

Kv7.5 potassium channel subunits are the primary targets for PKA-dependent enhancement of vascular smooth muscle Kv7 currents.

Bharath K. Mani, Christina Robakowski, Lyubov I. Brueggemann, Leanne L. Cribbs, Abhishek Tripathi, Matthias Majetschak, and Kenneth L. Byron

Department of Molecular Pharmacology & Therapeutics (BKM, CR, LIB, MM, KLB), Department of Cell and Molecular Physiology (LLC) and Department of Surgery (AT, MM), Loyola University Chicago, Maywood, IL 60153

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Corresponding author:

Kenneth L. Byron, Ph.D.
Loyola University Chicago
Stritch School of Medicine
2160 S. First Avenue
Maywood, IL 60153
tel.: 708-327-2819
fax.: 708-216-6596
kbyron@luc.edu

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Nonstandard abbreviations:

8Br-cAMP, 8-bromo cyclic adenosine monophosphate;
AC, adenylate cyclase;
cAMP, cyclic adenosine monophosphate;
GFP, Green Fluorescent Protein;
IBMX, 3-isobutyl-1-methylxanthine;
 I_{Ks} , slow delayed rectifier currents;
MASMC, mesenteric artery smooth muscle cell;
MLC₂₀, myosin light chain kinase 20;
MOI, multiplicity of infection;
PDE, phosphodiesterase;
PIP2, phosphatidylinositol 4,5-bisphosphate;
PKA, protein kinase A;
PKC, protein kinase C;
PLA, Proximal ligation assay
RCA, Rolling-circle amplification
VSMC, vascular smooth muscle cell;
 β AR, β -adrenergic receptor;

Abstract

Kv7 (KCNQ) channels, formed as homo- or hetero-tetramers 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. The physiological regulation of Kv7 channel activity, however, is still poorly understood. Here, we study the effect of cyclic adenosine monophosphate/protein kinase A (cAMP/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 hetero-tetrameric channels in A7r5 cells. Stimulation of G α_s -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 hetero-tetrameric 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 through 5), which are expressed in a variety of excitable cells, ranging from neurons (mainly expressing KCNQ2-5) and cardiac myocytes (mainly expressing KCNQ1), to smooth muscle cells of different origins (mainly expressing KCNQ1, KCNQ4 and KCNQ5) (Barhanin et al., 1996; Brueggemann et al., 2012; Brueggemann et al., 2011; Jepps et al., 2009; Kubisch et al., 1999; Lerche et al., 2000; Mackie et al., 2008; McCallum et al., 2009; Ohya et al., 2002; Schroeder et al., 2000; Sims et al., 1985; Svalø et al., 2013; Wang et al., 1998; Yeung et al., 2007). The KCNQ1-5 gene products (Kv7.1-Kv7.5 proteins) assemble as homo- or hetero-tetramers 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 (Brown, 2008; Wickenden, 2002). For example, the excitability of neurons is increased when Kv7 channel-mediated neuronal M-currents are suppressed upon G_{α_q} -coupled receptor activation. The mechanisms by which receptor activation leads to suppression of neuronal M-currents include depletion of phosphatidylinositol 4,5-bisphosphate (PIP_2), elevation of cytosolic Ca^{2+} concentration, and protein kinase C (PKC)-dependent phosphorylation of Kv7.2 channel subunits (Delmas and Brown, 2005). In cardiac myocytes, the amplitude of Kv7 channel-mediated slow delayed rectifier currents (I_{Kr}) currents can be increased via the activation of the β -adrenergic receptor (βAR)/ G_{α_s} /cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway (Chen and Kass, 2011). PKA-dependent regulation of the native neuronal M-current has not been reported

to date, though the activity of human Kv7.2 channels overexpressed in *Xenopus* oocytes was reportedly enhanced by PKA-dependent phosphorylation at Ser52 of the N-terminus (Schroeder et al., 1998). Treatment with a membrane-permeant cAMP analog or intracellular application of PKA catalytic subunit also enhanced human Kv7.4 current in an expression system (Chambard and Ashmore, 2005).

While the functional regulation of the neuronal and cardiac Kv7 channels has been investigated in considerable detail, physiological regulation of the Kv7 channels in smooth muscles remains sparsely studied. PKC-dependent suppression of native Kv7 currents in response to $G\alpha_q$ -coupled receptor activation was reported for vascular and airway smooth muscle cells, leading to vasoconstriction or bronchoconstriction, respectively (Brueggemann et al., 2012; Brueggemann et al., 2013; Brueggemann et al., 2007; Mackie et al., 2008; Mani et al., 2013). In terms of smooth muscle relaxation, there is some evidence that endogenous Kv7 channels are downstream targets for β AR-mediated vasodilation. Originally, β AR stimulation was found to enhance M-currents detected in toad gastric smooth muscle cells (Sims et al., 1988). Later, β AR stimulation was reported to enhance Kv7 currents detected in renal artery myocytes (Chadha et al., 2012). In contrast, activation of β ARs did not enhance native Kv7 currents in airway myocytes (Brueggemann et al., 2014b), and membrane permeable cAMP or cAMP-elevating agents failed to enhance native Kv7 currents in retinal pigment epithelial cells expressing Kv7.4/7.5 channels (Pattnaik and Hughes, 2012). In the present study, we investigate molecular mechanisms underlying the regulation of vascular Kv7 potassium channels, predominantly formed as homo- or hetero-tetramers of Kv7.4 and Kv7.5 (Brueggemann et al., 2013; Brueggemann et al., 2011), by the cAMP/PKA pathway.

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) (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 (MOI) 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 Green Fluorescent Protein (GFP) (co-expressed with the KCNQ products via the IRES-hrGFP element in the AdEasy™ Adenoviral Vector System (Stratagene)). To maintain endogenous β AR responsiveness, the cells were serum-deprived for 1-2 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 *Guide for the Care and Use of Laboratory Animals* (1996. National Academy of Sciences, Washington D.C.). 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 min 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., 2011; Brueggemann et al., 2007; 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 gluconate, 30 KCl, 5 HEPES, 1 K₂EGTA, pH 7.2. Osmolality was adjusted to 268 mOsm/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 mOsm/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 mOsm/l. 120 µg/ml Amphotericin B in the internal solution was used for membrane patch perforation. To isolate Kv7 currents, 100 µM GdCl₃ (sufficient to block L- and T-type Ca²⁺ channels, non-selective cation channels, and to shift activation of 4-AP-sensitive Kv channels to more positive voltages (Mani et al., 2011) was added to external solutions.

Voltage-clamp command voltages were generated using an Axopatch 200B amplifier under control of PCLAMP10 software and EPC10 amplifier under control of PATCHMASTER software (HEKA, Pfalz, 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.

K⁺ currents through overexpressed hKv7 channels were recorded using a 5 s voltage step protocol from a -74 mV holding voltage to test voltages ranging from -114 mV to -4 mV followed

by a 1 s step to -120 mV. The currents recorded during the last 1s of recording time for each voltage step were averaged and normalized by cell capacitance to obtain end pulse steady-state K^+ current. Stable currents were recorded for at least 15 min prior to drug application. Time courses of drug effects were recorded by applying 5 s voltage steps to -20 mV every 15 s. To measure endogenous currents in A7r5 cells, a 5 s voltage step protocol was used (from a -74 mV holding voltage to test voltages ranging from -94 mV to +36 mV) followed by a 1 s step to -120 mV 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 equation: $G = I_{tail}/(-120 - E_K)$, where I_{tail} is the instantaneous tail current amplitude, -120 mV is the tail current step potential and E_K is the reversal potential for potassium (-86 mV). Conductance plots in the absence (control) and in the presence of isoproterenol (1 μ M) for each experiment were fitted to a Boltzmann distribution: $G(V) = G_{max}/[1 + \exp(V_{0.5} - V)/k]$, where G is conductance, G_{max} is a maximal conductance, $V_{0.5}$ 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 s voltage step protocol (from a -74 mV holding potential to -20 mV) followed by 1 s repolarization to -120 mV.

Kv7 currents in MASMCS were recorded by application of 5 s voltage steps from a -4 mV holding voltage to test voltages ranging from -84 mV to +16 mV. Time courses of drug effects on Kv7 currents were recorded at -20 mV holding voltage.

Proximity ligation assays (PLA)

Duolink PLA assays (Sigma-Aldrich) were performed essentially as described previously (Brueggemann et al., 2013; Tripathi et al., 2015). A7r5 cells infected with Adv-hKCNQ4 or Adv-hKCNQ5-FLAG (Brueggemann et al., 2011) at a multiplicity of infection (MOI) of 100 were plated on 8-well tissue culture slides (Permanox®, Nunc) 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, 11.5 mM glucose, pH 7.3) and treated with vehicle (Control Buffer) or 1 μ M isoproterenol for 5 min (a subset of cells was pretreated with 1 μ M PKA inhibitor KT5720 for 30 min). After treatment, cells were fixed for 15 min with 2% paraformaldehyde in phosphate-buffered saline (PBS). The cells were permeabilized with 0.5% Triton X-100 in PBS for 20 min at room temperature and then blocked with 3% bovine serum albumin in PBS for 2 h. Primary mouse antibodies (anti-FLAG antibody (Sigma, F1804, at a dilution 1:500) for cells expressing hKv7.5, or anti-KCNQ4 antibodies (Abcam, ab84820, at dilution 1:500) for cells expressing hKv7.4) were applied in combination with rabbit anti-phospho-(Ser/Thr) PKA substrate (Cell Signaling, #9621, at dilution 1:100) in blocking buffer (3% bovine serum albumin in PBS) at 37°C for 2h in a humidifying chamber. As an antibody control, slides were incubated with PBS without primary antibody. Slides were then washed and incubated with secondary anti-rabbit/mouse antibodies conjugated with plus/minus Duolink II PLA probes (1:5). After washing, slides were incubated with a ligation-ligase solution to join the adjacent oligonucleotides by ligation into circular DNA molecules (30 min, 37°C). This was followed by incubation with amplification-polymerase solution (2 h, 37 °C), as per the manufacturer's protocol. Under these conditions, the circular DNA molecules were amplified by rolling-circle amplification (RCA) primed by one of the proximity probes, thus creating a concatemeric amplification product that remained covalently attached to the proximity probe (Söderberg et al., 2008). The RCA product was subsequently detected by hybridization of fluorescently-labeled oligonucleotides. Slides were mounted with a minimal volume of Duolink II Mounting medium with 4',6-diamidino-2-phenylindole (DAPI) for 15-30 min and PLA signals (Duolink In Situ Detection Reagents Red ($\lambda_{\text{excitation/emission}}$ 598/634 nm)) were identified as fluorescent dots under a Zeiss Axiovert 200M microscope with EC Plan-Neofluor 40X/1.30 oil or EC Plan- Neofluor 100X/1.30 oil objectives and

a Zeiss Axio CamMRC5 camera. A series of images (~8–10/cell) were acquired with z-axis scanning (1 μ m z-axis interval) using a wavelet fusion algorithm to combine those images into one single composite z-stack image (Zeiss AxioVision Rel.4.8.2 software). Images were captured and processed identically across all experimental groups. Quantification of PKA-dependent channel phosphorylation (as PLA signals per cell) was performed using the Duolink Image Tool software (Sigma-Aldrich). Images were imported in merged tiff formats containing both signal and nuclei channels. Merged images were checked visually and verified for analytical quality. A total of 21 fields (7 fields from each of 3 different cell preparations) were analyzed for each Kv7 channel subtype.

Materials

Cell culture media were from Gibco-BRL (Gaithersburg, MD) or MediaTech (Herndon, VA). Isoproterenol (4-[1-Hydroxy-2-[(1-methylethyl)amino]ethyl]-1,2-benzenediol hydrochloride), forskolin ([3*R*-(3 α ,4 α ,5 β ,6 β ,6 α ,10 α ,10 β ,10 β)]-5-(Acetyloxy)-3-ethenyldodecahydro-6,10,10b-trihydroxy-3,4a,7,7,10a-pentamethyl-1*H*-naphtho[2,1-*b*]pyran-1-one), IBMX (3-isobutyl-1-methylxanthine), papaverine hydrochloride (6,7-Dimethoxy-1-veratrylisoquinoline hydrochloride), collagenase, elastase, and Duolink PLA assay reagents were from Sigma-Aldrich (St. Louis, MO). XE-991 (10,10-bis(4-pyridinylmethyl)-9(10*H*)-anthracenone) dihydrochloride was from Ascent Scientific (Princeton, NJ). Rolipram (4-[3-(Cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone), KT5720 ((9*R*,10*S*,12*S*)-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-*i*][1,6]benzodiazocine-10-carboxylic acid, hexyl ester), ML213 (N-(2,4,6-Trimethylphenyl)-bicyclo[2.2.1]heptane-2-carboxamide), 8-bromo-cAMP sodium salt were from Tocris Bioscience (Bristol, UK). Amphotericin B was from Calbiochem (San Diego, CA). The vector pIRES2-EGFP was from Clontech (Mountain View, CA). The AdEasyTM Adenoviral Vector System was from Stratagene (La

Jolla, CA). The human KCNQ4 cDNA (accession number: AF105202, originally in the insect cell expression vector pMT) was a generous gift from Dr. Ian Wood at the University of Leeds, Leeds, UK. The human KCNQ5 cDNA (accession number: AF202977, originally in the insect cell expression vector pMT) was a generous gift from Prof. Thomas Jentsch at the Max-Delbrück-Centrum for Molecular Medicine, Berlin, Germany.

Statistics

Data are expressed as mean \pm S.E. SigmaStat (Systat 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 or ANOVA 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.

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 (Brueggemann et al., 2013; Brueggemann et al., 2011; Brueggemann et al., 2007). 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 RT-PCR (Brueggemann et al., 2011; Brueggemann et al., 2007), pharmacology (Brueggemann et al., 2011; Brueggemann et al., 2007), molecular approaches using shRNA (Brueggemann et al., 2007; Mani et al., 2009) and abolishment of the current upon expression of dominant negative Kv7.5 subunit (Brueggemann

and Byron, unpublished results). A7r5 cells have also been reported to express endogenous β ARs (Hirata et al., 1985). When we applied isoproterenol (1 μ M, to activate endogenous β ARs), 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 co-application of a pan Kv7 channel blocker, XE991 (1 μ M) (Fig. 1A, B, E). The isoproterenol effect was also prevented by propranolol (10 μ M), a β AR antagonist, or by pretreatment with a PKA inhibitor KT5720 (1 μ M for 30 min) (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), but it did slightly decrease the deactivation rate of endogenous Kv7.5 current measured at -120 mV, from 40.3 ± 0.9 ms 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 (8Br-cAMP; 1mM) also reversibly increased the amplitude of endogenous Kv7.5 currents by 2-3 fold in the voltage range from -54 mV 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 adenylate cyclase (AC) with forskolin (1 μ M; > 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 μ M to 10 μ M, enhancement of endogenous Kv7.5 was reversed to almost complete inhibition (data not shown). When activation of AC by forskolin (1 μ M) was combined with inhibition of phosphodiesterases (PDEs) by addition of the nonselective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX, 500 μ M), endogenous Kv7.5 currents were enhanced > 3-fold in the voltage range from -54 mV to +36 mV ($n = 4$, Fig. 2C). Another nonselective PDE inhibitor, papaverine, dose-dependently enhanced endogenous Kv7.5 currents by ~2 and ~3 fold at doses of 10 μ M and 100 μ M, respectively, even in the absence of forskolin (Fig. 2D).

The more selective inhibitor of PDE4 (cAMP phosphodiesterase isoform), rolipram, applied at increasing concentrations (1 nM - 1 μ M), dose-dependently and reversibly enhanced endogenous Kv7.5 current in A7r5 cells, with an $EC_{50} \sim 6$ nM and maximal enhancement (2.1 ± 0.5 fold) achieved at 100 nM (Fig. 3A, B). To test whether PDE4 inhibition by rolipram enhances endogenous Kv7.5 currents via activation of PKA, we used the PKA inhibitor, KT5720 (1 μ M). Pretreatment with KT5720 (1 μ M for 30 min) significantly inhibited the effect 100 nM rolipram, relative to vehicle pretreatment (Fig. 3C, 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., 2013; Brueggemann et al., 2011), whereas A7r5 cells only express Kv7.5 (Brueggemann et al., 2011; Brueggemann et al., 2007). 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., 2013). In the present study, the same was found to be true for PKA-dependent regulation. Direct activation of β ARs in MASMCs by isoproterenol (1 μ M) 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 non-selective Kv7.2-Kv7.5 activator ML213 (10 μ M, (Brueggemann et al., 2014a; Yu et al., 2011)) robustly enhanced the MASMC Kv7 currents and this effect was reversed by the selective Kv7 channel blocker XE991 (10 μ M) (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 AC with forskolin (1 μ M) in combination with the nonselective PDE inhibitor IBMX (500 μ M) induced a modest enhancement of Kv7 currents in MASMCs (by $66\% \pm 9\%$ in the voltage range from -39 mV 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 Kv7.4, homomeric Kv7.5 or heteromeric Kv7.4/7.5 channels. Consistent with our previous studies (Brueggemann et al., 2013; Brueggemann et al., 2011), the current densities following overexpression of the exogenous Kv7 channel subunits was 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 μ M) with IBMX (500 μ M) enhanced currents through exogenous Kv7.5 channels (by \sim 2.5-fold; Fig. 5A, B). On the other hand, neither rolipram (100 nM) nor the combination of forskolin (10 μ M) with IBMX (500 μ M) enhanced currents through exogenous Kv7.4 channels (Fig. 5C, D). Heteromeric Kv7.4/7.5 channels in A7r5 cells responded to application of rolipram and forskolin/IBMX similarly to endogenous Kv7 current in MASCs: rolipram had no effect on Kv7.4/7.5 currents, while forskolin/IBMX (10 μ M/500 μ M) induced a moderate enhancement of these currents (by $74\% \pm 4\%$; Fig. 5E, F).

Activation of endogenous β ARs with isoproterenol (1 μ M) also enhanced exogenous human Kv7.5 homomeric channels (by \sim 3.5-fold over the voltage range positive to -65 mV (n=6, Fig. 6A). In contrast, isoproterenol (1 μ M) only very slightly (but significantly) enhanced currents through exogenous Kv7.4 channels at voltages between -34 mV and -14 mV (n=6, Fig. 6B). A slightly greater enhancement was observed for the current through heteromeric Kv7.4/7.5 channels (\sim 50% enhancement; current amplitudes were significantly greater than control in the voltage range between -44 mV 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 μ M) slightly decreased the deactivation rate of exogenous Kv7.5 current measured at -120 mV, from 37.3 ± 7.1 ms 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 proximity ligation assays (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 μ M isoproterenol for 5 min (with or without 30 min pretreatment with 1 μ M 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 human Kv7.4 (for hKv7.4). Isoproterenol (1 μ M) 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 μ M; Fig. 7). No such increase in PLA signals was observed in A7r5 cells expressing hKv7.4 (Fig. 7), though the antibody against Kv7.4 had been validated for detection of these channels in a PLA assay in our previous study (Brueggemann et al., 2013).

Discussion

The results of the present study reveal clear differences in the regulation of VSMC Kv7 channel subtypes by the β AR-cAMP/PKA pathway. Kv7.5 channels are robustly enhanced by stimuli, including β AR activation, that elevate cAMP levels; this is a reversible effect which 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. Co-expression of Kv7.4 with Kv7.5, either exogenously (via expression vectors), or when they are natively co-expressed, as in MASCs, 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, as 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 (Cuinas et al., 2013; Maurice et al., 2003; Morgado et al., 2012; Yang et al., 1999; Zhao et al., 1998). Our findings add activation of Kv7.5 channels as another potential mechanism. We have previously demonstrated that activation of Kv7 channels is sufficient to induce dilation of rat mesenteric arteries (Mackie et al., 2008). We predict that cAMP/PKA-mediated activation of Kv7.5 currents in VSMCs would reverse or oppose further membrane depolarization and thereby decrease the open probability of voltage sensitive Ca^{2+} channels, resulting in decreased Ca^{2+} entry and decreased contractility. This prediction is supported by previous evidence that forskolin- and isoproterenol-induced vasorelaxation was reduced in the presence of linopirdine, 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, as 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 through 11), 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 (Maurice et al., 2003; Polson

and Strada, 1996). Rolipram, a selective PDE4 inhibitor (Ahmad et al., 2015), robustly activated Kv7 currents in A7r5 cells (Figure 3), but not in MASMCs (Figure 4). A7r5 cells natively express only Kv7.5 channels (Brueggemann et al., 2011; Brueggemann et al., 2007) whereas MASMCs predominantly express Kv7.4/Kv7.5 channels (Brueggemann et al., 2014a; Brueggemann et al., 2011). 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 (Dunkerley et al., 2002; Maurice et al., 2003; Polson and Strada, 1996).

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/Kv7.5 channels in A7r5 cells, but rolipram failed to elicit a response (Figure 4, 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 cyclic guanosine monophosphate, 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 proximity ligation assays, we detected an increase in PKA-dependent phosphorylation of Kv7.5 in response to activation of β ARs with isoproterenol (Fig. 7), but 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. So far the only physiological 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 channels 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 (Chadha et al., 2012; Joshi et

al., 2009; Ng et al., 2011; Yeung et al., 2007; Zhong et al., 2010). However, functional assembly of Kv7 channel protein subunits within vascular myocytes is just beginning to be understood. Use of biochemical methods, including proximity ligation assays, fluorescence resonance energy transfer, and co-immunoprecipitation, 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., 2014a; 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 β ARs would only modestly increase outward currents (e.g. Figure 6C), so 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 β AR-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 towards a predominantly Kv7.5 channel phenotype. Therefore, in hypertensive states, which are typically associated with increased sympathetic drive, the β AR-cAMP/PKA mediated activation of Kv7.5 channels in myocytes described here (Figure 1, 2 and 6) would play a protective role in preventing a hyper-contracted state of vascular myocytes. This interpretation is lent support by the result that coronary artery preparations which 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 $G\alpha_q$ -coupled receptors on A7r5 cells) was dependent on the subunit composition of the channels (Brueggemann et al., 2013). Overexpressed hKv7.5 channels in A7r5 cells were found to be highly sensitive to this PKC-dependent suppression of channel activity, but, 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., 2013).

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., 2011; Brueggemann et al., 2007) may relate to the developmental stage of the tissue from which these cells were isolated. As noted above, 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 vascular smooth muscle cells. 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 co-expressed, 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 Contribution

Participated in research design: Mani, Brueggemann, Cribbs, and Byron

Conducted experiments: Mani, Robakowski, Brueggemann, and Tripathi

Contributed new reagents or analytic tools: Cribbs

Performed data analysis: Mani, Robakowski, Brueggemann, and Tripathi

Wrote or contributed to the writing of the manuscript: Mani, Robakowski, Brueggemann, Tripathi, Majetschak, and Byron

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Footnotes

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Bharath K. Mani is currently at University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390.

Figure Legends

Figure 1. Enhancement of endogenous Kv7.5 current in A7r5 cells by isoproterenol depends on activation of endogenous β -adrenergic receptors and PKA.

A. Representative traces of endogenous Kv7.5 currents recorded in an A7r5 cell before (control, left panel), after 5 min in the presence of 1 μ M isoproterenol (middle panel) and following addition of 1 μ M XE991 (right panel) ($C = 151$ pF, representative of 11 similar experiments). B. Current-Voltage (I-V) relationships of endogenous Kv7.5 current densities recorded in A7r5 cells before (control, filled circles), after 5 min treatment with 1 μ M isoproterenol (open circles) and after 10 min of treatment with 1 μ M isoproterenol in combination with 1 μ M XE991 (filled triangles). * indicates significant difference from control, $P < 0.05$, One Way Repeated Measure ANOVA, $n=5-11$. C. Conductance-voltage relationships of endogenous Kv7.5 channels normalized to maximal conductance (G_{max}) before (filled circles, $n=5$) and after addition of 1 μ M isoproterenol (open circles, $n=5$) fitted to the Boltzmann equation (solid lines). Absolute values of conductance densities are shown in the inset. D. Deactivation tail currents recorded at -120 mV before (black) and after addition of 1 μ M isoproterenol (red), fitted by a single exponential function (solid lines). Endogenous Kv7.5 channels were activated by 5-s steps to -20 mV, followed by 1s step to -120 mV; 5 traces were averaged for each condition. The inset shows tail currents normalized to peak amplitude, to highlight the differences in deactivation kinetics. E. Normalized currents recorded at -20mV in control (black bar), in the presence of 1 μ M isoproterenol (ISO, open bar, $n = 5$), in the presence of 10 μ M propranolol (P, dark grey bar, $n=4$), in the presence of 10 μ M propranolol plus 1 μ M isoproterenol (P+ISO, dark grey striped bar, $n=4$), in the presence of 1 μ M KT5720 (KT, light grey bar, $n=4$) and in the presence of 1 μ M KT5720 plus 1 μ M isoproterenol (KT+ISO, light grey striped bar, $n=4$). * indicates significant difference from control ($P < 0.05$, one way ANOVA on Ranks).

Figure 2. Enhancement of endogenous Kv7.5 current in A7r5 cells by elevation of intracellular [cAMP].

A. I-V relationships of endogenous Kv7.5 currents recorded in A7r5 cells before (control, filled circles), after 15 min treatment with 1 mM 8Br-cAMP (open circles) and after 10 min of washout (filled triangles). Currents were normalized to current recorded at -20 mV before application of 8Br-cAMP (n= 4, * indicates significant difference from control, $P < 0.05$, One Way Repeated Measures ANOVA). B. I-V relationships of endogenous Kv7.5 currents recorded in A7r5 cells before (control, filled circles), after 5 min treatment with 1 μ M forskolin (open circles) and after 10 min of washout (filled triangles). Currents were normalized to current recorded at -20 mV before application of forskolin (n= 4, * 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, filled circles), after 5 min treatment with 1 μ M forskolin in the presence of 500 μ M IBMX (open circles) and after 10 min of washout (filled triangles). Currents were normalized to current recorded at -20 mV before application of forskolin/IBMX (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, filled circles) and after treatment with papaverine (10 μ M open circles, 100 μ M open triangles). Current were normalized to currents recorded at -20 mV before application of papaverine (n= 4, * indicates significant difference from control, $P < 0.01$, One Way Repeated Measures ANOVA).

Figure 3. Inhibition of PDE4 with rolipram enhances endogenous Kv7.5 current in A7r5 cells in a PKA-dependent manner.

A. I-V relationships of endogenous Kv7.5 currents recorded in A7r5 cells before (control, filled circles) and during treatment with increasing concentrations of rolipram (open symbols: circle- 1nM, triangle- 10 nM, reversed triangle- 30 nM, square- 100 nM, diamond- 1 μ M). B. Cumulative rolipram dose-response curve for Kv7.5 current enhancement, presented as fold increase in amplitude of current recorded at -20 mV, fitted to the Hill equation. (n= 4-5, * indicates significant difference from control, $P < 0.001$, One Way Repeated Measures ANOVA). C, D. I-V relationships of Kv7.5 currents recorded in A7r5 cells before (control, filled circles), after 30 min pre-treatment with either vehicle for KT5720 (0.01% DMSO, filled triangles) (C) or 1 μ M KT5720 (D) and after 5 min treatment with 100 nM rolipram (open circles). Current were normalized to currents recorded at -20 mV before application of rolipram (n= 4, * indicates significant difference from control, $P < 0.05$, One Way Repeated Measures ANOVA)

Figure 4. Regulation of endogenous Kv7 currents in mesenteric artery myocytes by isoproterenol, rolipram, and forskolin/IBMX.

A. I-V relationships of endogenous Kv7 currents recorded in MASMCs before (control, filled circles) and after 5 min treatment with 1 μ M isoproterenol (open circles, n=6). B. I-V relationships of endogenous Kv7 currents recorded in MASMCs before (control, filled circles) and after 5 min treatment with 100 nM rolipram (open circles, n=5), followed by application of ML213 (10 μ M, open triangles, n=5) and by application of 10 μ M XE991 in the presence of 10 μ M ML213 (filled triangles, n=4). * indicates significant difference from control, $P < 0.05$, One Way Repeated Measures ANOVA. D. I-V relationships of endogenous Kv7 currents recorded in MASMCs before (control, filled circles) and after 5 min treatment with 1 μ M forskolin in the presence of 500 μ M IBMX (open circles, n=5, * indicates significant difference from control, $P <$

0.05, paired Student's t-test) and after 10 min of washout (filled upward triangles), followed by application of 1 μ M XE991 (filled downward triangles, n=4).

Figure 5. Differential regulation of hKv7.5, hKv7.4 and hKv7.4/7.5 by forskolin/IBMX and rolipram.

A, D. I-V curves of steady-state Kv currents recorded in A7r5 cells overexpressing hKv7.5 before (control, filled circles) and after 5 min treatment with 10 μ M forskolin in the presence of 500 μ M IBMX (A, open circles, n=7) or 100 nM rolipram (D, open circles, n=7). B, E. I-V curves of steady-state Kv currents recorded in A7r5 cells overexpressing hKv7.4 before (control, filled circles) and after 5 min treatment with 10 μ M forskolin in the presence of 500 μ M IBMX (B, open circles, n=3) or 100 nM rolipram (E, open circles, n=5). C, F. I-V curves of steady-state Kv currents recorded in A7r5 cells overexpressing hKv7.4/7.5 before (control, filled circles) and after 5 min treatment with 10 μ M forskolin in the presence of 500 μ M IBMX (C, open circles, n=6) or 100 nM rolipram (F, open circles, n=5). * indicates significant difference from control, $P < 0.05$, paired Student's t-test.

Figure 6. Activation of endogenous β ARs in A7r5 cells enhances exogenous hKv7.5, hKv7.4 and hKv7.4/7.5 to varying degrees.

A-C. I-V curves of steady-state Kv currents recorded in A7r5 cells overexpressing hKv7.5 (A, n=6), hKv7.4 (B, n=6), or hKv7.4/7.5 (C, n=5) before (control, filled circles) and after 5 min treatment with 1 μ M isoproterenol (open circles). * indicates significant difference from control, $P < 0.05$, paired Student's t-test. D-F. Conductance-voltage relationships of exogenous hKv7.5 (D), Kv7.4 (E) and Kv7.4/7.5 (F) channels normalized to maximal conductance (G_{max}) before (filled circles, n=5-6) and after addition of 1 μ M isoproterenol (open circles, n=5-6) fitted to the Boltzmann

equation (solid lines). Conductances normalized to the maximal control conductance (G_{\max_c}) are shown in insets.

Figure 7. Activation of endogenous β ARs in A7r5 cells enhances PKA-dependent phosphorylation of exogenous hKv7.5 but not hKv7.4 channels.

A. Representative images of A7r5 cells exogenously expressing hKv7.5 channels (top panels) and hKv7.4 channels (bottom panels); untreated (control), treated with 1 μ M isoproterenol for 5 min (ISO) and treated with 1 μ M isoproterenol for 5 min after pretreatment with 1 μ M KT5720 for 30 min (ISO+KT). Proximity ligation assays (PLAs) were conducted on hKv7.5-expressing cells labeled with a combination of mouse anti-FLAG antibody and rabbit anti-phospho-Ser/Thr PKA substrate antibody or on hKv7.4-expressing cells labeled with a combination of mouse anti-KCNQ4 antibody and rabbit anti-phospho-Ser/Thr PKA substrate antibody. B, C. Bar graphs summarizing the number of PLA signals/cell in A7r5 cells exogenously expressing hKv7.5 (B) and hKv7.4 (C) under control conditions (c), after treatment for 30 min with 1 μ M KT5720 (c+KT) , after treatment for 5 min with 1 μ M isoproterenol in the absence (ISO) or presence of pretreatment with 1 μ M KT5720 (ISO+KT), and in cells where the primary antibodies were omitted (-1°ab). *, # $P < 0.001$ from all groups ANOVA on Ranks, $n=12-21$.

Table 1. Effect of 1 μ M isoproterenol on the voltage dependence of endogenous Kv7.5 and exogenous homomeric hKv7.4, homomeric hKv7.5, and heteromeric hKv7.4/7.5 channels.

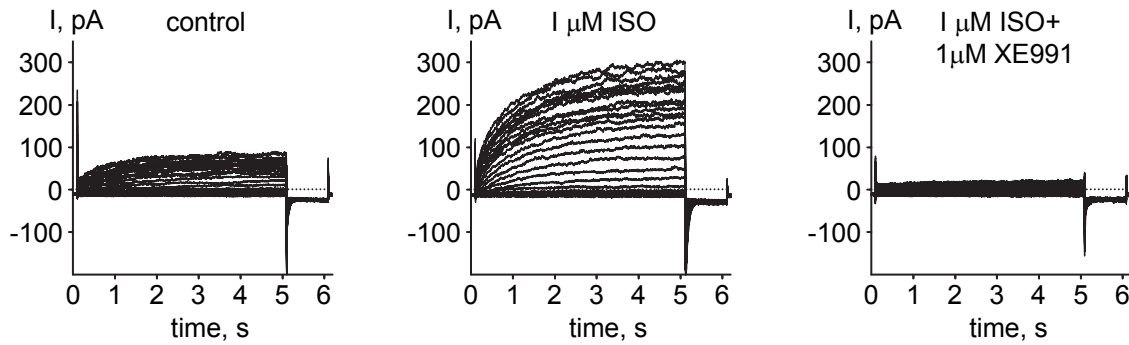
Kv7 isoforms	G _{max} , % of control	V _{0.5} , mV control	V _{0.5} , mV 1 μ M ISO	k, mV control	k, mV 1 μ M ISO
Endogenous Kv7.5, n=10	259.3 \pm 33.8*	-48.5 \pm 2.5	-47.8 \pm 2.7	9.5 \pm 1.0	11.3 \pm 0.8
Exogenous hKv7.5, n=5	267.4 \pm 16.6**	-46.2 \pm 2.5	-46.1 \pm 2.1	12.3 \pm 0.8	13.6 \pm 1.7
Exogenous hKv7.4, n=5	98.1 \pm 3.1	-28.6 \pm 2.0	-28.2 \pm 1.6	10.8 \pm 0.7	10.7 \pm 0.7
Exogenous hKv7.4/7.5, n=5	134.9 \pm 9.3*	-34.0 \pm 3.9	-35.5 \pm 5.6	11.3 \pm 0.8	12.1 \pm 0.9

G_{max} - maximal conductance, V_{0.5}- voltage of half-maximal activation, k- slope factor.

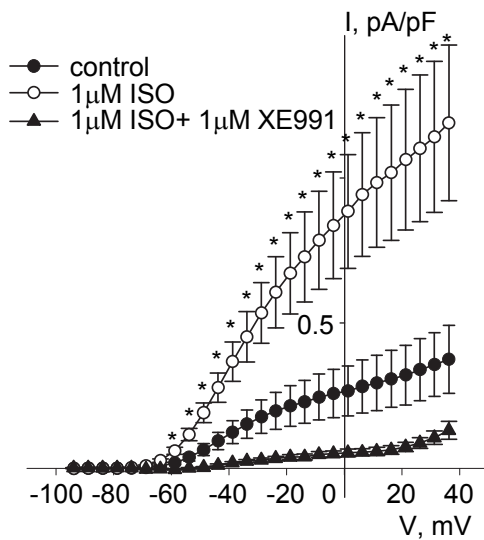
** significantly different from control, paired Student's t-test, p< 0.001

* significantly different from control, paired Student's t-test, p< 0.05

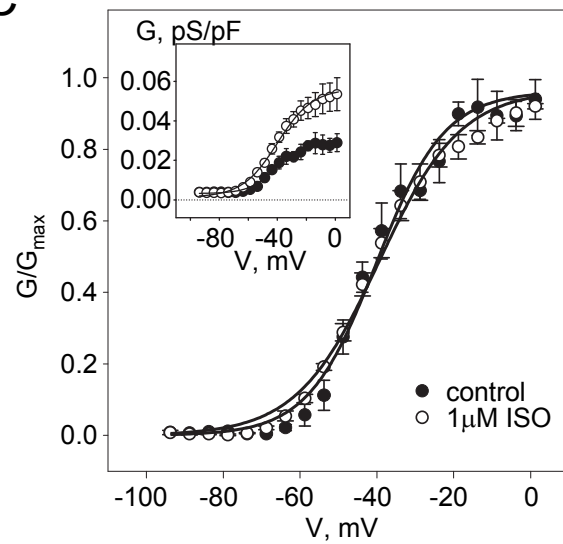
A



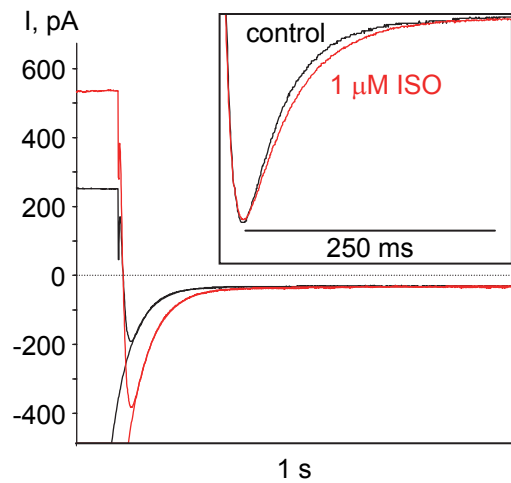
B



C



D



E

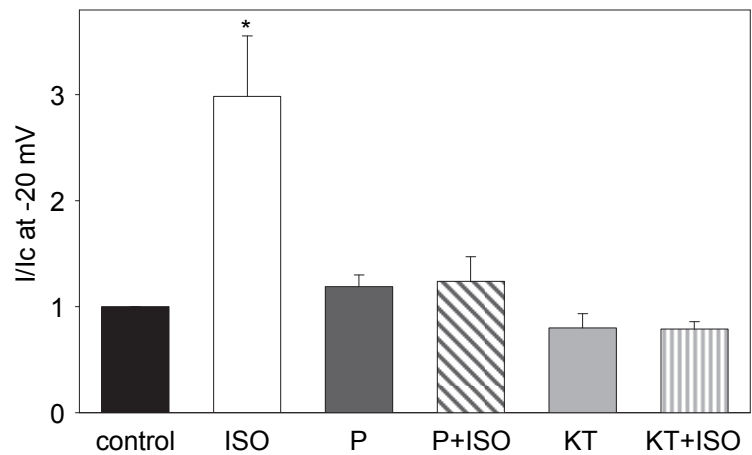


Figure 1

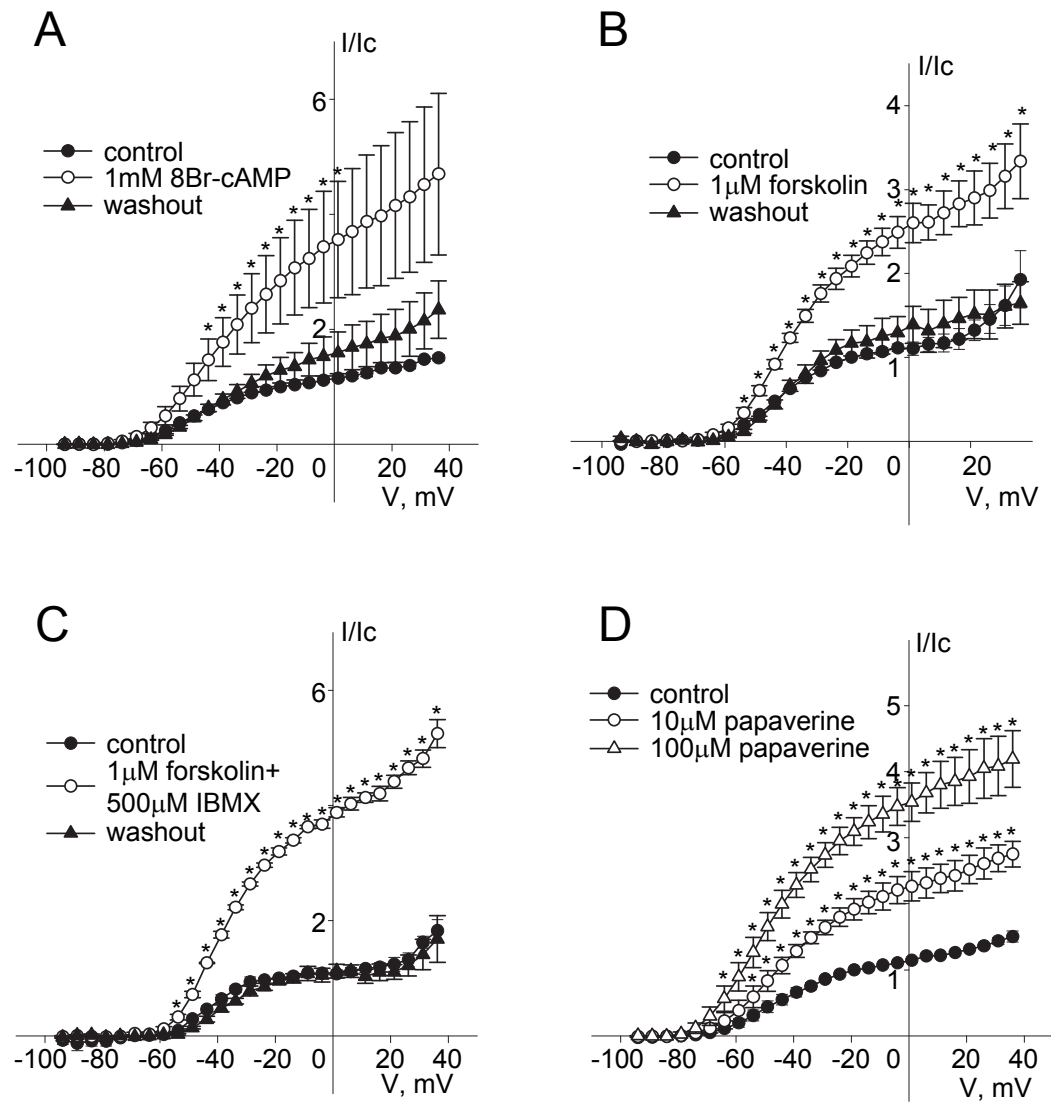


Figure 2

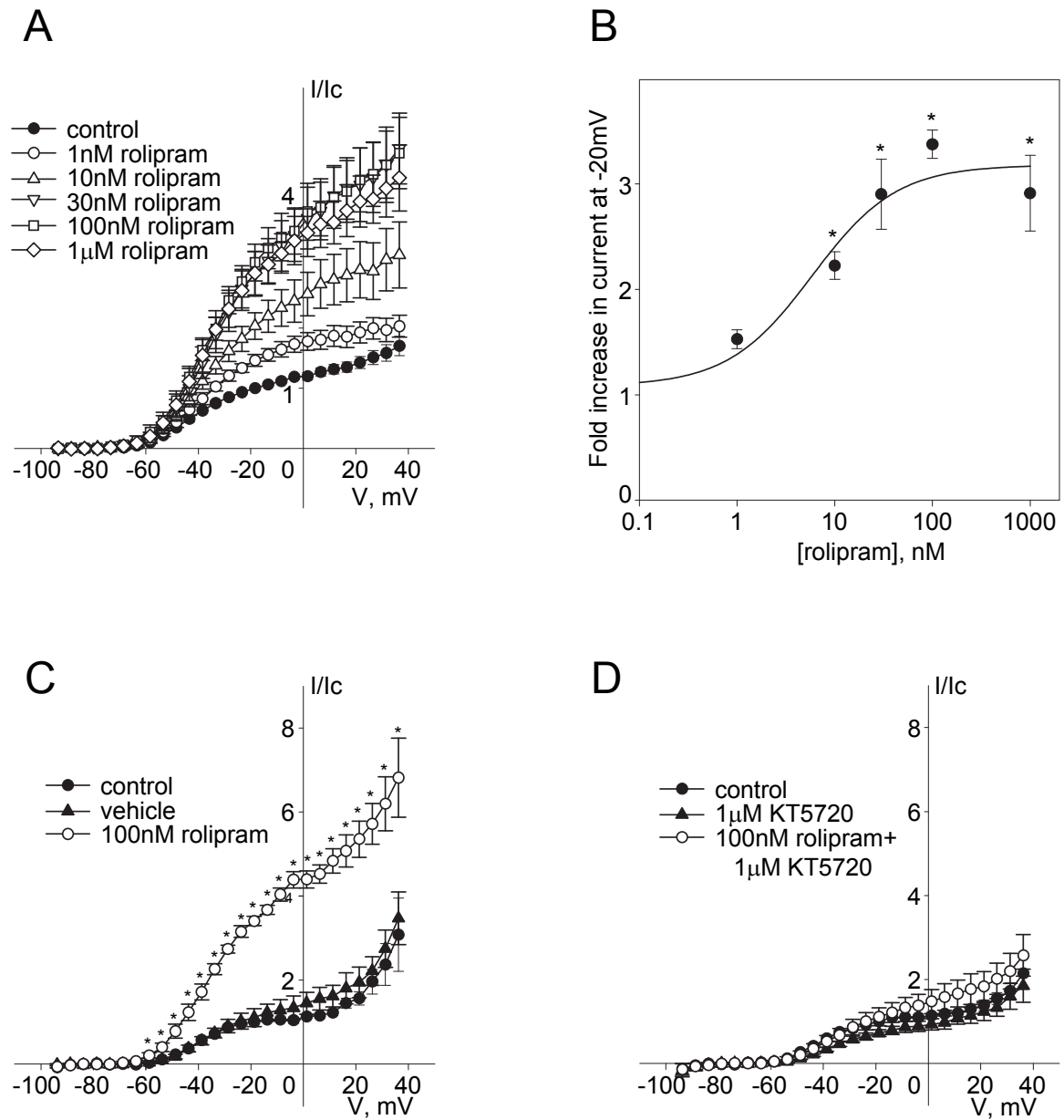


Figure 3

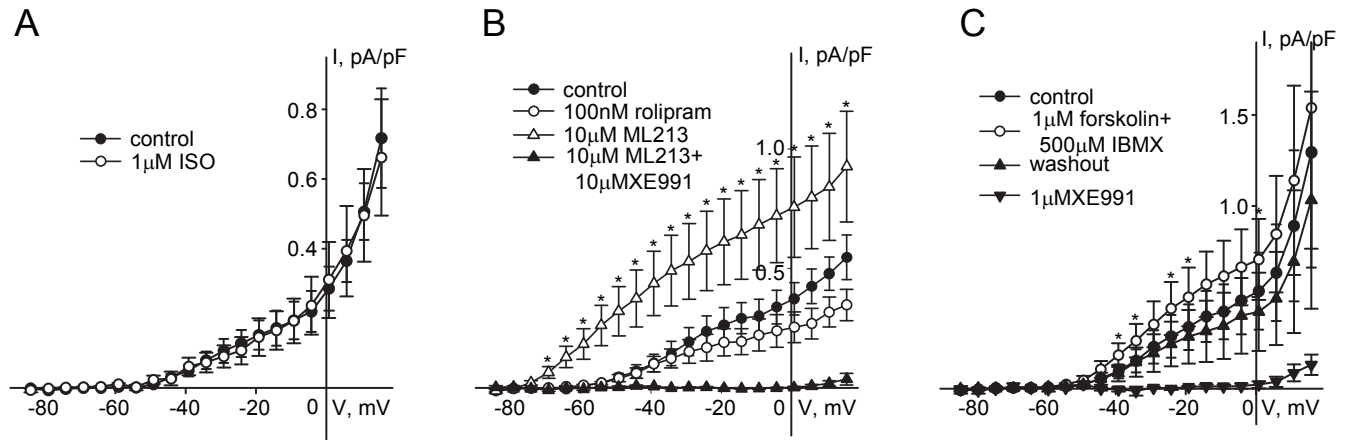


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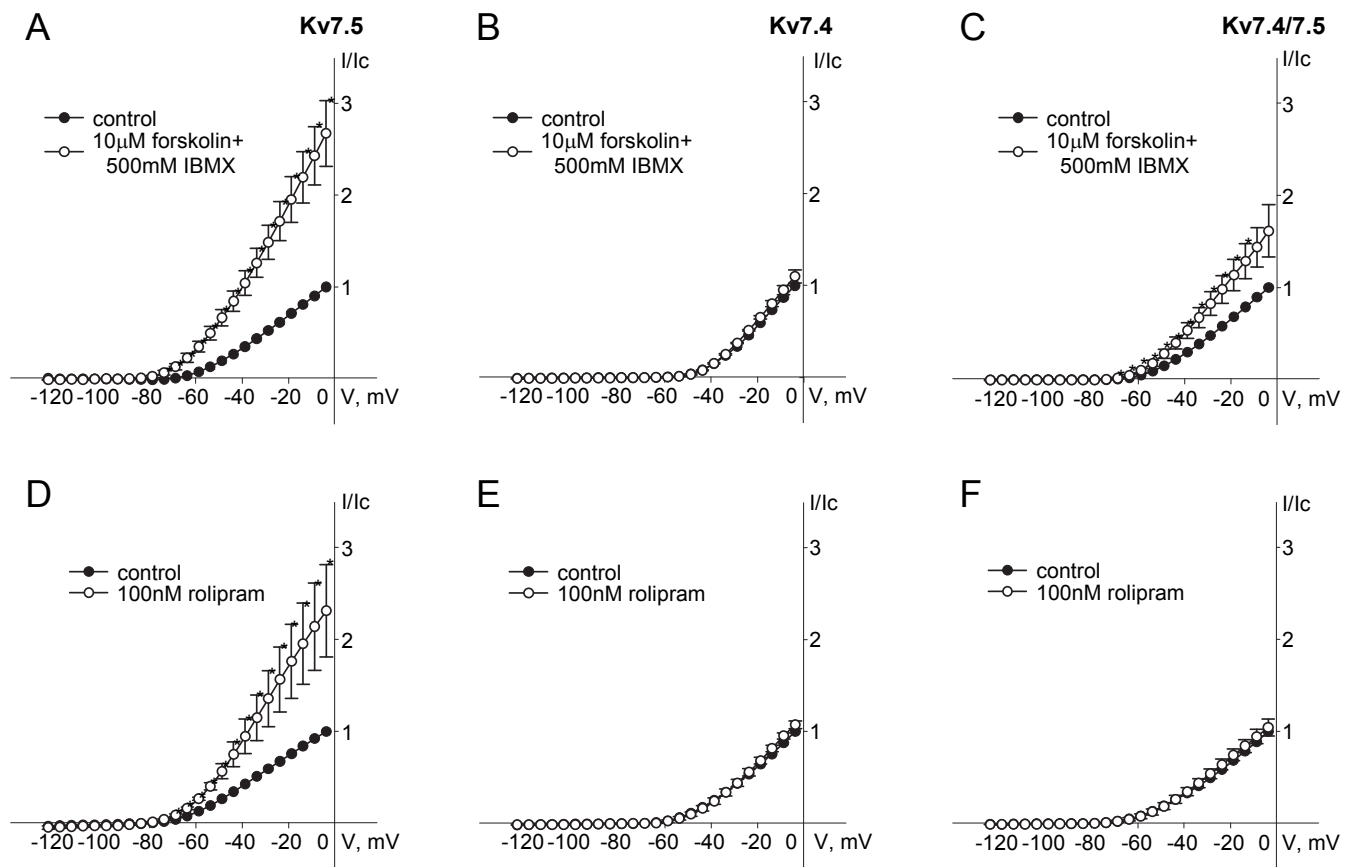


Figure 5

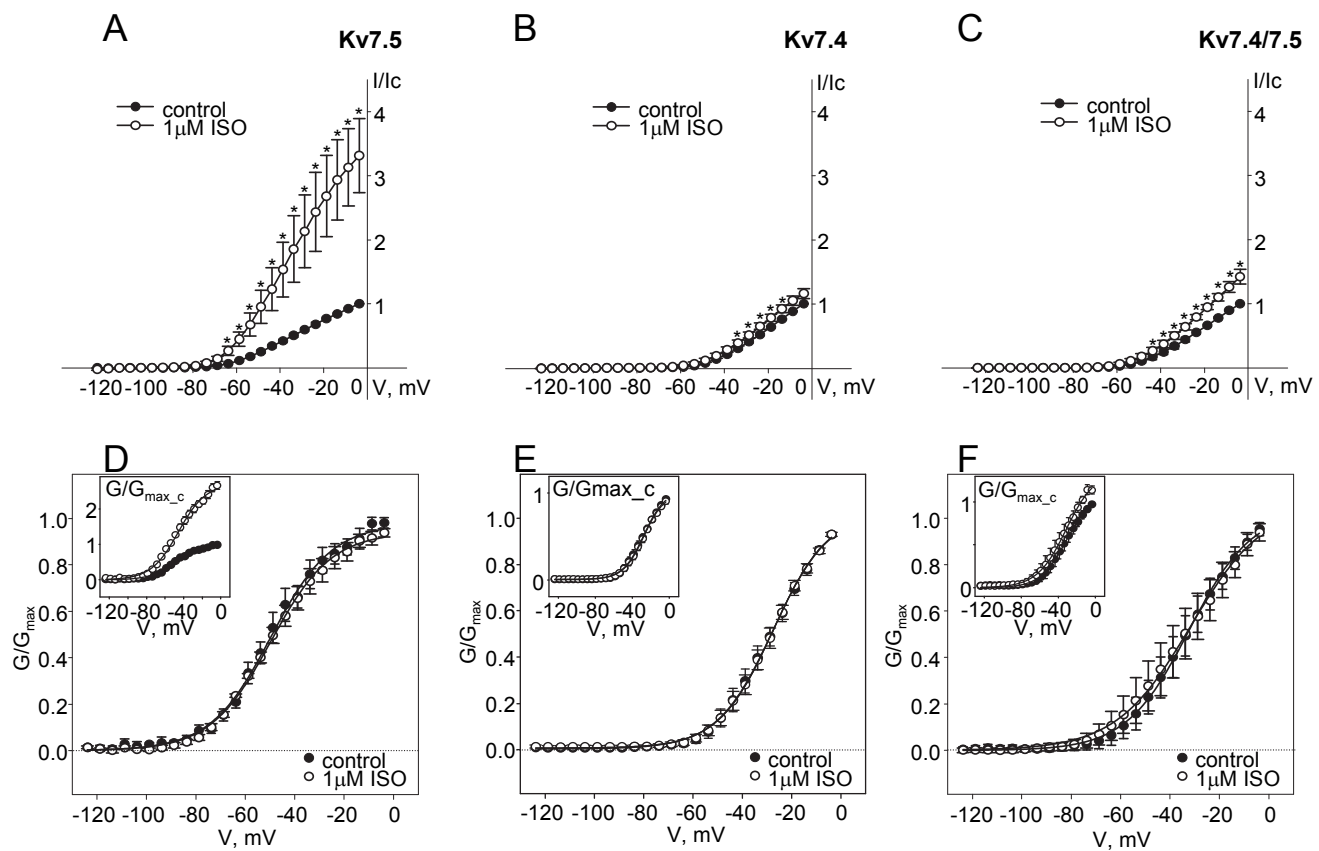


Figure 6

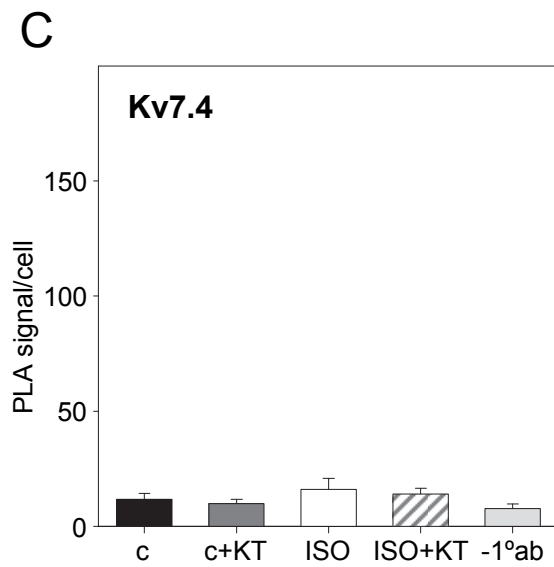
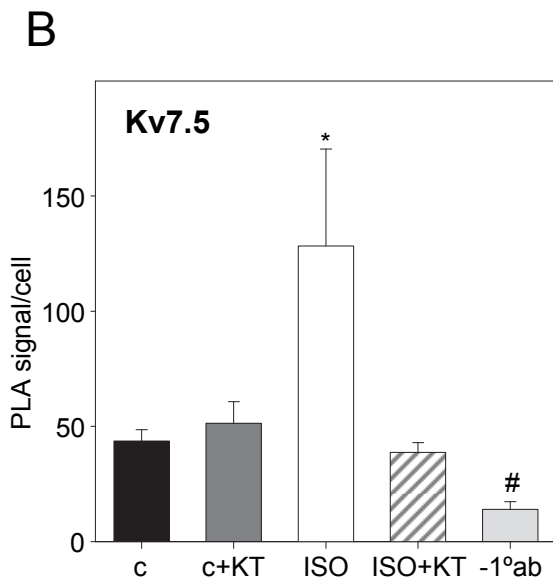
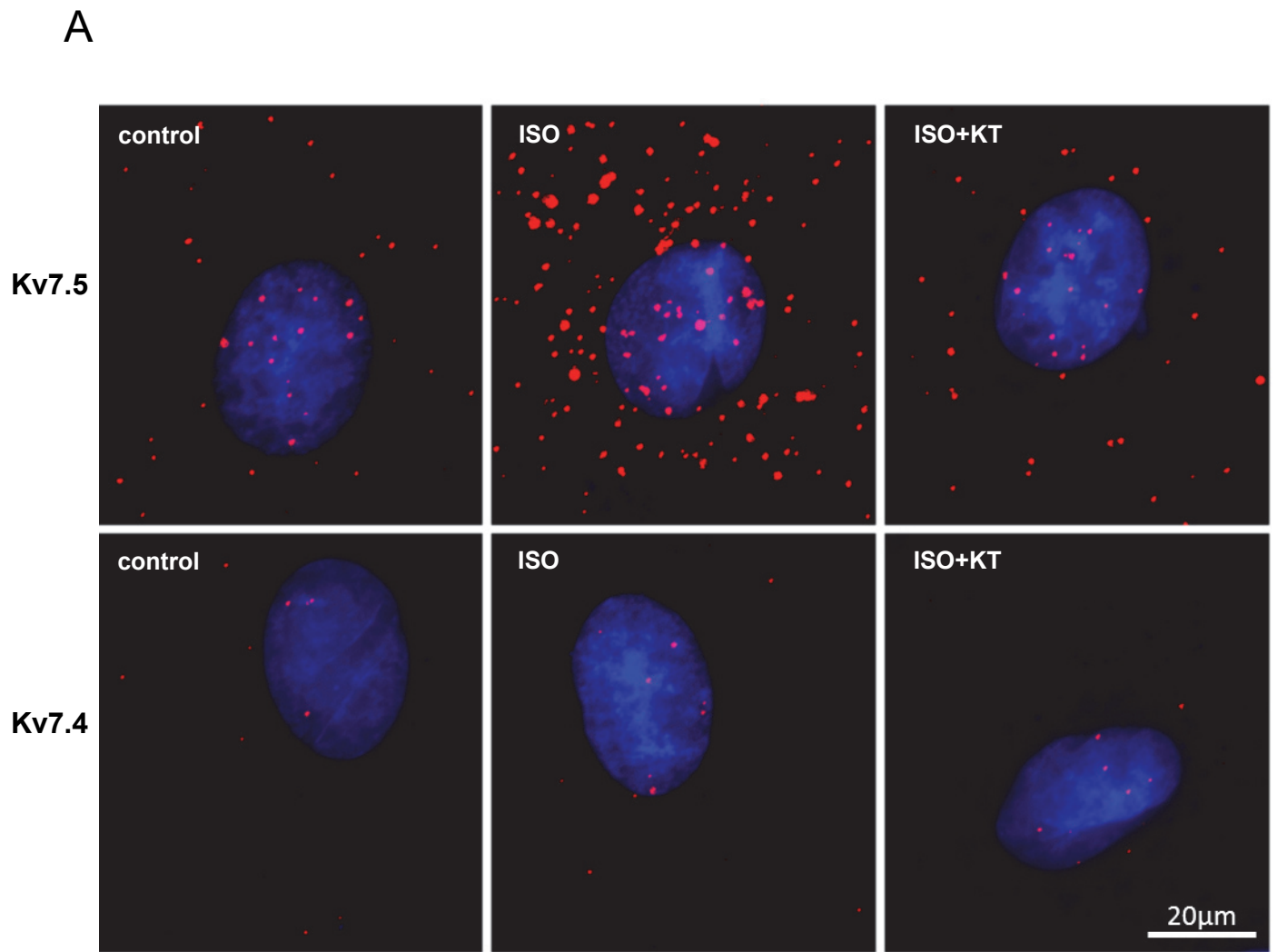


Figure 7