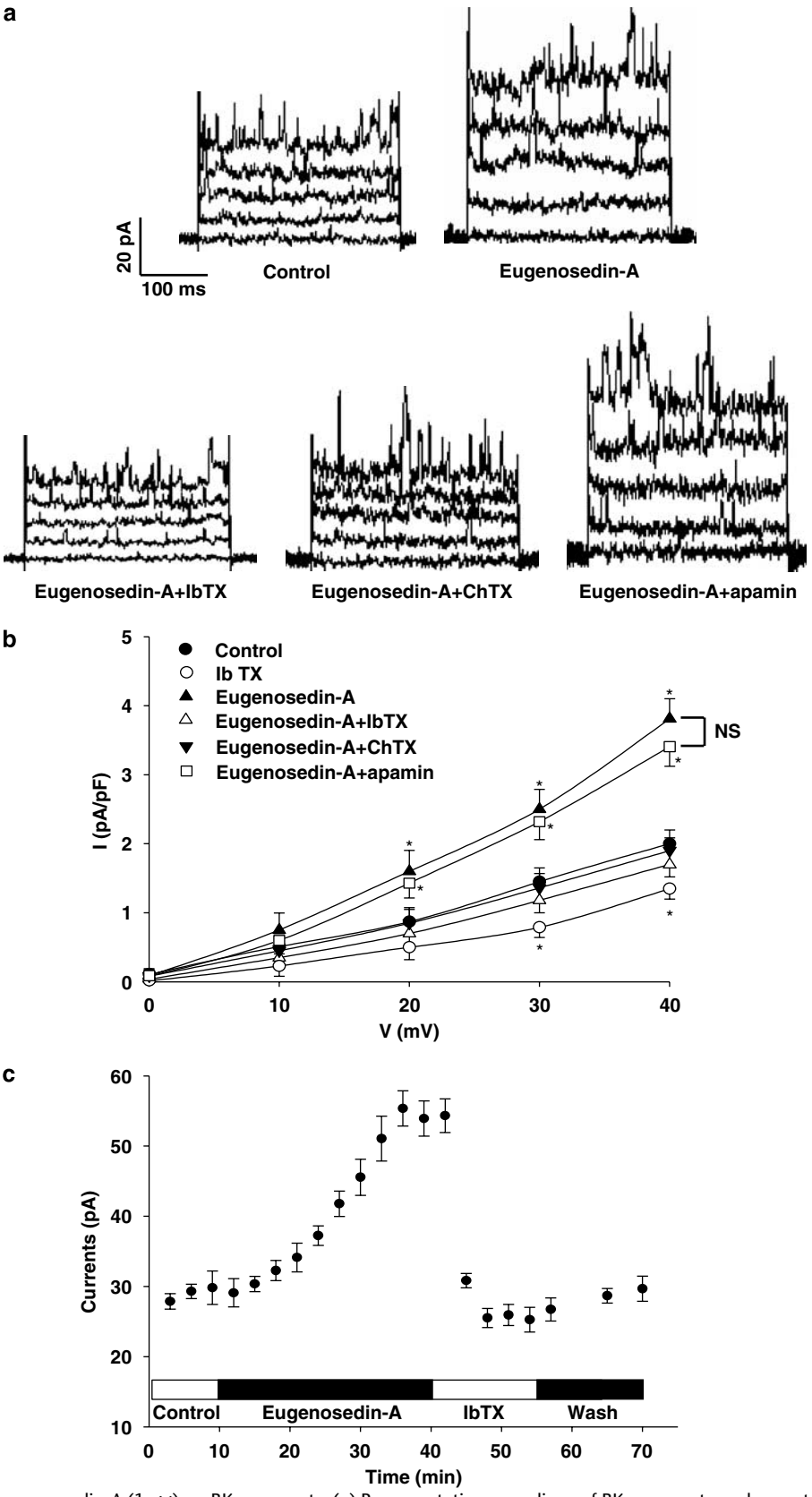

Figure 4 Effects of eugenosedin-A (1 μM) on BK_{Ca} currents. (**a**) Representative recordings of BK_{Ca} currents under control conditions and in the presence of eugenosedin-A, eugenosedin-A + IbTX (0.1 μM), eugenosedin-A + ChTX (0.1 μM) or eugenosedin-A + apamin (100 μM). (**b**) Average *I–V* relationships under control conditions and in the presence of eugenosedin-A, eugenosedin-A + IbTX, eugenosedin-A + ChTX or eugenosedin-A + apamin. (**c**) Time course of eugenosedin-A on BK_{Ca} currents. The horizontal bars of the diagram indicate the periods of drug perfusion ($n = 7$). *denotes significant difference from control. BK_{Ca}, large-conductance Ca^{2+}-activated potassium; ChTX, charybdotoxin; IbTX, iberiotoxin; NS, nonsignificant.

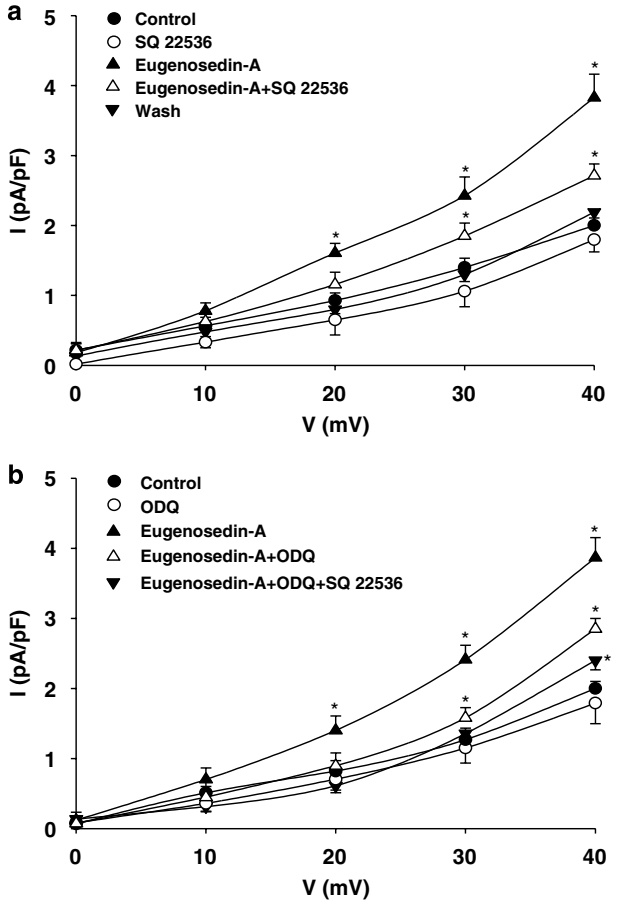

Figure 5 BK_{Ca} current activation by eugenosedin-A (1 μM) is dependent on adenylate cyclase and soluble guanylate cyclase. (**a**) Average *I–V* relationships under control conditions and in the presence of eugenosedin-A or eugenosedin-A + SQ 22536 (10 μM, $n=6$). (**b**) Average *I–V* relationships under control conditions and in the presence of eugenosedin-A, eugenosedin-A + ODQ (10 μM) or eugenosedin-A + ODQ + SQ 22536 ($n=7$). *denotes significant difference from control. BK_{Ca}, large-conductance Ca^{2+}-activated potassium; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; SQ 22536, 9-(terahydro-2-furanyl)-9H-purin-6-amine.

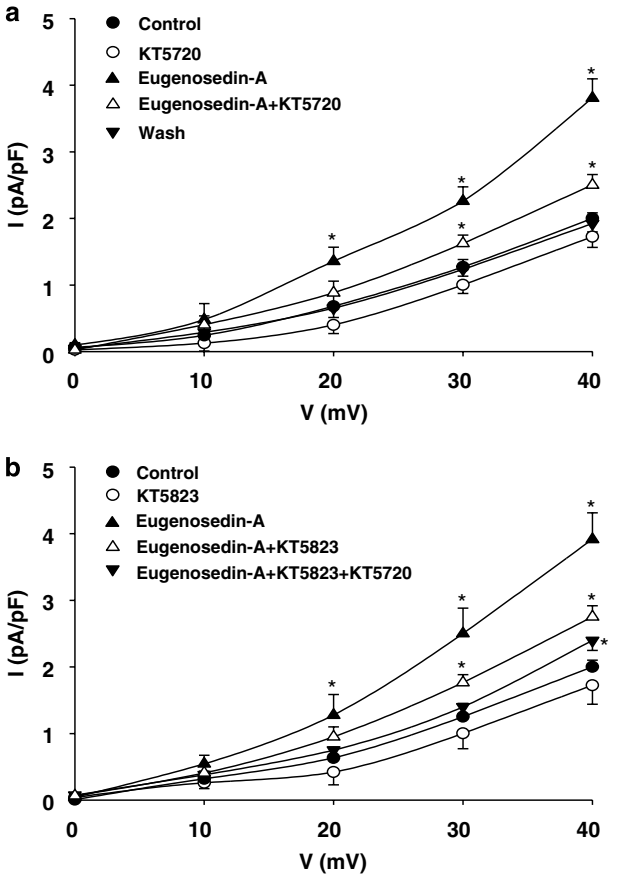

Figure 6 BK_{Ca} current activation by eugenosedin-A (1 μM) is dependent on PKA and PKG. (**a**) Average *I–V* relationships under control conditions and in the presence of eugenosedin-A or eugenosedin-A + KT5720 (0.3 μM) ($n=6$). (**b**) Average *I–V* relationships under control conditions and in the presence of eugenosedin-A, eugenosedin-A + KT5823 (0.3 μM) or eugenosedin-A + KT5823 + KT5720 ($n=6$). *denotes significant difference from control. BK_{Ca}, large-conductance Ca^{2+}-activated potassium; KT5720, (9R,10S,12S)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-l][1,6]benzodiazocine-10-carboxylic acid hexyl ester; KT5823, (9S,10R,12R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1*H*-diindolo- [1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-l][1,6]benzodiazocine-10-carboxylic acid methyl ester; PKA, protein kinase A; PKG, protein kinase G.

pathways, we sought to investigate whether eugenosedin-A could activate BK_{Ca} channels in the complete absence of intracellular signalling factors. Eugenosedin-A regulation of BK_{Ca} channel activity was measured in excised inside-out membrane patches with 0.3 μM free Ca^{2+} present in the bath solution. At 0 mV, eugenosedin-A (1 μM) increased mean BK_{Ca} channel open probability ~3.7-fold (Figure 9). This increase in channel opening was abolished by a BK_{Ca} inhibitor IbTX (0.1 μM in the pipette), as shown in Figure 9. These results suggest that eugenosedin-A could directly activate BK_{Ca} channels located on the membrane of the cerebral artery myocytes, without the involvement of the enzyme systems contained in the cytosol.

Inhibition of L-type Ca^{2+} channels by eugenosedin-A

These experiments were conducted with a Cs^{+}-containing solution. The peak amplitude of voltage-dependent L-type Ca^{2+} currents ($I_{Ca,L}$) was at 10 mV in rat basilar artery myocytes. Perfused eugenosedin-A (1 μM) was found to significantly suppress the $I_{Ca,L}$ (Figure 10). Eugenosedin-A reduced the amplitude of $I_{Ca,L}$ (from 140 ± 11 to 100 ± 9 pA at 10 mV, $n=6$, $P<0.05$) when cells were depolarized from −40 to 50 mV, but it did not modify the voltage-dependence of the current in these cells (Figure 10c). The increase in $I_{Ca,L}$ was observable 5–10 min after the addition of eugenosedin-A, with the peak steady-state level occurring by 30 min (Figure 10d).

Accumulation of cAMP and cGMP by eugenosedin-A

cAMP and cGMP levels were examined in endothelium-denuded cerebral arteries. The basal content of cAMP or

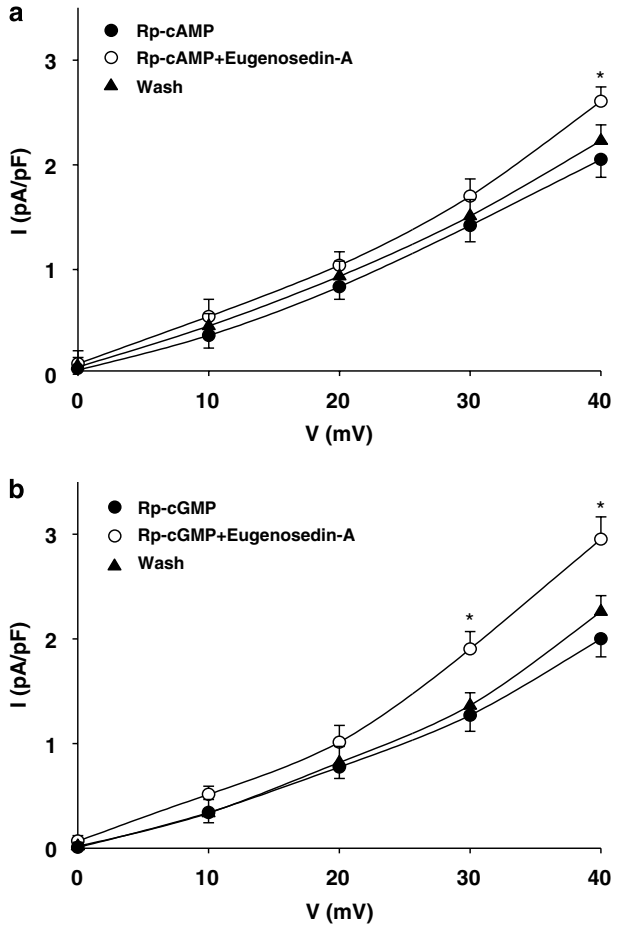

Figure 7 BK_{Ca} current activation by eugenosedin-A (1 μM) is dependent on cAMP/PKA and cGMP/PKG activities. Rp-cAMP or Rp-cGMP (100 μM) was added in the pipette solution. (**a**) Average *I–V* relationships in the presence of Rp-cAMP, Rp-cAMP + eugenosedin-A, and washout ($n=6$). (**b**) Average *I–V* relationships in the presence of Rp-cGMP, Rp-cGMP + eugenosedin-A, and washout ($n=6$). *denotes significant difference from Rp-cAMP or Rp-cGMP. BK_{Ca}, large-conductance Ca^{2+}-activated potassium; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; PKA, protein kinase A; PKG, protein kinase G; Rp-cAMP, Rp-adenosine 3′,5′-cyclic monophosphorothioate; Rp-cGMP, Rp-guanosine 3′,5′-cyclic monophosphorothioate.

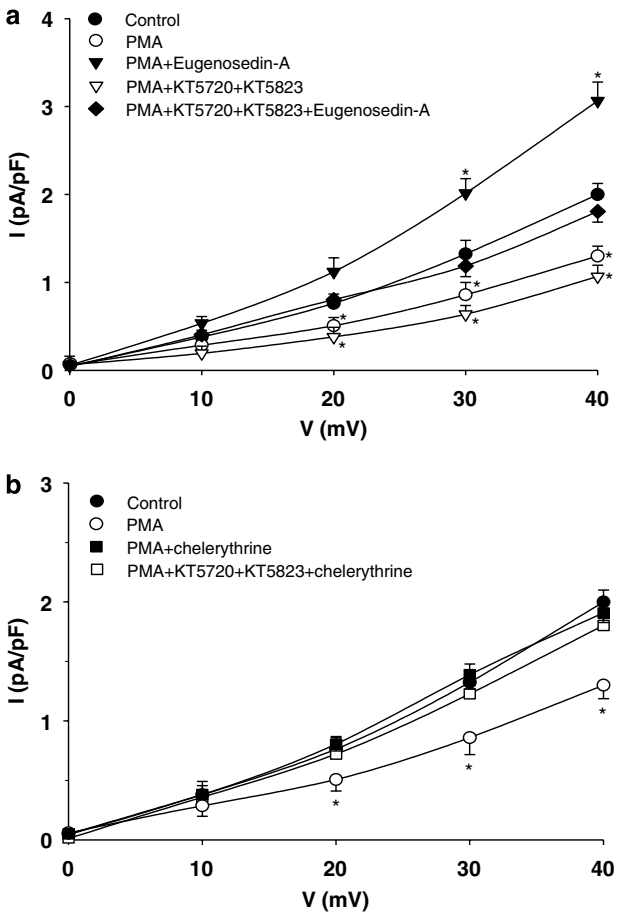

Figure 8 Eugenosedin-A (1 μM) reversed the inhibition of BK_{Ca} currents induced by activation of PKC by PMA. (**a**) Average *I–V* relationships under control conditions and in the presence of PMA, PMA + eugenosedin-A, PMA + KT5720 + KT5823 or PMA + KT5720 + KT5823 + eugenosedin-A ($n=7$). (**b**) Average *I–V* relationships under control conditions and in the presence of PMA, PMA + chelerythrine or PMA + KT5720 + KT5823 + chelerythrine ($n=6$). *denotes significant difference from control. BK_{Ca}, large-conductance Ca^{2+}-activated potassium; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; KT5720, (9R,10S,12S)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-l][1,6]benzodiazocine-10-carboxylic acid hexyl ester; KT5823, (9S,10R,12R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1*H*-diindolo-[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-l][1,6]benzodiazocine-10-carboxylic acid methyl ester.

cGMP in the vessels was 7.0 ± 0.8 and 3.9 ± 0.4 pmol mg^{-1}, respectively ($n=3$). Eugenosedin-A, at two concentrations (1, 10 μM), significantly increased the tissue content of both cAMP (54.5 ± 4.9 (1 μM); 80.3 ± 6.1 (10 μM) pmol mg^{-1}) and cGMP (20.4 ± 2.1 (1 μM); 28.6 ± 3.5, (10 μM) pmol mg^{-1}), compared with basal values.

Discussion

The eugenosedin-A-induced increase in BK_{Ca} current in cerebral SMCs occurs primarily through the activation of cAMP/PKA and cGMP/PKG. This would hyperpolarize SMCs, thereby closing L-type Ca^{2+} channels, reducing intracellular Ca^{2+} and thereby relaxing SMCs (Nelson *et al.*, 1995). In addition, the increase in cellular cyclic nucleotides induced by eugenosedin-A could also contribute to its relaxation of vessels, which is important because dilation to cyclic nucleotides does not require BK_{Ca} activation. In other words, some part of the relaxation may result from the activation of cAMP/cGMP independent of changes in membrane potential. Previous work (Shen *et al.*, 2004) showed that the vasocontractile inhibitory activity of eugenosedin-A (0.01–1 μM) was mainly a result of antagonism of 5-HT_{2A} receptors ($pA_2=8.86\pm0.32$) and α_1-adrenoceptors ($pA_2=7.88\pm0.13$). It is unlikely that the increase in cyclic nucleotides observed herein could be explained by receptor blockade, as these pathways were not first stimulated in the isolated SMC and therefore could not be inhibited. However, the actual

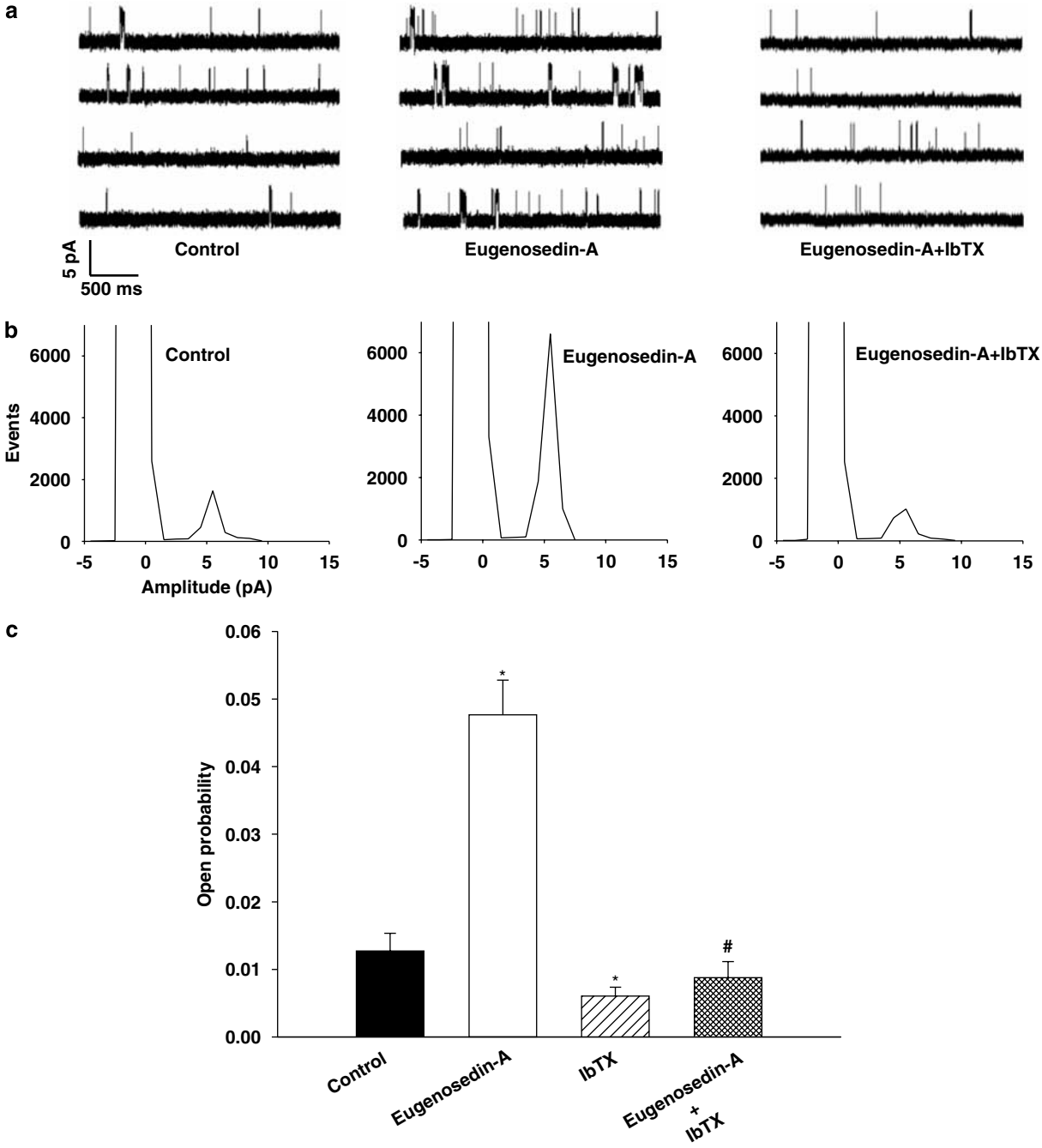

Figure 9 Eugenosedin-A activates BK_{Ca} channels in excised inside-out membrane patches. (**a**) Original current recording illustrating BK_{Ca} channels activation by eugenosedin-A (1 μM), voltage clamped at 0 mV. IbTX (0.1 μM) was added in the pipette solution. (**b**) Current amplitude histograms constructed from the traces shown in (**a**). (**c**) Bar graph showing the relative open probability of BK_{Ca} channels in controls and in the presence of eugenosedin-A, IbTX or eugenosedin-A + IbTX. *denotes significant difference from control. #denotes significant difference from eugenosedin-A ($n = 7$). BK_{Ca}, large-conductance Ca^{2+}-activated potassium; IbTX, iberiotoxin.

pathway by which eugenosedin-A enhanced cellular levels of cyclic nucleotides remains to be investigated.

Alterations in BK_{Ca} channel activity play a central role in mediating vasoconstriction and vasodilatation. One previous study has shown BK_{Ca} channels to be involved in mediating vascular relaxation in response to agents that elevate cGMP (Zhao *et al.*, 1997). Another study found 8-bromo-guanosine 3′,5′-cyclic monophosphate (8-Br-cGMP)-induced relaxation to be attenuated by IbTX (Tanaka *et al.*, 1998), a finding showing that BK_{Ca} channels are indeed a target of cGMP signalling. One of our previous studies has confirmed that 8-Br-cGMP increases BK_{Ca} currents in basilar artery myocytes (Wu *et al.*, 2005). Likewise, agents that elevate cAMP have also been shown to modulate this current

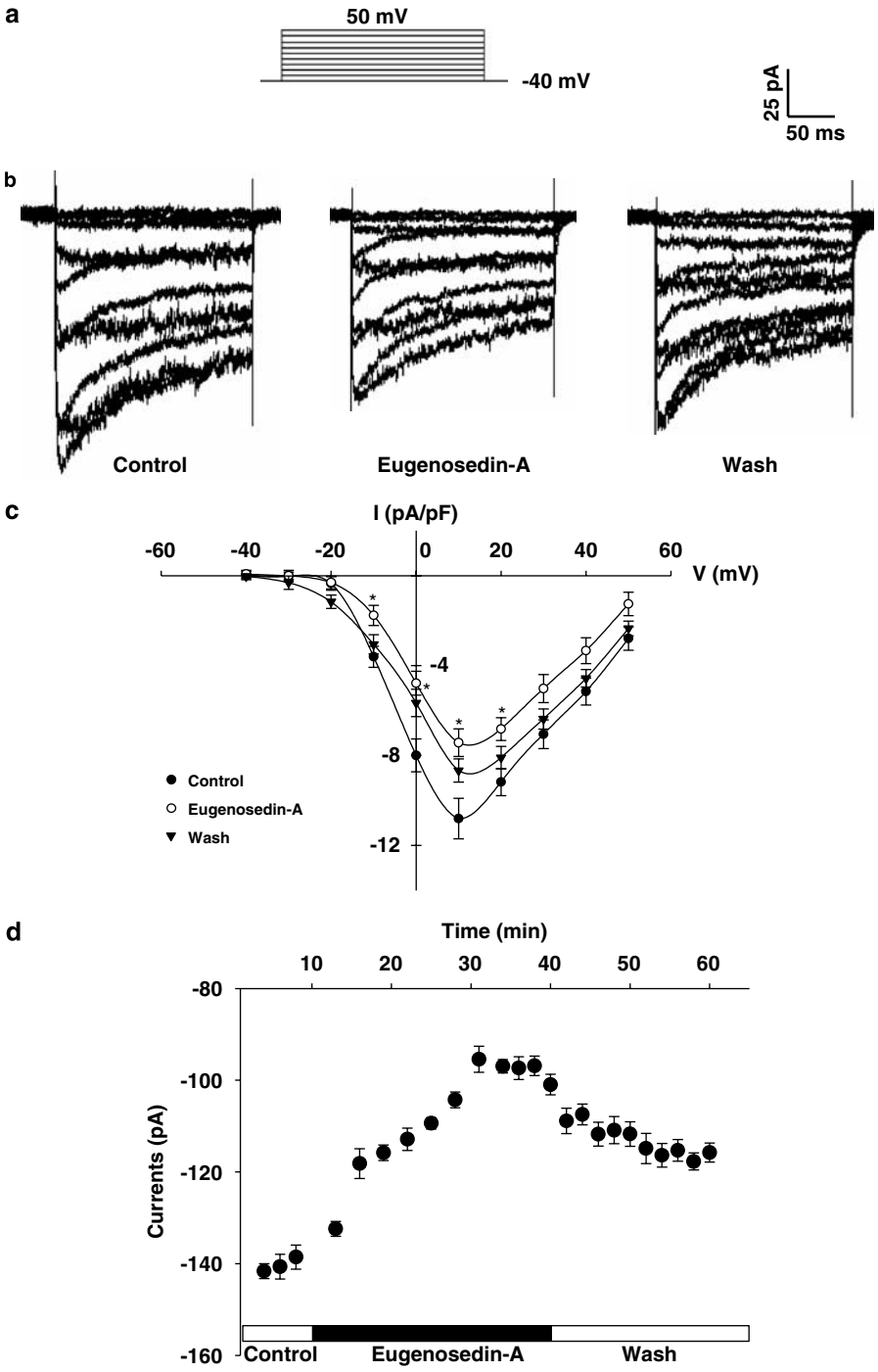

Figure 10 Effects of eugenosedin-A on voltage-dependent L-type Ca^{2+} currents ($I_{Ca,L}$) in rat basilar artery myocytes. (**a**) Voltage protocol. (**b**) Representative recordings of $I_{Ca,L}$ in the absence and presence of eugenosedin-A (1 μM), and washout. (**c**) Average *I–V* relationships of the peak $I_{Ca,L}$ in the absence and presence of eugenosedin-A, and washout. (**d**) Time course of eugenosedin-A on $I_{Ca,L}$. The horizontal bars of the diagram indicate the periods of drug perfusion ($n=6$). *denotes significant difference from control.

(White *et al.*, 2000; Wu *et al.*, 2005) and initiate vascular relaxation (Paterno *et al.*, 1996). Increases in cAMP and cGMP simultaneously activate PKA and PKG pathways resulting in the opening of BK_{Ca} channels (Torphy, 1994). In contrast, as PKC is known to inhibit BK_{Ca} channel activity (Bayguinov *et al.*, 2001; Barman *et al.*, 2004), any activation of PKA/PKG would be likely to cause a reduction in PKC activation and an increase in BK_{Ca} activity through cross-talk. Thus, cyclic nucleotides are required to initiate the cross-talk with PKC, thereby enhancing the BK_{Ca} current.

Eugenosedin-A-induced increases in BK_{Ca} currents are strongly reduced, but not abolished, by combining inhibitors of AC (SQ 22536) and sGC (ODQ), and by combining inhibitors of PKA (KT5720) and PKG (KT5823) in basilar

artery myocytes. To our knowledge, these compounds are selective inhibitors; they have some inhibition on basal BK_{Ca} currents. Fortunately, their effect is not significantly different from the control. Any lack of selectivity would lead to some doubts regarding the relative role of the AC/cAMP and sGC/cGMP pathways. In cyclic nucleotide assays, eugenosedin-A did enhance both cAMP and cGMP levels in endothelium-denuded cerebral arteries. Smooth muscle relaxants that increase cAMP and cGMP have been shown to activate BK_{Ca} channels through direct phosphorylation effects on the channel protein (Robertson *et al*., 1993) and through elevation of Ca^{2+} spark frequency. PKA and PKG may in part increase Ca^{2+} sensitivity of Ca^{2+} sparks by increasing sarcoplasmic reticulum Ca^{2+} load, which may enhance the BK_{Ca} activity (for review see Jaggar *et al*., 2000). Based on our observations of eugenosedin-A-modulated channel activity, we believe that the activation of BK_{Ca} channel is mainly dependent on both cAMP/PKA and cGMP/PKG. We also found that PKC activation, by PMA, inhibited BK_{Ca} current. Eugenosedin-A reversed PMA-mediated inhibition of the BK_{Ca} channel, and it also prevented the combination of PMA, KT5720 and KT5823 from inhibiting BK_{Ca} channels as well. Therefore, we suggest that eugenosedin-A-induced BK_{Ca} channel activation occurs not only as a result of PKA/PKG, but also as a result of cross-interaction with PKC.

We used inside-out patch clamp electrophysiology in a number of experiments to explore whether eugenosedin-A could directly affect the BK_{Ca} channel or a closely associated site. We found that its action could be explained at least in part by a direct effect on the channel rather than via protein kinases. Under whole-cell recording conditions, there was a substantial delay ($\sim$10 min) between the addition of eugenosedin-A and the activation of BK_{Ca} currents, compared with the rapid activation of BK_{Ca} channels in excised patches. A possible explanation for this discrepancy is that eugenosedin-A's primary site of action is intracellular. The delay in activation of BK_{Ca} channels by eugenosedin-A in whole-cell configurations might be rationally explained by the additional time required to penetrate the cell membrane and to activate the second messenger cascades. This would lead to the stimulation of K^+ effluxes and consequent membrane hyperpolarization.

In this study, we provide the first evidence that eugenosedin-A activates BK_{Ca} currents in rat basilar arteries. We also found it to be able to reduce the activity of Ca^{2+} channels directly though its Ca^{2+} channel blocking action and indirectly by activating BK_{Ca} channels producing hyperpolarization, which would decrease the open probability of Ca^{2+} channels via their voltage-dependence. Functionally, these properties would be likely to lead to the relaxation of SMCs at the concentrations of eugenosedin-A employed in the present study. Some of the vasorelaxation could result from the direct stimulation of cyclic nucleotides, which are independent of changes in membrane potential. In conclusion, eugenosedin-A-induced activation of BK_{Ca} channels is mainly mediated by PKA and PKG, which phosphorylate the channels or associated regulatory proteins. This in turn enhances the K^+ effluxes and leads to membrane hyperpolarization and closure of voltage-dependent L-type Ca^{2+} channels.

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Conflict of interest

The authors state no conflict of interest.

References

Alioua A, Mahajan A, Nishimaru K, Zarei MM, Stefani E, Toro L (2002). Coupling of c-Src to large conductance voltage- and Ca^{2+}-activated K^+ channels as a new mechanism of agonist-induced vasoconstriction. *Proc Natl Acad Sci USA* **99**: 14560–14565.

Barman SA, Zhu S, White RE (2004). Protein kinase C inhibits BK_{Ca} channel activity in pulmonary arterial smooth muscle. *Am J Physiol Lung Cell Mol Physiol* **286**: L149–L155.

Bayguinov O, Hagen B, Kenyon JL, Sanders KM (2001). Coupling strength between localized Ca^{2+} transients and K^+ channels is regulated by protein kinase C. *Am J Physiol Cell Physiol* **281**: C1512–C1523.

Brayden JE, Nelson MT (1992). Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science* **256**: 532–535.

Jaggar JH, Leffler CW, Cheranov SY, Tcheranova DES, Cheng X (2002). Carbon monoxide dilates cerebral arterioles by enhancing the coupling of Ca^{2+} sparks to Ca^{2+}-activated K^+ channels. *Circ Res* **91**: 610–617.

Jaggar JH, Porter VA, Lederer WJ, Nelson MT (2000). Calcium sparks in smooth muscle. *Am J Physiol Cell Physiol* **278**: C235–C256.

Luykenaar KD, Brett SE, Wu BN, Wiehler WB, Welsh DG (2004). Pyrimidine nucleotides suppress K_{DR} currents and depolarize rat cerebral arteries by activating Rho kinase. *Am J Physiol Heart Circ Physiol* **286**: H1088–H1100.

Malomvolgyi B, Tothfalusi L, Tekes K, Magyar K (1991). Comparison of serotonin agonistic and antagonistic activities of a new antidepressant agent Trelibet (EGYT-475) and its metabolite EGYT-2760 on isolated rat fundus. *Acta Physiol Hung* **78**: 201–209.

Nelson MT, Cheng H, Rubart M, Santana LF, Bonev AD, Knot HJ *et al*. (1995). Relaxation of arterial smooth muscle by calcium sparks. *Science* **270**: 633–637.

Nelson MT, Quayle JM (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *Am J Physiol Cell Physiol* **268**: C799–C822.

Paterno R, Faraci FM, Heistad DD (1996). Role of Ca^{2+}-dependent K^+ channels in cerebral vasodilatation induced by increases in cyclic GMP and cyclic AMP in the rat. *Stroke* **27**: 1603–1607.

Robertson BE, Schubert R, Hescheler J, Nelson MT (1993). cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. *Am J Physiol Cell Physiol* **265**: C299–C303.

Schubert R, Serebryakov VN, Engel H, Hopp HH (1996). Iloprost activates K_{Ca} channels of vascular smooth muscle cells: role of cAMP-dependent protein kinase. *Am J Physiol Cell Physiol* **271**: C1203–C1211.

Shen KP, Chiu CC, Chen SJ, Chen IJ, Wu BN (2004). Hypotensive effects of eugenosedin-A with serotonin, alpha- and beta-adrenoceptor antagonistic activities in spontaneously hypertensive and normotensive rats. *Pharmacology* **71**: 91–101.

Somlyo AV, Somlyo AP (1968). Electromechanical and pharmacomechanical coupling in vascular smooth muscle. *J Pharmacol Exp Ther* **159**: 129–145.

Tanaka Y, Aida M, Tanaka H, Shigenobu K, Toro L (1998). Involvement of maxi-K_{Ca} channel activation in atrial natriuretic peptide-induced vasorelaxation. *Naunyn Schmiedebergs Arch Pharmacol* **357**: 705–708.

Torphy TJ (1994). Beta-adrenoceptors, cAMP and airway smooth muscle relaxation: challenges to the dogma. *Trends Pharmacol Sci* **15**: 370–374.

White RE, Kryman JP, El-Mowafy AM, Han G, Carrier GO (2000). cAMP-dependent vasodilators cross-activate the cGMP-dependent protein kinase to stimulate BK_{Ca} channel activity in coronary artery smooth muscle cells. *Circ Res* **86**: 897–905.

Wu BN, Chen CW, Liou SF, Yeh JL, Chung HH, Chen IJ (2006). Inhibition of pro-inflammatory TNF-α-induced iNOS by xanthine-based KMUP-1 and KMUP-3 in rat trachea: the involvement of soluble guanylate cyclase and protein kinase G. *Mol Pharmacol* **70**: 977–985.

Wu BN, Lin RJ, Lin CY, Shen KP, Chiang LC, Chen IJ (2001). A xanthine-based KMUP-1 with cyclic GMP enhancing and K^+ channels opening activities in rat aortic smooth muscle. *Br J Pharmacol* **134**: 265–274.

Wu BN, Luykenaar KD, Brayden JE, Giles WR, Corteling RL, Wiehler WB *et al*. (2007). Hyposmotic challenge inhibits inward rectifying K^+ channel in cerebral arterial smooth muscle cells. *Am J Physiol Heart Circ Physiol* **292**: H1085–H1094.

Wu BN, Tu HF, Welsh DG, Chen IJ (2005). KMUP-1 activates BK_{Ca} channels in basilar artery myocytes via cyclic nucleotide-dependent protein kinases. *Br J Pharmacol* **146**: 862–871.

Xi Q, Tcheranova D, Parfenova H, Horowitz B, Leffler CW, Jaggar JH (2004). Carbon monoxide activates K_{Ca} channels in newborn arteriole smooth muscle cells by increasing apparent Ca^{2+} sensitivity of alpha-subunits. *Am J Physiol Heart Circ Physiol* **286**: H610–H618.

Zhao YJ, Wang J, Rubin LJ, Yuan XJ (1997). Inhibition of K_V and K_{Ca} channels antagonizes NO-induced relaxation in pulmonary artery. *Am J Physiol Heart Circ Physiol* **272**: H904–H912.