Kv7.5 Potassium Channel Subunits Are the Primary Targets for PKA-Dependent Enhancement of Vascular Smooth Muscle Kv7 Currents

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ABSTRACT

Kv7 (KCNQ) channels, formed as homo- or heterotetramers of Kv7.4 and Kv7.5 α-subunits, are important regulators of vascular smooth muscle cell (VSMC) membrane voltage. Recent studies demonstrate that direct pharmacological modulation of VSMC Kv7 channel activity can influence blood vessel contractility and diameter. However, the physiologic regulation of Kv7 channel activity is still poorly understood. Here, we study the effect of cAMP/protein kinase A (PKA) activation on whole cell K⁺ currents through endogenous Kv7.5 channels in A7r5 rat aortic smooth muscle cells or through Kv7.4/Kv7.5 heteromeric channels natively expressed in rat mesenteric artery smooth muscle cells. The contributions of specific α-subunits are further dissected using exogenously expressed human Kv7.4 and Kv7.5 homo- or heterotetrameric channels in A7r5 cells. Stimulation of Gαs-coupled β-adrenergic receptors with isoproterenol induced PKA-dependent activation of endogenous Kv7.5 currents in A7r5 cells. The receptor-mediated enhancement of Kv7.5 currents was mimicked by pharmacological agents that increase [cAMP] (forskolin, rolipram, 3-isobutyl-1-methylxanthine, and papaverine) or mimic cAMP (8-bromo-cAMP); the 2- to 4-fold PKA-dependent enhancement of currents was also observed with exogenously expressed Kv7.5 channels. In contrast, exogenously-expressed heterotetrameric Kv7.4/7.5 channels in A7r5 cells or native mesenteric artery smooth muscle Kv7.4/7.5 channels were only modestly enhanced, and homo-tetrameric Kv7.4 channels were insensitive to this regulatory pathway. Correspondingly, proximity ligation assays indicated that isoproterenol induced PKA-dependent phosphorylation of exogenously expressed Kv7.5 channel subunits, but not of Kv7.4 subunits. These results suggest that signal transduction-mediated responsiveness of vascular smooth muscle Kv7 channel subunits to cAMP/PKA activation follows the order of Kv7.5 >> Kv7.4/Kv7.5 > Kv7.4.

Introduction

Voltage-dependent Kv7 potassium channels, encoded by KCNQ genes, are involved in the regulation of cell excitability (Jentsch, 2000; Robbins, 2001). There are five known members of the KCNQ gene family (KCNQ1–KCNQ5), which are expressed in a variety of excitable cells, ranging from neurons (mainly expressing KCNQ2–KCNQ5) and cardiac myocytes (mainly expressing KCNQ1), to smooth muscle cells of different origins (mainly expressing KCNQ1, KCNQ4, and KCNQ5) (Simons et al., 1985; Barhanin et al., 1996; Wang et al., 1998; Kubisch et al., 1999; Lerche et al., 2000; Schroeder et al., 2000; Ohya et al., 2002; Yeung et al., 2007; Mackie et al., 2008; Jepps et al., 2009; McCallum et al., 2009; Brueggemann et al., 2011, 2012; Svala et al., 2013). The KCNQ1–KCNQ5 gene products (Kv7.1–Kv7.5 proteins) assemble as homo- or heterotetramers to form functional channels (Schwake et al., 2003).

As modulators of cell excitability, Kv7 channels are tightly regulated. Regulatory mechanisms can control the activity of the channels acutely on a rapid time scale (seconds to minutes). Acute suppression of Kv7 channel activity increases cell excitability, whereas augmentation of the Kv7 channel activity decreases excitability (Wickenden, 2002; Brown, 2008). For example, the excitability of neurons is increased when Kv7 channel–mediated neuronal M-currents are suppressed upon Gαs-coupled receptor activation. The mechanisms by which receptor activation leads to suppression of neuronal M-currents include depletion of phosphatidylinositol 4,5-bisphosphate (PIP2), elevation of cytosolic Ca²⁺ concentration, and protein kinase C (PKC)–dependent phosphorylation of Kv7.2 channel subunits (Delmas and Brown, 2005). In cardiac myocytes, the amplitude of Kv7.1 channel-mediated slow delayed rectifier currents can be increased via the activation of the β-adrenergic receptor (βAR)/Gαs/cAMP/protein kinase A (PKA) pathway (Chen and Kass, 2011). PKA-dependent regulation of the native neuronal M-current
Materials and Methods

Constructs. The adenoviruses to express human KCNQ4 (Adv-hKCNQ4) and human KCNQ5 (Adv-hKCNQ5-FLAG) were created previously using the AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA) (Brueggemann et al., 2011).  

Cell Culture. A7r5 cells were cultured as described previously (Byron and Taylor, 1993). For overexpression studies, A7r5 cells, subcultured at 70% confluence, were infected with Adv-hKCNQ4 or Adv-hKCNQ5-FLAG or both at a multiplicity of infection of 100 and used for electrophysiological experiments 7–10 days after infection. Cells expressing the exogenous channels were identified based on detection of the fluorescence of GFP (coexpressed with the KCNQ products via the IRES-hrGFP element in the Stratagene AdEasy Adenoviral Vector System). To maintain endogenous βAR responsiveness, the cells were serum deprived for 1 to 2 days before use in patch-clamp experiments.

Isolation of Myocytes. All animal studies were approved by the Loyola University Chicago Institutional Animal Care and Use Committee and were conducted in accordance with the 1996 Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (Washington, DC). Adult male Sprague-Dawley rats were anesthetized by inhalation of isoflurane, and segments of small intestinal mesentery were surgically removed as described previously (Henderson and Byron, 2007). Methods for isolation of mesenteric artery smooth muscle cells (MASMCs) were described previously (Mackie et al., 2008). Freshly isolated MASMCs were kept on ice until use. The cells were then dispensed onto a glass coverslip base of the recording chamber and allowed to adhere for at least 15 minutes at room temperature.  

Patch-Clamp Electrophysiology. The whole cell perforated patch configuration was used to measure membrane currents under voltage-clamp conditions. All experiments were performed at room temperature with continuous perfusion of bath solution as described previously (Brueggemann et al., 2007, 2011; Mackie et al., 2008). The standard bath solution for A7r5 cells contained (in mM): 5 KCl, 130 NaCl, 10 HEPES, 2 CaCl2, 1.2 MgCl2, 5 D-glucose, pH 7.3. The standard internal (pipette) solution for A7r5 cells contained (in mM): 110 K-glutamate, 30 KCl, 5 HEPES, 1 K2EGTA, pH 7.2. Osmolality was adjusted to 268 mosmol/l with D-glucose. The standard bath solution for MASMCs contained (in mM): 140 NaCl, 5.36 KCl, 1.2 MgCl2, 2 CaCl2, 10 HEPES, 10 D-Glucose, pH 7.3, 298 mosmol/l. Standard internal (pipette) solution for MASMCs contained (in mM): 135 KCl, 5 NaCl, 10 HEPES, 0.05 K2EGTA, 1 MgCl2, 20 D-Glucose, pH 7.2, 298 mosmol/l. Amphotericin B (120 μg/ml) was used in the internal solution for membrane patch perforation. To isolate Kv7 currents, 100 μM GdCl3, sufficient to block L- and T-type Ca2+ channels and nonelectroactive cation channels, and to shift activation of 4-AP-sensitive Kv channels to more positive voltages (Mansi et al., 2011), was added to external solutions.

Voltage-clamp command voltages were generated using an Axopatch 200B amplifier under control of (Molecular Devices, Sunnyvale, CA) and EPC10 amplifier under control of PATCHMASTER software (HEKA, Pflaz, Germany). Series resistances after amphotericin perforation were 8–15 MΩ and were compensated by 60% in cells overexpressing Kv7 channels. Whole-cell currents were digitized at 2 or 5 kHz and filtered at 1 or 2.9 kHz, respectively.

The Kv7 currents through overexpressed hKv7 channels were recorded using a 5-second voltage step protocol from a −74 mV holding voltage to test voltages ranging from −114 to −4 mV, followed by a 1-second step to −120 mV. The currents recorded during the last 1 second of recording time for each voltage step were averaged and normalized by cell capacitance to obtain end-pulse steady-state Kv7 currents. Stable currents were recorded for at least 15 minutes prior to drug application. Time courses of drug effects were recorded by applying 5-second voltage steps to −20 mV every 15 seconds. To measure endogenous currents in A7r5 cells, a 5-second voltage step protocol was used (from a −74 mV holding voltage to test voltages ranging from −94 to +36 mV), followed by a 1-second step to −120 or −30 mV. To analyze the voltage dependence of channel activation the instantaneous tail current amplitude (estimated from an exponential fit of current deactivation measured at −120 mV) was converted to conductance according to the following equation: $G = \frac{I_{\text{tail}}}{\Delta V - E_g}$, where $I_{\text{tail}}$ is the instantaneous tail current amplitude, −120 mV is the tail current step potential, and $E_g$ is the reversal potential for potassium (−86 mV). Conductance plots in the absence (control) and in the presence of isoproterenol (1 μM) for each experiment were fitted to a Boltzmann distribution: $G = G_{\text{max}} / \left(1 + \exp \left(\frac{V - V_{50}}{k}\right)\right)$, where $G$ is conductance, $G_{\text{max}}$ is a maximal conductance, $V_{50}$ is the voltage of half-maximal activation, and $k$ is the slope factor. Deactivation kinetics were analyzed by applying single exponential fits to the tail currents recorded using a 5-second voltage step protocol (from a −74 mV holding potential to −20 mV), followed by 1-second repolarization to −120 mV.

The Kv7 currents in MASMCs were recorded by application of 5-second voltage steps from a −4 to −74 mV holding voltage to test voltages ranging from −94 to +36 mV, followed by a 1-second step to −120 mV. Time courses of drug effects were recorded during the last 1 second of recording time for each voltage step. The voltage protocol consisted of 1-second voltage steps from −94 mV to −120 mV, followed by a 1-second step to −120 mV.

Proximity Ligation Assays (PLAs). Duolink PLA assays (Sigma-Aldrich, St. Louis, MO) were performed essentially as described previously (Brueggemann et al., 2014c; Tripathi et al., 2015). A7r5 cells infected with Adv-hKCNQ4 or Adv-hKCNQ5-FLAG (Brueggemann et al., 2011) at a multiplicity of infection of 100 were plated on Permanox 8-well tissue culture slides (Nunc, Thermo Fisher Scientific, Waltham, MA) 7–10 days after infection. On the next day, cells were washed with control buffer (5.9 mM KCl, 135 mM NaCl, 10 mM HEPES, 1.5 mM CaCl2, 1.2 mM MgCl2, 1.15 mM glucose, pH 7.3) and treated with vehicle (control buffer) or 1 μM isoproterenol for 5 minutes (a subset of cells was pretreated with 1 μM PKA inhibitor...
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Materials. Cell culture media were from Gibco-BRL (Gaithersburg, MD) or MediaTech (Herndon, VA). Isoproterenol (4-[1-hydroxy-2-[(1-methylethylamino)ethyl]-1,2-benzenediol hydrochloride), forskolin (3R,3a,4a,5β,6,6a,7a,10a,10α,10β)-5-(Acetyl oxy)-3-ethenyldecahydro-6,10,10b-trihydroxy-3,4a,7,7a-pentamethyl-1H-naphtho[2,1-b]pyryran-1-one, 3-isobutyl-1-methylxanthine (IBMX), papaverine hydrochloride, (6,7-dimethoxy-1-veratrylisoquinoline hydrochloride), collagenase, elastase, and Duolink PLA assay reagents were from Sigma-Aldrich. XE991 (10,10-bis(4-pyridylmethyl)-9(1H)-antraechene) dihydrochloride was from Ascent Scientific (Princeton, NJ). Rolipram (4-[3-(Cyclopropylxoy)-4-methoxyphenyl]-2-pyrrolidinone), KT5720 (9R,10H,12S)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-dinoloid [1,2,3-fg-3′2′,1′-klipyr rolo[3′,4′,3,4,3,5]benzodiazocine-10-carboxylic acid, hexyl ester], ML213 (N-[2,4,6-trimethylphenyl]-bicyclo[2,2,1]heptane-2-carboxamide), and 8-bromo-cAMP sodium salt were from Tocris Bioscience (Bristol, United Kingdom). Amphotericin B was from Calbiochem (San Diego, CA). The vector pIRE2-EFPP was from Clontech (Mountain View, CA). The AdEasy Adenoviral Vector System was from Stratagene. The human KCNQ4 cDNA (accession number: AF105292, originally in the insect cell expression vector pMT) was a generous gift from Dr. I. Wood at the University of Leeds (Leeds, United Kingdom). The human KCNQ5 cDNA (accession number: AF209277, originally in the insect cell expression vector pMT) was a generous gift from Dr. T. Jentsch at the Max-Delbrück-Centrum for Molecular Medicine (Berlin, Germany).

Results.

We previously used the A7r5 embryonic rat aortic cell line as a model system to investigate the regulation of native Kv7.5 channels and as an expression system for functional vascular Kv7.4, Kv7.5, and Kv7.4/7.5 channels (Bruegge mann et al., 2007, 2011, 2014c). Evidence for the presence of functional native Kv7.5 channels in A7r5 cells as a sole source of conductance in the voltage range from −60 to +20 mV under the recording conditions used here were obtained previously based on reverse transcription polymerase chain reaction (Bruegge mann et al., 2007, 2011), pharmacology (Bruegge mann et al., 2007, 2011), molecular approaches using shRNA (Bruegge mann et al., 2007; Mani et al., 2009), and abolishment of the current upon expression of the dominant negative Kv7.5 subunit (L.I. Bruegge mann and K.L. Byron, unpublished results). A7r5 cells have also been reported to express endogenous βARs (Hirata et al., 1985). When we applied isoproterenol (1 μM, to activate endogenous βARs), it induced a 3-fold increase in the amplitude of endogenous Kv7.5 currents in serum-deprived A7r5 cells. The isoproterenol-induced increase in current amplitude was abolished by coapplication of a pan Kv7 channel blocker, XE991 (1 μM) (Fig. 1, A, B). The isoproterenol effect was also prevented by propranolol (10 μM, a βAR antagonist, or by pretreatment with a PKA inhibitor KT5720 (1 μM for 30 minutes) (Fig. 1E). Isoproterenol induced neither a shift in voltage dependence of activation nor a change in the slope of the conductance plot of native Kv7.5 channels (Fig. 1C; Table 1); however, it did slightly decrease the deactivation rate of endogenous Kv7.5 current measured at −120 mV, from 40.3 ± 0.9 to 49.7 ± 3.3 ms (P < 0.05, n = 4, paired Student’s t test) (Fig. 1D).

Application of a membrane permeant cAMP analog, 8-bromo cAMP (1 mM) also reversibly increased the amplitude of endogenous Kv7.5 currents by 2- to 3-fold in the voltage range from −54 to +36 mV (n = 4) (Fig. 2A). A similar reversible enhancement of endogenous Kv7.5 currents in A7r5 cells was observed upon activation of endogenous adenylyl cyclase with forskolin (1 μM; >2-fold increase in amplitude in the voltage range positive to −54 mV; n = 6) (Fig. 2B). It is worth noting that when the concentration of forskolin was increased from 1 to 10 μM, enhancement of endogenous Kv7.5 was reversed to almost complete inhibition (data not shown). When activation of adenylyl cyclase by forskolin (1 μM) was combined with inhibition of phosphodiesterases (PDEs) by addition of the nonsel ective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX, 500 μM), endogenous Kv7.5 currents were enhanced >3-fold in the voltage range from −54 to +36 mV (n = 4) (Fig. 2C). Another nonsel ective PDE inhibitor, papaverine, dose dependently enhanced endogenous

Statistics. Data are expressed as mean ± S.E. SigmaStat (Sigmat Software, Inc.) was used for all statistical analyses. The 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 followed by a Holm-Sidak post hoc test or analysis of variance on ranks followed by multiple comparisons versus control group (Dunn’s method). Differences associated with two-tailed P values < 0.05 were considered statistically significant.
Kv7.5 currents by ∼2- and ∼3-fold at doses of 10 and 100 μM, respectively, even in the absence of forskolin (Fig. 2D).

The more selective inhibitor of PDE4 (cAMP PDE isoform), rolipram, applied at increasing concentrations (1 nM–1 μM), dose dependently and reversibly enhanced endogenous Kv7.5 current in A7r5 cells, with an EC_{50} ~6 nM and maximal enhancement (2.1-fold ± 0.5-fold) achieved at 100 nM (Fig. 3, A and B). To test whether PDE4 inhibition by rolipram enhances endogenous Kv7.5 currents via activation of PKA, we used the PKA inhibitor KT5720 (1 μM). Pretreatment with
KT5720 (1 μM for 30 minutes) significantly inhibited the effect of 100 nM rolipram, relative to vehicle pretreatment (Fig. 3, C and D).

Freshly isolated rat MASMCs differ from A7r5 cells in that they express both Kv7.4 and Kv7.5 subunits, which predominantly form heteromeric channels (Brueggemann et al., 2011, 2014c), whereas A7r5 cells only express Kv7.5 (Brueggemann et al., 2007, 2011). We previously found that PKC-dependent regulation of heteromeric Kv7.4/7.5 channels in MASMCs differs from the regulation of homomeric Kv7.5 channels in A7r5 cells (Brueggemann et al., 2014c). In the present study, the same was found to be true for PKA-dependent regulation. Direct activation of βARs in MASMCs by isoproterenol (1 μM) did not significantly enhance endogenous Kv7 current (n = 5) (Fig. 4A). Similarly, rolipram (100 nM) also failed to enhance endogenous Kv7 current in MASMCs (Fig. 4B); a slight suppression of the current observed in the presence of rolipram was not statistically significant. Subsequent application of the nonselective Kv7.2–Kv7.5 activator ML213 (10 μM) (Yu et al., 2011; Brueggemann et al., 2014a) robustly enhanced the MASMC Kv7 currents and this effect was reversed by the selective Kv7 channel blocker XE991 (10 μM) (Fig. 4B), demonstrating the expected pharmacological characteristics of the Kv7 currents and confirming our ability to detect an increase in current amplitude in these cells. Only direct activation of adenylate cyclase with forskolin (1 μM) in combination with the nonselective PDE inhibitor IBMX (500 μM) induced a modest

### TABLE 1

<table>
<thead>
<tr>
<th>Kv7 isoform</th>
<th>$G_{\text{max}}$ (ISO)</th>
<th>$V_{\text{0.5}}$ (ISO)</th>
<th>$k$ (ISO)</th>
<th>$G_{\text{max}}$ (Control)</th>
<th>$V_{\text{0.5}}$ (Control)</th>
<th>$k$ (Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous Kv7.5, n = 10</td>
<td>259.3 ± 33.8a</td>
<td>−48.5 ± 2.5</td>
<td>9.5 ± 1.0</td>
<td>113.3 ± 0.8</td>
<td>12.3 ± 0.8</td>
<td>13.6 ± 1.7</td>
</tr>
<tr>
<td>Exogenous hKv7.5, n = 5</td>
<td>267.4 ± 16.6b</td>
<td>−46.2 ± 2.5</td>
<td>12.3 ± 0.8</td>
<td>13.6 ± 1.7</td>
<td>13.6 ± 1.7</td>
<td>13.6 ± 1.7</td>
</tr>
<tr>
<td>Exogenous hKv7.4, n = 5</td>
<td>98.1 ± 3.1</td>
<td>−28.6 ± 2.0</td>
<td>10.8 ± 0.7</td>
<td>16.7 ± 0.7</td>
<td>10.7 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Exogenous hKv7.4/7.5, n = 5</td>
<td>134.9 ± 9.3c</td>
<td>−34.0 ± 3.9</td>
<td>11.3 ± 0.8</td>
<td>121.9 ± 0.9</td>
<td>121.9 ± 0.9</td>
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</table>

$G_{\text{max}}$, maximal conductance; ISO, isoproterenol; $k$, slope factor; $V_{\text{0.5}}$, voltage of half-maximal activation.

*Significantly different from control, paired Student’s t test, P < 0.05.

**Significantly different from control, paired Student’s t test, P < 0.001.

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**Fig. 2.** Enhancement of endogenous Kv7.5 current in A7r5 cells by elevation of intracellular cAMP. (A) Current-voltage (I-V) relationships of endogenous Kv7.5 currents recorded in A7r5 cells before (control, solid circles), after 15 minutes of treatment with 1 mM 8-bromo cAMP (8Br-cAMP) (open circles), and after 10 minutes of washout (solid triangles). Currents were normalized to currents recorded at −20 mV before application of 8Br-cAMP [n = 4, * indicates significant difference from control, P < 0.05, one-way repeated-measures analysis of variance (ANOVA)]. (B) I-V relationships of endogenous Kv7.5 currents recorded in A7r5 cells before (control, solid circles), after 10 minutes of treatment with 1 μM forskolin (open circles), and after 10 minutes of washout (solid triangles). Currents were normalized to currents recorded at −20 mV before application of forskolin [n = 6, * indicates significant difference from control, P < 0.05, one-way repeated-measures ANOVA]. (C) I-V relationships of endogenous Kv7.5 currents recorded in A7r5 cells before (control, solid circles), after 5 minutes of treatment with 1 μM forskolin (open circles), and after 10 minutes of washout (solid triangles). Currents were normalized to currents recorded at −20 mV before application of forskolin [n = 4, * indicates significant difference from control, P < 0.05, one-way repeated-measures ANOVA]. (D) I-V relationships of endogenous Kv7.5 currents recorded in A7r5 cells before (control, solid circles), after 5 minutes of treatment with 1 μM forskolin in the presence of 500 μM IBMX (open circles), and after 10 minutes of washout (solid triangles). Currents were normalized to currents recorded at −20 mV before application of forskolin/IBMX [n = 4, * indicates significant difference from control, P < 0.01, one-way repeated-measures ANOVA].
enhancement of Kv7 currents in MASMCs, by 66% ± 9% in the voltage range from −39 to −19 mV (Fig. 4C), in comparison with the 3.5-fold enhancement of endogenous Kv7.5 current in A7r5 cells with the same treatment (Fig. 2C).

To more directly test the ability of PKA to regulate individual vascular Kv7 channel isoforms we turned back to the A7r5 cell line, using it as an expression system. Human Kv7.4 and Kv7.5 were expressed alone or together to form homomeric

![Fig. 4](https://molpharm.aspetjournals.org)
Kv7.4, homomeric Kv7.5, or heteromeric Kv7.4/7.5 channels. Consistent with previous studies (Brueggemann et al., 2011, 2014c), the current densities following overexpression of the exogenous Kv7 channel subunits were approximately 100- to 200-fold higher than the average native Kv7.5 current density in A7r5 cells (data not shown), allowing evaluation of effects on the exogenous currents with little contamination of endogenous currents. Both rolipram (100 nM) and the combination of forskolin (10 μM) with IBMX (500 μM) enhanced currents through exogenous Kv7.5 channels by ~2.5-fold (Fig. 5, A and D). On the other hand, neither rolipram (100 nM) nor the combination of forskolin (10 μM) with IBMX (500 μM) enhanced currents through exogenous Kv7.4 channels (Fig. 5, B and E). Heteromeric Kv7.4/7.5 channels in A7r5 cells responded to application of rolipram and forskolin/IBMX similarly to endogenous Kv7 current in MASMCs; rolipram had no effect on Kv7.4/7.5 currents, while forskolin/IBMX (10 μM/500 μM) induced a moderate enhancement of these currents (by 74% ± 4%) (Fig. 5, C and F).

Activation of endogenous βARs with isoproterenol (1 μM) also enhanced exogenous human Kv7.5 homomeric channels by ~3.5-fold over the voltage range positive to −65 mV (n = 6) (Fig. 6A). In contrast, isoproterenol (1 μM) only very slightly (but significantly) enhanced currents through exogenous Kv7.4 channels at voltages between −34 and −14 mV (n = 6) (Fig. 6B). A slightly greater enhancement was observed for the current through heteromeric Kv7.4/7.5 channels (~50% enhancement; current amplitudes were significantly greater than control in the voltage range between −44 and −4 mV, n = 5) (Fig. 6C). Similar to the finding for endogenous Kv7.5 channels, there were no changes in the voltage dependence of activation or slopes of conductance plots for exogenous Kv7 channels in the presence of isoproterenol (Fig. 6, D–F; Table 1). Application of isoproterenol (1 μM) slightly decreased the deactivation rate of exogenous Kv7.5 current measured at −120 mV, from 37.3 ± 7.1 to 43.0 ± 7.5 ms (P < 0.05, n = 6, paired Student’s t test).

To determine whether activation of endogenous β-adrenergic receptors by isoproterenol results in PKA-dependent phosphorylation of the Kv7.5 and Kv7.4 channel subunits we used PLAs to visualize and quantify channel phosphorylation at single molecule resolution. A7r5 cells overexpressing FLAG-tagged hKv7.5 or hKv7.4 were treated with 1 μM isoproterenol for 5 minutes (with or without 30 minutes of pretreatment with 1 μM KT5720), fixed, and stained with primary antibodies raised in rabbit against phospho-Ser/Thr PKA substrate and with antibodies raised in mouse against FLAG (for FLAG-tagged hKv7.5) or against amino acids 2–77 of

![Fig. 5. Differential regulation of hKv7.5, hKv7.4, and hKv7.4/7.5 by forskolin/IBMX and rolipram.](https://molpharm.aspetjournals.org)
Isoproterenol (1 μM) induced a significant increase in the number of punctal fluorescent PLA signals in A7r5 cells expressing FLAG-tagged hKv7.5 channels; this effect was abolished by pretreatment with the PKA inhibitor KT5720 (1 μM; Fig. 7). No such increase in PLA signals was observed in A7r5 cells expressing hKv7.4 (Fig. 7), although the antibody against Kv7.4 had been validated for detection of these channels in a PLA assay in a previous study (Brueggemann et al., 2014c).

Discussion

The results of the present study reveal clear differences in the regulation of vascular smooth muscle cell (VSMC) Kv7 channel subtypes by the βAR-cAMP/PKA pathway. Kv7.5 channels are robustly enhanced by stimuli, including βAR activation, that elevate cAMP levels; this is a reversible effect that appears to be dependent on the activation of PKA and direct phosphorylation of the channel subunits. In contrast, Kv7.4 channels are remarkably insensitive to the same treatments. Coexpression of Kv7.4 with Kv7.5, either exogenously (via expression vectors) or when they are natively coexpressed, as in MASMCs, results in heteromeric Kv7.4/Kv7.5 channels that are very weakly sensitive to an elevation of [cAMP]. These differences in response may have important ramifications in terms of vascular reactivity since expression patterns among Kv7 channel subtypes may differ in different vascular beds or they may change during development or with disease.

Elevation of cytosolic [cAMP] in VSMCs, by activation of cell surface receptors or by inhibition of PDEs, is well known to induce vasodilation. Multiple mechanisms for cAMP-mediated vasodilatory responses have been proposed (Zhao et al., 1998; Yang et al., 1999; Maurice et al., 2003; Morgado et al., 2012; Cuiñas et al., 2013). Our findings add activation of Kv7.5 channels as another potential mechanism. Mackie et al. (2008) previously demonstrated that activation of Kv7 channels is sufficient to induce dilation of rat mesenteric arteries. We predict that cAMP/PKA-mediated vasodilatory responses would reverse or oppose further membrane depolarization and thereby decrease the open probability of voltage-sensitive Ca²⁺ channels, resulting in decreased Ca²⁺ entry and decreased contractility. This prediction is supported by previous evidence that forskolin- and isoproterenol-induced vasorelaxation was reduced in the presence of pinacidil, a selective Kv7 channel blocker (Chadha et al., 2012; Lee et al., 2015).

Our findings suggest that inhibition of PDE is sufficient to activate the cAMP/PKA/Kv7.5 channel signaling pathway in A7r5 cells since several PDE inhibitors (rolipram, papaverine,
and IBMX) were found to robustly enhance Kv7.5 currents in these cells. Of the known members of the heterogeneous PDE superfamily (PDE1–PDE11), PDE1, PDE3, PDE4, and PDE5 are the predominant enzyme families expressed in vascular myocytes, with PDE3 and PDE4 accounting for the majority of the cAMP-hydrolyzing activity (Polson and Strada, 1996; Maurice et al., 2003). Rolipram, a selective PDE4 inhibitor (Ahmad et al., 2015), robustly activated Kv7 currents in A7r5 cells (Fig. 3) but not in MASMCs (Fig. 4). A7r5 cells natively express only Kv7.5 channels (Brueggemann et al., 2007, 2011), whereas MASMCs predominantly express Kv7.4/Kv7.5 channels (Brueggemann et al., 2011, 2014a). Insensitivity of MASMC Kv7.4/7.5 channels to rolipram was replicated when this same subunit combination was expressed in A7r5 cells, suggesting that the difference in regulation of native Kv7 currents between A7r5 cells and MASMCs relates primarily to the Kv7 channel subunit stoichiometry rather than to differences in PDE isoforms (Polson and Strada, 1996; Dunkerley et al., 2002; Maurice et al., 2003).

IBMX, in combination with the direct activator of adenylate cyclase, forskolin, induced a modest enhancement of currents through Kv7.4/Kv7.5 channels in MASMCs, and through exogenously expressed hKv7.4/hKv7.5 channels in A7r5 cells;
however, rolipram failed to elicit a response (Figs. 4 and 5). The pan inhibitor of PDEs, IBMX, may increase [cAMP] to a greater extent (particularly when it is combined with forskolin), and thus induce a greater enhancement of Kv7.4/7.5 currents. We cannot rule out a potential role of cGMP, which may be elevated via the inhibition of PDE5 by IBMX. IBMX and other methylxanthine PDE inhibitors also have a number of documented off-target effects (Wells and Kramer, 1981), which might account for the differences between IBMX and rolipram. In general, our results suggest that modest elevation of cytosolic [cAMP] is sufficient to open Kv7.5 homomeric channels, whereas a greater elevation of cAMP levels is required to open Kv7.4/Kv7.5 heteromeric channels, and Kv7.4 homomeric channels are insensitive to the cAMP/PKA pathway.

A mechanism that could account for the difference in enhancement of Kv7.5 versus Kv7.4 channels in response to cAMP/PKA activation would be the presence of consensus site(s) for PKA-mediated phosphorylation in Kv7.5, but not in Kv7.4 channels. The Kv7.5 channel contains one reported PKA phosphorylation site (Schroeder et al., 2000). However, evaluation of amino acid sequences using MIT Scansite software (Obenauer et al., 2003) revealed 11 putative PKA phosphorylation sites in Kv7.5, only two of which have homologous residues in Kv7.4. Using PLAs, we detected an increase in PKA-dependent phosphorylation of Kv7.5 in response to activation of βARs with isoproterenol (Fig. 7); however, it has yet to be determined which sites might be phosphorylated to elicit the increase in current amplitude that we observed in the present study. We failed to detect PKA-dependent phosphorylation of Kv7.4 channel subunits, supporting the possibility that the Kv7.5 and Kv7.4 subunits may be differentially phosphorylated, and hence have different sensitivities to PKA activation.

The mechanism by which PKA-dependent phosphorylation of Kv7.5 channel subunits enhances current amplitude remains unknown. Thus far, the only physiologic mechanism proposed for positively regulating the activity of Kv7 channels is an increase of membrane concentration of PIP2 (Suh and Hille, 2007). PIP2 is a minor membrane phospholipid that associates with Kv7 channels and stabilizes their open state (Li et al., 2005). PKA-dependent phosphorylation of the Kv7.1 channel was suggested to increase apparent affinity of the channel to PIP2 (Lopes et al., 2007). Considering that the isoproterenol-induced change of conductance-voltage relationships of Kv7.5 channels is similar to the effect of increased PIP2 concentration on open probability of Kv7 channels (no shifts or changes of slope of conductance plots) (Zaydman and Cui, 2014), we speculate that PKA-dependent phosphorylation of the Kv7.5 channel α-subunits increases their apparent PIP2 affinity and thus increases channel open probability.

At the mRNA level, almost all vascular myocytes tested have shown predominant expression of KCNQ4 transcripts, followed by KCNQ1 and KCNQ5 (Yeung et al., 2007; Joshi et al., 2009; Zhong et al., 2010; Ng et al., 2011; Chadha et al., 2012). However, functional assembly of Kv7 channel protein subunits within vascular myocytes is just beginning to be understood. Use of biochemical methods, including PLAs, fluorescence resonance energy transfer, and coinmunoprecipitation, has suggested the existence of Kv7.4/Kv7.5 heterotetramers in mesenteric and cerebral artery myocytes as well as Kv7.1/Kv7.5 heterotetramers in aortic myocytes (Brueggemann et al., 2014c; Chadha et al., 2014; Oliveras et al., 2014). Pharmacological and siRNA knockdown approaches also suggest that functional channels in mesenteric and cerebral artery myocytes are predominantly Kv7.4/Kv7.5 heterotetramers, rather than Kv7.4 or Kv7.5 homotetramers (Brueggemann et al., 2011; Chadha et al., 2014). Our results suggest that activation of vascular Kv7.4/7.5 channels by stimulation of βARs would only modestly increase outward currents (e.g., Fig. 6C), thus this mechanism would therefore be expected to be a minor contributor to vasodilation. However, to the extent that some vascular beds may express Kv7.5 homomeric channels, there remains some potential for βAR-mediated vasodilation via this pathway.

Systemic and pulmonary hypertensive conditions have been reported to be associated with a reduction in KCNQ4 mRNA and Kv7.4 protein in arterial myocytes, while KCNQ5 mRNA levels were unaltered (Chadha et al., 2012; Khanamiri et al., 2013; Sedivy et al., 2015). The selective loss of Kv7.4 subunits would likely shift the stoichiometry of the functional Kv7 channels toward a predominantly Kv7.5 channel phenotype. Therefore, in hypertensive states, which are typically associated with increased sympathetic drive, the βAR-cAMP/PKA-mediated activation of Kv7.5 channels in myocytes described here (Figs. 1, 2, and 6) would play a protective role in preventing a hypercontracted state of vascular myocytes. This interpretation is lent support by the result that coronary artery preparations that exhibited higher expression of KCNQ5 mRNA/protein relaxed more in response to forskolin than artery preparations containing less KCNQ5, when KCNQ4 mRNA levels were comparable between them (Morales-Cano et al., 2015).

We previously found that PKC-dependent phosphorylation and suppression of Kv7 activity by arginine vasopressin (acting through Goq-coupled receptors on A7r5 cells) were dependent on the subunit composition of the channels (Brueggemann et al., 2014c). Overexpressed hKv7.5 channels in A7r5 cells were found to be highly sensitive to this PKC-dependent suppression of channel activity; however, as in the present study, Kv7.4 homomeric channels were resistant to this form of regulation, while heteromeric Kv7.4/7.5 channels displayed intermediate sensitivity (Brueggemann et al., 2014c).

It is worth noting that the A7r5 cell line is derived from embryonic rat thoracic aorta (Kimes and Brandt, 1976); the robust expression of KCNQ5 with no detectable KCNQ4 (Brueggemann et al., 2007; 2011) may relate to the developmental stage of the tissue from which these cells were isolated. As noted previously, vascular myocytes from adult arteries have almost uniformly been found to express higher levels of KCNQ4 than KCNQ5. Although no studies have been conducted to examine the developmental changes in KCNQ gene expression or Kv7 channel function in the vasculature, it is intriguing to speculate that developmental changes in Kv7 channel subunit stoichiometry might be associated with differences in channel sensitivities to regulatory pathways.

The findings of the present study are consistent with previous research in implicating Kv7.5 subunits as the primary regulatory target in Kv7 channels of VSMCs. To the extent that these subunits are predominant, as in the embryonic rat aorta–derived A7r5 cells, the channels formed are highly responsive to both positive (cAMP/PKA) and negative (PKC) regulatory pathways. To the extent that Kv7.4 subunits are coexpressed, as in MASMCs from adult
rats or the more extreme case when human KCNQ4 is overexpressed in A7r5 cells, the channels become progressively less responsive. Future studies will determine whether changing expression of Kv7 channel subtypes, as may occur during development or in pathologies such as hypertension, results in altered Kv7 channel–dependent vascular responsiveness. This could provide important clues for designing appropriate Kv7 channel–targeted therapeutic regimens to treat cardiovascular diseases.

**Authorship Contributions**

- **Participated in research design:** Mani, Brueggemann, Cribbs, Byron.
- **Conducted experiments:** Mani, Robakowski, Brueggemann, Tripathi.
- **Collected new reagents or analytic tools:** Cribbs.
- **Conducted data analysis:** Mani, Robakowski, Brueggemann, Tripathi.
- **Wrote or contributed to the writing of the manuscript:** Mani, Robakowski, Brueggemann, Majetschak, Byron.

**References**


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