Vascular KCNQ potassium channels as novel targets for the control of mesenteric artery constriction by vasopressin.

Based on studies in single cells, pressurized arteries and in vivo measurements of mesenteric vascular resistance

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Running Title Page

KCNQ channels: targets of AVP in mesenteric artery myocytes

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Abbreviations: AVP, arginine vasopressin; K⁺, potassium; K, currents, voltage-sensitive K⁺ currents; MAP, mean arterial pressure; MASMCs, mesenteric artery smooth muscle cells; MVR, mesenteric vascular resistance; PKC, protein kinase C; PCR, polymerase chain reaction; PIP₂, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; RT, reverse transcriptase; Vᵐ, membrane voltage; VSMCs, vascular smooth muscle cells.

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Abstract

Pressor effects of the vasoconstrictor hormone arginine vasopressin (AVP), observed when systemic AVP concentrations are less than 100 pM, are important for the physiological maintenance of blood pressure and are also the basis for therapeutic use of vasopressin to restore blood pressure in hypotensive patients. However, the mechanisms by which circulating AVP induces arterial constriction are unclear. We examined the novel hypothesis that KCNQ potassium channels mediate the physiological vasoconstrictor actions of AVP. Reverse transcriptase polymerase chain reaction revealed expression of KCNQ1, KCNQ4 and KCNQ5 in rat mesenteric artery smooth muscle cells (MASMCs). Whole-cell perforated patch recordings of voltage-sensitive K\(^+\) (K\(_v\)) currents in freshly isolated MASMCs revealed linopirdine- and XE-991-sensitive KCNQ currents that were electrophysiologically and pharmacologically distinct from other K\(_v\) currents. Suppression of KCNQ currents by AVP (100 pM) was associated with significant membrane depolarization and was abolished by the protein kinase C (PKC) inhibitor calphostin C (250 nM). The KCNQ channel blocker linopirdine (10 µM) inhibited KCNQ currents in MASMCs and induced constriction of isolated rat mesenteric arteries. The vasoconstrictor responses were not additive when combined with 30 pM AVP and were prevented by the L-type Ca\(^{2+}\) channel blocker verapamil. Flupirtine significantly enhanced KCNQ currents and reversed constrictor responses to 30 pM AVP. In vivo, intravenous administration of linopirdine induced a dose-dependent increase in mesenteric artery resistance and blood pressure, whereas flupirtine had the opposite effects. We conclude that physiological concentrations of AVP induce mesenteric artery constriction via PKC-dependent suppression of KCNQ currents and L-type Ca\(^{2+}\) channel activation in MASMCs.
Introduction

Membrane voltage ($V_m$) determines the open probability of L-type Ca$^{2+}$ channels in vascular smooth muscle cells (VSMCs) and potassium (K$^+$) channels represent a primary effector for adjusting $V_m$. To the extent that K$^+$ channels are open in resting VSMCs, the outward flux of K$^+$ through these channels (measured as K$^+$ current) will tend to stabilize the resting $V_m$ at negative (hyperpolarized) voltages and prevent opening of voltage-sensitive Ca$^{2+}$ channels. Conversely, reduction of outward K$^+$ currents in VSMCs results in a shift to more positive $V_m$ (membrane depolarization) leading to activation of L-type Ca$^{2+}$ channels and entry of Ca$^{2+}$ into the cell. Elevation of the cytosolic Ca$^{2+}$ concentration in this manner can trigger VSMC contraction and vasoconstriction.

KCNQ channels (Kv7 family) are voltage-sensitive K$^+$ (K$\text{v}$) channels that have been recognized as mediators of neuronal “M-currents”: non-inactivating, outwardly rectifying K$\text{v}$ currents. Their inhibition in response to muscarinic receptor activation results in increased neuronal excitation (Jentsch, 2000). Suppression of KCNQ channel-mediated neuronal M-currents is generally considered to involve phosphatidylinositol 4,5-bisphosphate (PIP$_2$) hydrolysis. PIP$_2$ is thought to stabilize the open state of KCNQ channels; activation of phospholipase C (PLC), e.g. via m1 muscarinic receptor activation, leads to hydrolysis of PIP$_2$ and suppression of channel activity (Delmas and Brown, 2005). In addition to this mechanism, bradykinin at high concentrations may suppress neuronal M-currents via PLC-mediated inositol trisphosphate formation and release of intracellular Ca$^{2+}$ stores (Cruzblanca et al., 1998; Brown et al., 2007). Another proposed mechanism for suppression of M-currents is via PKC activation and potentially, direct PKC-mediated KCNQ channel phosphorylation (Hoshi et al., 2003; Nakajo and Kubo, 2005; Surti et al., 2005).
KCNQ channels have very recently been found to be expressed in VSMCs (Ohya et al., 2003; Yeung and Greenwood, 2005; Joshi et al., 2006; Brueggemann et al., 2007; Yeung et al., 2007), but little is known about their regulation or function in these cells. In A7r5 cells (a rat aortic smooth muscle cell line), we found that a physiological concentration of the vasoconstrictor hormone arginine vasopressin (AVP, 100 pM) can suppress native KCNQ5 currents via protein kinase C (PKC) activation and this effect is sufficient to induce membrane depolarization and action potential firing (Brueggemann et al., 2007). We speculated that KCNQ channel suppression might represent the depolarizing mechanism responsible for the vasoconstrictor actions of physiological concentrations of AVP (Brueggemann et al., 2007).

The previous identification of KCNQ channels as regulators of neuronal excitation has led to the use of activators or blockers of KCNQ channels for treatment of epilepsy, neuropathic pain, and Alzheimer’s disease (Passmore et al., 2003; Surti and Jan, 2005; Rogawski, 2006). Although a reduction in systolic blood pressure and heart rate was noted in patients treated chronically with the KCNQ channel activator flupirtine (Herrmann et al., 1987), the effects of KCNQ channel modulators on arterial resistance have not been evaluated. Vasodilator/vasoconstrictor actions might have important implications for the use of KCNQ channel modulators in existing therapies as well as for their potential use in the treatment of cardiovascular diseases.

AVP-induced constriction of mesenteric arteries is thought to be essential for its physiological pressor effects (Altura, 1975; Banks et al., 1985). Mesenteric artery constriction also contributes to the clinical actions of AVP, which is increasingly used as a pressor agent to treat patients during cardiopulmonary resuscitation, for septic/vasodilatory shock, or intraoperative hypotension (Altura, 1976; Holmes et al., 2001; Treschan and Peters, 2006;
Barrett et al., 2007). In disease states such as hypertension, heart failure, and vasospasm, elevated circulating [AVP] may contribute to disease progression (Cowley et al., 1981; Delgado et al., 1988; Nakamura et al., 2006). Considering our previous findings that physiological concentrations of AVP regulate KCNQ channel function in cultured vascular smooth muscle cells, if these channels were present in mesenteric arteries they might represent a novel therapeutic target for blood pressure regulation or for treatment of cardiovascular diseases in which AVP levels are altered.

In the present study we extend our previous findings by evaluating the expression and functional contribution of native mesenteric artery myocyte KCNQ channels. Our results indicate that the vasoconstrictor actions of vasopressin involve PKC-dependent suppression of mesenteric artery KCNQ currents. We also present evidence that isolated mesenteric arteries are sensitive to clinically used KCNQ channel blockers and activators (linopirdine and flupirtine, respectively), and that systemic administration of these KCNQ channel modulators influences mesenteric vascular resistance and systemic blood pressure in vivo.
Methods

All studies involving animal use were approved by the Institutional Animal Care and Use Committee of Loyola University Medical Center.

**RT PCR:** Primers for KCNQ mRNAs were adapted from Ohya et al. (KCNQ1-3, KCNQ5 (Ohya et al., 2002)) and Yeung et al. (KCNQ4 (Yeung et al., 2007)) to correspond to the rat sequences. Preparation of RNA from freshly isolated mesenteric artery smooth muscle cells (MASMCs) (selected individually based on morphology) and PCR procedures have been published previously (Brueggemann et al., 2006; Brueggemann et al., 2007). For rat brain positive controls and MASMC RNA, a second round of PCR was carried out using the same primers and 2 µl of PCR product. PCR products were confirmed for each primer pair by DNA sequencing. For KCNQ4, the second round of PCR was performed using a nested forward primer with the same reverse primer. Minus-RT controls using the same reaction conditions with MASMC RNA were negative for all KCNQ primer pairs (not shown). PCR analysis of three different MASMC preparations produced similar results.

**Isolation of Myocytes and Patch Clamp Recording:** Segments of mesenteric artery were prepared as described previously (Henderson and Byron, 2007) and subjected to enzymatic digestion for isolation of MASMCs as described by Berra-Romani et al. (Berra-Romani et al., 2005): MASMCs were kept on ice until use. For use, the cells were dispensed onto a glass coverslip base of the recording chamber and allowed to adhere for at least 15 min at room temperature. Methods for recording isolated $K_v$ currents in MASMCs were adapted from previous A7r5 cell studies (Brueggemann et al., 2007) and are described in detail in the Supplemental Materials.

**Pressure Myography:** Methods used for isolated artery pressure myography have been described previously (Henderson and Byron, 2007).
In Vivo Cardiovascular Experiments: Adult male Sprague-Dawley rats were anesthetized with thiobutabarbital (100mg/kg i.p). Following catheterization (unilateral femoral arterial and venous catheters for measurement of arterial pressure and drug injections), a blood flow probe (Transonic Systems Inc, Ithaca NY) was placed around the superior mesenteric artery through a mid-line laparotomy. Basal blood pressure/blood flow values were recorded (30 min) prior to drug administration. Vehicle for linopirdine and flupirtine was a 1:1 mixture of polyethylene glycol-400 (PEG-400) and physiological saline. Vehicle responses (same sequence of infusion volumes as used for drug administration) were measured in each rat prior to administering the test drug. Each dose was administered over 5 seconds at 5 minute intervals. Parameters measured during the last 120 seconds of each 5 minute time period were averaged for the linopirdine responses whereas peak values were averaged for flupirtine because of the more transient nature of the latter responses.

Statistics: SigmaStat (SysStat Software Inc) was used for all statistical analyses. Paired Student’s t-test was used for comparisons of parameters measured before and after treatments. Comparisons among multiple treatment groups were evaluated by analysis of variance (ANOVA) followed by a Holm-Sidak post hoc test. Cumulative concentration-response data were analyzed by repeated measures ANOVA and post hoc Holm-Sidak. Comparisons of constrictor responses to 10 nM AVP in the presence or absence of 10 µM linopirdine were evaluated using a Mann-Whitney Rank Sum Test. Differences associated with p values < 0.05 were considered statistically significant.

Materials: Linopirdine, flupirtine, glibenclamide, iberiotoxin, tetraethylammonium chloride, tetrodotoxin, collagenase, elastase, [Arg⁸]-vasopressin and verapamil were from Sigma-Aldrich (St. Louis, MO, USA). Calphostin C was from Biomol (Plymouth Meeting, PA, USA). 4β-
phorbol 12-myristate 13-acetate and amphotericin B were from Calbiochem (San Diego, CA, USA). XE-991 was from TOCRIS (Ellisville, MO, USA).
Results

Using reverse transcriptase polymerase chain reaction (RT-PCR) to evaluate gene expression of KCNQ channels in isolated rat mesenteric artery smooth muscle cells (MASMCs), we found that of the 5 known KCNQ channel subtypes, messages for KCNQ1, KCNQ4, and KCNQ5 were detected (Fig. 1).

To evaluate the function of KCNQ channels in MASMCs, KCNQ currents were assessed using perforated patch whole cell voltage clamp techniques. As shown in Figure 2A, a relatively small outwardly rectifying K_α current was measured during the last 500 ms of 5 s voltage steps from a -4 mV holding voltage. The voltage-dependence of activation was well fit by a single Boltzmann distribution (V_{0.5} of -34.3 mV and slope factor of +6.5 mV; Fig. 3C). The selective KCNQ channel activator flupirtine (10 µM) enhanced the currents approximately 2-fold (mean current measured at -20 mV, Fig. 2A,B,D). Treatment with selective KCNQ channel blockers linopirdine (10 µM) or XE-991 (10 µM), almost completely abolished the isolated currents at all voltages negative to -15 mV (Figs. 2 and 3). Pharmacological studies using inhibitors of other classes of vascular K^+ channels confirmed that the KCNQ currents are effectively isolated from other K^+ currents under our recording conditions (2C,D).

Treatment of freshly isolated MASMCs with a physiological concentration of AVP (100 pM) led to a reduction of the KCNQ current amplitude by ~50% (Fig. 3A) without a significant shift in the conductance-voltage curve (not shown). Subsequent addition of the KCNQ channel activator flupirtine (10 µM) completely restored the currents. Much larger voltage-sensitive K^+ currents can be detected using a different voltage step protocol and omitting GdCl_3 from the external solution (Fig. 3B). These currents were activated at more positive voltages (V_{0.5} = +5.1 mV, Fig. 3C) than KCNQ currents and were inhibited by 4-aminopyridine (4-AP, 2 mM), but
were not sensitive to treatment with 100 pM AVP (Fig. 3B) or 10 µM linopirdine (Supplemental Fig. 1A).

Pretreatment of MASMCs with a selective PKC inhibitor, calphostin C (250 nM), prevented the suppression of KCNQ currents by 100 pM AVP (Fig. 3D). There was no significant difference in resting current density between untreated and calphostin C-treated MASMCs. Direct activation of PKC with phorbol-12-myristate-13-acetate (PMA, 1 nM) was sufficient to significantly suppress KCNQ currents (Fig. 3E). This effect was manifested as both a significant decrease in current amplitude (by 70 ± 7% at -20 mV) and a significant positive shift of the conductance-voltage curve (by 7 ± 2 mV, Fig. 3F).

If KCNQ channels are active in resting MASMCs then suppression of KCNQ currents is expected to induce membrane depolarization. We measured membrane voltage in isolated myocytes using whole cell current clamp. AVP (100 pM) significantly depolarized the membrane of freshly isolated MASMCs from an average resting voltage of -61.5 ± 2.4 mV to -38.4 ± 5.2 mV (Fig. 4A,C). Treatment with linopirdine (10 µM) also significantly depolarized the resting membrane of MASMCs to -38.5 ± 3.9 mV, but this effect was not significantly enhanced by subsequent addition of 100 pM AVP (Fig. 4B,C).

Results presented above indicate that KCNQ channels are present and functional in freshly isolated MASMCs and that treatment with a physiological concentration of AVP (100 pM) leads to suppression of KCNQ currents and membrane depolarization. To investigate the physiological relevance of these responses, we measured constriction/dilation of isolated mesenteric arterial segments (MAs) maintained at 37°C and pressurized to a physiological pressure of 80 mmHg (Fenger-Gron et al., 1995). The KCNQ channel blocker linopirdine (10 µM) caused significant constriction of MAs (Fig. 5B) compared to vehicle control
(dimethylsulfoxide (DMSO)), which had no effect (Fig. 5A). Doubling the linopirdine concentration to 20 µM did not induce greater constriction (35 ± 3% of maximal constriction compared with 35 ± 2% induced by 10 µM linopirdine, n=6 for each, Fig. 5C).

The maximal response to linopirdine (~35% constriction) was achieved at a concentration of 10 µM and we have previously shown that 30 pM AVP induces a similar degree of constriction of rat MAs (Henderson and Byron, 2007). As shown in Fig. 5A-C, the constrictions induced by 30 pM AVP and 10 µM linopirdine were not additive. The inability of 30 pM AVP to significantly augment the constrictor responses to 10 µM linopirdine was not due to a limitation of L-type Ca²⁺ channel availability, because 60 mM external K⁺ elicited a maximal constrictor response when applied in the presence of linopirdine (Supplemental Fig. 2A,B).

If physiological concentrations of AVP constrict MAs via suppression of KCNQ currents, then the KCNQ channel activator flupirtine, which we found could reverse the AVP-induced suppression of KCNQ currents in isolated myocytes (Fig. 3A), would be expected to reverse the constrictor effects of AVP. This prediction was borne out by results shown in Fig. 5D demonstrating that flupirtine reproducibly exerted a concentration-dependent vasodilatory effect on MAs pre-constricted with 30 pM AVP.

A supraphysiological concentration of AVP (10 nM) induced a maximal constrictor response (i.e. complete occlusion of the vessel lumen) regardless of the absence or presence of 10 µM linopirdine (outer vessel diameters were 156 ± 11 µm vs. 158 ± 5 µm, respectively, p = 0.589, Mann-Whitney Rank Sum Test). This is consistent with our previous finding that the acute constrictor responses of MAs to 10 nM AVP involve different signal transduction
mechanisms when compared to responses activated by 30 pM AVP (Henderson and Byron, 2007).

The L-type Ca$^{2+}$ channel blocker verapamil (10 µM) completely abolished constrictor responses of mesenteric arteries to 10 µM linopirdine and 30 pM AVP, but had no impact on the maximal constriction induced by supraphysiological AVP (10 nM; Fig. 6A,B). Pretreatment of mesenteric arteries with 250 nM calphostin C, which was previously shown to prevent MA constriction in response to 10 nM PMA or 30 pM AVP (Henderson and Byron, 2007), did not significantly affect the constrictor responses to 10 µM linopirdine (Fig. 6C,D). In agreement with our previous report (Henderson and Byron, 2007), acute responses to 10 nM AVP were not altered by calphostin C treatment.

Our voltage clamp results (Figs. 2-3) distinguish linopirdine-sensitive K$_v$ currents (detected at voltages negative to -40 mV and suppressed in response to physiological concentrations of AVP) from 4-AP-sensitive K$_v$ currents (activated at more positive voltages and not inhibited by 100 pM AVP). In pressurized MAs, we found that 2 mM 4-AP was ineffective as a vasoconstrictor, but the same arteries constricted robustly when, in the continued presence of 4-AP, the KCNQ channel blocker linopirdine was added (Supplemental Fig. 1B,C). Arteries constricted more rapidly (Supplemental Fig. 1D,E), and to a significantly greater extent (approximately 10% greater constriction) when linopirdine was added in the presence of 4-AP (Supplemental Fig. 1C).

To assess the role of KCNQ channels in mesenteric vascular resistance in vivo, we measured superior mesenteric artery blood flow and systemic blood pressure in anesthetized rats. Mesenteric vascular resistance (MVR) was determined by dividing perfusion pressure (estimated as mean arterial blood pressure (MAP)) by superior mesenteric artery blood flow. As seen in
Figure 7, linopirdine (0.01 – 3 mg/kg i.v.) produced a concentration-dependent increase in both MAP and MVR. Conversely, the KCNQ channel activator flupirtine (0.01 – 3 mg/kg i.v.) produced a dose-dependent decrease in both MAP and MVR. Flupirtine also induced a modest dose-dependent decrease in heart rate, whereas linopirdine failed to significantly alter heart rate.
Discussion

Mesenteric arteries are highly sensitive to the vasoconstrictor actions of AVP and are important targets for its pressor effects (Altura, 1975; Banks et al., 1985). Our results suggest that 1) KCNQ channels expressed in mesenteric artery myocytes are active at resting membrane potentials and are negatively regulated by physiological concentrations of AVP; 2) suppression of KCNQ currents is sufficient to induce membrane depolarization and L-type Ca\textsuperscript{2+} channel-dependent arterial constriction; 3) regulation of KCNQ channels is downstream of PKC activation in the signaling cascade activated by AVP; and 4) the effects of KCNQ channel modulation in arterial myocytes are apparent at the level of the single cell, isolated artery, and intact vasculature of a live animal. These findings are significant for the elucidation of the biochemical mechanisms by which physiological concentrations of AVP exert their pressor effects, and also because they implicate KCNQ channels, which until very recently were not considered among the cohort of vascular potassium channels.

Other classes of Kv channels have been postulated to play important roles in vasoconstrictor actions. Several Kv1-Kv4 subtypes, which are inhibited to varying extents by 4-aminopyridine (4-AP) (Xu et al., 1999; Cox, 2005), have been implicated as mediators of VSMC Kv currents. Evidence suggests that inhibition of 4-AP-sensitive Kv currents by circulating vasoconstrictor hormones may contribute to vasoconstrictor actions (Nelson and Quayle, 1995; Cole et al., 1996; Jackson, 2005), but the link between inhibition of these currents and activation of L-type Ca\textsuperscript{2+} channels is not clearly established.

The threshold for voltage-dependent activation of L-type Ca\textsuperscript{2+} channels and 4-AP-sensitive Kv channels in VSMCs is positive to -40 mV (Rubart et al., 1996; Xu et al., 1999). However, \( V_m \) measured in VSMCs in arteries subjected to normal intravascular pressures in vitro
or from arteries in vivo are more negative, generally between -40 and -60 mV (Nelson and Quayle, 1995). 4-AP-sensitive currents were undetectable in MASMCs at voltages negative to ~-30 mV (Fig. 3B,C), making them unlikely to contribute appreciably at the resting $V_m$ (-61.5 ± 2.4 mV) of isolated MASMCs. Their insensitivity to 100 pM AVP further suggests that suppression of these 4-AP-sensitive currents cannot mediate the membrane depolarization required for the vasoconstrictor responses to physiological concentrations of AVP. On the other hand, KCNQ channels are active at voltages negative to -40 mV and might therefore be effective targets for vasoconstrictor signaling.

In addition to voltage-activated $K^+$ ($K_v$) channels, $Ca^{2+}$-activated ($K_{Ca}$), inward rectifier ($K_{IR}$) and ATP-sensitive ($K_{ATP}$) $K^+$ channels are also known to be expressed in VSMCs (Nelson and Quayle, 1995). Relevant to our findings, AVP has been shown to negatively regulate $K_{ATP}$ channel activity (Martin et al., 1989; Wakatsuki et al., 1992; Shi et al., 2007). This effect has been measured experimentally in VSMCs when $K_{ATP}$ channels are pre-activated pharmacologically, and has been posited as a potential mechanism by which AVP raises blood pressure in patients with prolonged vasodilatory shock (Holmes et al., 2001; Barrett et al., 2007). Importantly, the concentrations of AVP needed to detectably inhibit $K_{ATP}$ currents in these experimental systems (Martin et al., 1989; Wakatsuki et al., 1992; Shi et al., 2007) exceed, by at least an order of magnitude, the plasma concentrations of AVP achieved with effective clinical therapy (approximately 100 pM; (Holmes et al., 2001)). Our finding that glibenclamide (a $K_{ATP}$ channel blocker) did not affect AVP-sensitive $K_v$ currents in MASMCs (Fig. 2C,D) and did not induce constriction of rat MAs (Supplemental Figure 2C,D), suggests that $K_{ATP}$ channels do not contribute significantly to the AVP effect in MASMCs from healthy rats (in agreement with
previous studies of rat arteries (Dumont and Lamontagne, 1995; Sanz et al., 2003). Instead, we attribute the effects of physiological [AVP] to the suppression of KCNQ channel activity.

Different vascular beds express different complements of the five known KCNQ subtypes. KCNQ1 and KCNQ3 genes were previously found to be expressed in vascular smooth muscle of murine portal vein and rat pulmonary arteries (Ohya et al., 2003; Yeung and Greenwood, 2005; Joshi et al., 2006) and a more quantitative analysis recently revealed KCNQ4 and KCNQ5 to be the most abundantly expressed subtypes in mouse carotid and femoral arteries (Yeung et al., 2007). We previously reported that rat aortic smooth muscle cells express KCNQ1 and KCNQ5 (Brueggemann et al., 2007), and determined in the present study that KCNQ1, KCNQ4 and KCNQ5 are expressed in rat MASMCs (Fig. 1). Although each of the five KCNQ subtypes can form functional Kv channels, it is unknown whether vascular KCNQ channels form homo- or heteromeric tetramers of the individual gene products (Jentsch, 2000; Schwake et al., 2003). It also remains to be determined whether the heterogeneity of expression patterns among different vascular beds is related to differences in resting tone, autoregulatory mechanisms, or in reactivity to vasoactive agents.

KCNQ channel blockers (linopirdine and XE-991) were shown to constrict both rat and mouse intrapulmonary arteries (Joshi et al., 2006), but were without effect on mesenteric arteries from the same animals. The reasons for this discrepancy with our current findings are not immediately apparent. One notable difference was that Joshi et al. measured constriction using a wire myograph as opposed to the pressurized artery preparation used in our experiments. Constrictor responses of pulmonary arteries to linopirdine were similar to the responses we observed with pressurized mesenteric arteries: maximal responses were obtained with 10 µM
linopirdine and these responses were abolished by a blocker of L-type Ca\(^{2+}\) channels (nifedipine, 1 µM) (Joshi et al., 2006).

Our *in vivo* studies suggest that vascular KCNQ channels contribute to MVR, in agreement with our in vitro electrophysiological and functional studies. Dose-dependent increases in MAP and MVR in response to the KCNQ channel blocker linopirdine may be expected considering our in vitro results demonstrating that linopirdine depolarizes the membrane of isolated MASMCs and constricts mesenteric arteries. The opposite effects of flupirtine on both MAP and MVR are in accordance with its hyperpolarizing influence on MASMCs and its concentration-dependent vasodilatory effects on mesenteric arteries (Fig 5D). A modest decrease in heart rate in response to flupirtine may be a consequence of non-vascular KCNQ channel activation (e.g. a reduction of sympathetic ganglionic nerve activity). In contrast, linopirdine did not affect heart rate and the in vitro constriction of artery segments in response to linopirdine was not affected by treatment with the neuronal voltage-sensitive Na\(^{+}\) channel blocker tetrodotoxin (100 nM, results not shown). Although we cannot rule out some contribution of non-vascular KCNQ channels to the systemic effects of linopirdine or flupirtine, our findings are consistent with our hypothesis that modulation of vascular KCNQ channel activity (by physiological agonists or pharmacological agents) will affect mesenteric vascular resistance and systemic blood pressure in vivo. These findings suggest that direct pharmacological modulation of vascular KCNQ channels may prove useful in clinical settings where acute blood pressure regulation is required.

KCNQ channels are targeted by neurotransmitters to regulate neuronal excitation, but signal transduction pathways for the regulation of neuronal versus vascular KCNQ channels may be different. Suppression of KCNQ channel-mediated neuronal M-currents in response to
receptor-mediated signal transduction is not generally considered to involve PKC (Bosma and Hille, 1989; Cruzblanca et al., 1998; Ma et al., 2006). Instead, evidence favors hydrolysis of PIP2 as an essential mechanism (Delmas and Brown, 2005). This mechanism is robustly activated during cholinergic synaptic transmission, where release of acetylcholine results in very high local concentrations that are likely to saturate muscarinic receptors and maximally activate PLC to deplete PIP2. Bradykinin-induced suppression of neuronal M-currents may involve PLC-mediated inositol trisphosphate formation and release of intracellular Ca2+ stores, but this mechanism has also only been observed at very high agonist concentrations (Cruzblanca et al., 1998; Brown et al., 2007). In contrast, the endocrine actions of AVP are evident at very low concentrations that will occupy only a small fraction of the V1a vasopressin receptors. These concentrations are at least an order of magnitude below the EC50 for AVP-stimulated inositol trisphosphate formation or release of intracellular Ca2+ stores (Doyle and Ruegg, 1985; Byron, 1996). We speculate that physiological vasoconstrictor concentrations of AVP will not induce significant depletion of PIP2, and that the observed suppression of KCNQ channel-mediated Kv currents at these low concentrations of AVP must be attributed to other mechanisms (e.g. PKC-mediated channel phosphorylation (Hoshi et al., 2003; Nakajo and Kubo, 2005; Surti et al., 2005)). Our findings (Fig. 3D,E) suggest that PKC activation is both sufficient to reduce KCNQ currents in MASMCs and necessary for KCNQ current suppression by physiological concentrations of AVP.

In summary, we have demonstrated expression of KCNQ channels in rat MASMCs and revealed non-inactivating Kv currents that contribute to resting Vm and exhibit pharmacological and electrophysiological characteristics of KCNQ currents. These vascular KCNQ currents display an exquisite sensitivity to AVP at concentrations that are both physiologically and
clinically relevant for regulation of MVR and systemic blood pressure. We propose a novel physiological mechanism for AVP-induced constriction of mesenteric arteries that involves PKC-dependent suppression of KCNQ channel activity at resting voltages, and results in membrane depolarization, activation of L-type Ca\(^{2+}\) channels, and myocyte contraction (Fig. 8). We also demonstrate \textit{in vivo} effects of KCNQ channel modulators on systemic blood pressure and MVR that may have important implications for the clinical use of these drugs.
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Yeung SY, Pucovsky V, Moffatt JD, Saldanha L, Schwake M, Ohya S and Greenwood IA

Footnotes

1. This work was supported by the National Heart Lung & Blood Institute (R01 HL070670 to KLB) and the American Heart Association (0715618Z to ARM).

2. The chemical structures for the KCNQ channel modulators (linopirdine, XE-991 and flupirtine) are provided in the following reference. Munro, G. and W. Dalby-Brown, K(\(v\))7 (KCNQ) channel modulators and neuropathic pain. J Med Chem., 2007. 50(11): 2576-82.
Legends for Figures:

**Figure 1 | KCNQ channel expression in MASMCs**

Total RNA prepared from rat mesenteric artery smooth muscle cells (MASMCs) or adult rat brain (as a positive control) was reverse transcribed and subjected to PCR using primers specific for rat KCNQ1 through KCNQ5. Molecular weight marker (M) is a 100-bp ladder (New England Biolabs); 500 bp is indicated to the left of each panel. Expected sizes of each reaction product are: KCNQ1, 453 bp; KCNQ2, 372 bp; KCNQ3, 424 bp; KCNQ4, 359 bp; KCNQ5, 240 bp.

**Figure 2 | KCNQ currents in MASMCs**

A. Representative current traces recorded from a cluster of 4-5 MASMCs (capacitance = 208.5 pF): control (untreated, left panel), 10 µM flupirtine (middle panel), 10 µM XE-991 (right panel). B. Summarized I/V relationship from 6-8 recordings from cells treated as in panel A. Currents measured at all membrane potentials between -34 mV to +1 mV following XE-991 and flupirtine treatment are significantly different from control treatment (p<0.05, one-way repeated measures ANOVA, post hoc Holm-Sidak method). C. Summarized I/V relationships recorded in the presence of conventional VSMC K⁺ channel blockers (at the concentrations indicated on the figure; 10 min treatment followed by a 10 min washout) and after subsequent exposure for 10 min to 10 µM linopirdine. Currents measured at all membrane potentials positive to -34 mV following linopirdine treatment are significantly different from control treatment (n=4, p<0.05, one-way repeated measures ANOVA, post hoc Holm-Sidak method). D. Mean currents recorded at -20 mV normalized to control currents (CTL). Numbers below treatment indicate n for each group. *** indicates significant difference from control, p<0.001, based on one-way ANOVA and Holm Sidak post hoc analysis.
Figure 3 | AVP suppresses KCNQ currents in a PKC-dependent manner but does not influence 4-AP sensitive Kᵥ currents.

A. Summarized I/V relationships for KCNQ currents recorded from MASMCs. The currents are decreased following treatment for 15 min with 100 pM AVP. This effect is reversed by 10 min treatment with 10 µM flupirtine. Subsequent exposure to 10 µM linopirdine for 10 min significantly reduces the current. Currents measured at all membrane potentials between -34 mV to +1 mV following AVP and linopirdine treatment are significantly different from control and flupirtine treatment (n=4, p<0.05, one-way repeated measures ANOVA, post hoc Holm-Sidak method).

B. 4-AP-sensitive Kᵥ currents were recorded as described in the Supplemental Materials. Treatment for 15 min with 100 pM AVP had no significant effect, whereas the currents were significantly reduced following 10 min treatment with 2 mM 4-AP (n=3).

C. Steady-state activation curves fitted by single Boltzmann functions for KCNQ currents and 4-AP-sensitive Kᵥ currents.

D. Summarized I/V relationships for KCNQ currents in MASMCs pretreated with 250 nM calphostin C (PKC inhibitor) for 1 hour at room temperature. Pretreatment with calphostin C completely abolished the negative regulation by 100 pM AVP. However, subsequent application of 10 µM flupirtine and 10 µM XE-991 enhanced and suppressed KCNQ currents, respectively (n=4).

E. Summarized I/V relationships for KCNQ currents normalized to control currents measured at -20 mV. Application of 1 nM PMA for 15 min significantly reduced KCNQ current at all voltages positive to -39 mV (n=4, paired Student’s t-test, p<0.05). Subsequent application of 10 µM linopirdine suppressed the remaining current.

F. Steady-state activation curves fitted by single Boltzmann functions. Treatment with 1 nM PMA induced a significant positive shift of the activation curve (V₀.5 = -27.5 ± 1.7 mV vs. -
20.8 ± 0.3 mV for control and 1 nM PMA treatment, respectively, n=4, p<0.05 using paired Student’s t-test).

**Figure 4 | Membrane depolarization by AVP and the KCNQ channel blocker linopirdine.**

A. Representative time-course of membrane depolarization in response to 100 pM AVP recorded from a single MASMC. B. Representative time-course of membrane depolarization in response to 10 µM linopirdine followed by additional application of 100 pM AVP. C. Average membrane voltage values in MASMCs measured before treatment and after stabilization in the presence of 100 pM AVP, 10 µM linopirdine, or both 10 µM linopirdine and 100 pM AVP. All treatments were significantly different from control (p<0.001), but differences among treatment groups were not statistically significant.

**Figure 5 | Constriction of mesenteric arteries: effects of KCNQ channels blockers or activators.**

A,B. Representative recordings of MA constriction in response to 30 pM AVP and 10 nM AVP in an artery pretreated with dimethylsulfoxide (DMSO, vehicle control; (A), or KCNQ channel blocker linopirdine (10 µM), followed by addition of 30 pM AVP and then 10 nM AVP (B). C. Summarized results (mean ± SE) expressed as percent of maximal constriction. Significant constriction in response to 10 or 20 µM linopirdine is not significantly augmented by 30 pM AVP. Total occlusion of the vessel lumen (100 % maximal constriction) was induced by 10 nM AVP under all conditions. DMSO alone (0 µM linopirdine) failed to induce any detectable constriction such that the error bars are not visible. D. Concentration-dependent vasodilation in response to the KCNQ channel activator flupirtine (10-40 µM) following pre-constriction of...
MAs with 30 pM AVP. Inset: mean concentration-response data expressed as percent of maximal dilation (n=3).

**Figure 6 | Constriction of mesenteric arteries by linopirdine depends on L-type Ca\(^{2+}\) channels but not protein kinase C activation.**

A. A representative recording of mesenteric arterial constriction in response to the sequential addition of the L-type Ca\(^{2+}\) channel blocker verapamil (10 µM, 20 min), 10 µM linopirdine, 30 pM AVP and 10 nM AVP. B. Summarized results from MAs treated with or without the L-type Ca\(^{2+}\) channel blocker verapamil (10 µM) followed by additions of linopirdine (10 µM), 30 pM AVP, and 10 nM AVP. *** indicates a significant difference between treated and untreated groups (p<0.001). Full arterial occlusion was induced by 10 nM AVP in the presence and absence of verapamil. C. A representative recording of mesenteric arterial constriction in response to the sequential addition of the PKC inhibitor calphostin C (250 nM, 30 min), 10 µM linopirdine, 30 pM AVP and 10 nM AVP. D. Summarized results from MAs pretreated with or without the PKC inhibitor calphostin C (250 nM) followed by exposures to 10 µM linopirdine, 30 pM AVP, and 10 nM AVP. Responses to linopirdine with or without 30 pM or 10 nM AVP were not significantly affected by calphostin C (p>0.1 for each). Statistical comparisons are based on Student’s t-test.

**Figure 7 | Effects of KCNQ channel modulators on blood pressure, mesenteric vascular resistance and heart rate measured in vivo.**

Dose-dependent MAP, MVR and HR responses (expressed as a percentage of basal value ± SE) to: A, linopirdine (open symbols, n=3) and B, flupirtine (open symbols, n=3). Effects of injecting
vehicle alone are shown for comparison (closed symbols, n=6). Average pretreatment values for each variable were as follows; MAP: 90.3 ± 2.7 mmHg, MVR: 7.8 ± 0.8 mmHg·min·ml⁻¹, HR: 276.3 ± 7.3 beats/min. * indicates significant differences from control, p<0.05 using a two-way repeated measures ANOVA and Holm-Sidak post hoc test.

**Figure 8 | Working model for AVP-induced MA constriction.**

Coordinated regulation of three types of voltage-gated ion channels in MASMCs determines contractile responses to physiological concentrations of AVP. Our working model attempts to explain our present findings, along with our previous observation that constrictor responses of MAs to physiological concentrations of AVP are prevented by pretreatment with PKC inhibitors or by treatment with verapamil, a blocker of L-type Ca²⁺ channels (Henderson and Byron, 2007). We propose that AVP induces PKC-dependent suppression of KCNQ channel activity at resting membrane voltages (typically negative to -50 mV), resulting in membrane depolarization (ΔV_m) and activation of L-type Ca²⁺ channels. Ca²⁺ influx via L-type channels then provides the contractile stimulus for the vasoconstrictor effects of AVP. 4-AP-sensitive Kᵥ channels will also activate at membrane voltages positive to -30 mV, and their activity will tend to limit Ca²⁺ influx and oppose constriction (see Supplementary Fig. 1). Our finding that verapamil, but not calphostin C, abolished constrictor responses to linopirdine supports our working model and suggests that by blocking KCNQ channels directly, linopirdine by-passes the PKC-dependent signal transduction step.
Figure 1

KCNQ Subtype

Rat Brain

MASMC
Figure 2

A

Control

10 µM Flupirtine

10 µM XE-991

B

I (pA/pF) vs. V (mV)

Control (n=8)

10 µM Flupirtine (n=8)

10 µM XE-991 (n=6)

C

I/Ic at -20 mV

CTL

IbTx

Glib

TEA

4-AP

Lino

XE

Flup

D

Graph showing I/Ic at -20 mV with various treatments:

CTL

IbTx

Glib

TEA

4-AP

Lino

XE

Flup
Figure 3
Figure 5

A

B

C

D

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Figure 7
Figure 8

AVP

PKC

KCNQ Currents

\[ \Delta V \]

\[ \Delta V_{mm} \]

4-AP-sensitive K\textsubscript{i} currents

L-type Ca\textsuperscript{2+} currents

↑Ca\textsuperscript{2+} influx

Contraction