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Heteromeric complexes of aldo-keto reductase auxiliary $K_V\beta$ subunits (AKR6A) regulate sarcolemmal localization of $K_V1.5$ in coronary arterial myocytes

Matthew A. Nystoriak*, Deqing Zhang, Ganapathy Jagatheesan, Aruni Bhatnagar

Diabetes and Obesity Center, Institute of Molecular Cardiology, Department of Medicine, University of Louisville, Louisville, KY 40202, United States

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ABSTRACT

Redox-sensitive potassium channels consisting of the voltage-gated K^+ (K_V) channel pore subunit $K_V1.5$ regulate resting membrane potential and thereby contractility of vascular smooth muscle cells. Members of the K_V1 family associate with cytosolic auxiliary β subunits, which are members of the aldo-keto reductase (AKR) superfamily (AKR6A subfamily). The Kvβ subunits have been proposed to regulate Kv1 gating via pyridine nucleotide cofactor binding. However, the molecular identity of $K_V\beta$ subunits that associate with native $K_V1.5$ channels in the vasculature is unknown. Here, we examined mRNA and protein expression of $K_V\beta$ subunits and tested whether $K_V\beta$ isoforms interact with $K_V1.5$ channels in murine coronary arteries. We detected $K_V\beta1$ (AKR6A3), $K_V\beta2$ (AKR6A5) and $K_V\beta3$ (AKR6A9) transcripts and $K_V\beta1$ and $K_V\beta2$ protein in left anterior descending coronary arteries by real time quantitative PCR and Western blot, respectively. *In situ* proximity ligation assays indicated abundant protein-protein interactions between $K_V1.5/K_V\beta1$, $K_V1.5/K_V\beta2$ and $K_V\beta1/\beta2$ in coronary arterial myocytes. Confocal microscopy and membrane fractionation analyses suggest that arterial myocytes from $K_V\beta2$-null mice have reduced abundance of sarcolemmal $K_V1.5$. Together, data suggest that in coronary arterial myocytes, $K_V1.5$ channels predominantly associate with $K_V\beta1$ and $K_V\beta2$ proteins and that $K_V\beta2$ performs a chaperone function for $K_V1.5$ channels in arterial myocytes, thereby facilitating $Kv1\alpha$ trafficking and membrane localization.

1. Introduction

Voltage-gated potassium (K_V) channels, via regulation of membrane potential, control numerous physiological processes such as muscle contraction, neurotransmitter release and hormone secretion. These channels are octameric complexes consisting of membrane-bound α pore-subunits and cytoplasmic auxiliary β subunits [1]. The β subunits can promiscuously assemble as either homo- or hetero-tetramers, with each member (β1-3) capable of interacting with the N-terminal T1 docking motif (in K_V1 channels) or the C-terminus (in K_V4 channels) of the adjacent α subunit early in channel biosynthesis [2–5]. The $K_V\beta$ proteins belong to the aldo-keto reductase superfamily (AKR6A subfamily) and possess a core catalytic site and NADPH binding site common to those found in other AKR proteins. Whereas $K_V\beta$ subunits demonstrate weak enzymatic activity with aldehydes and ketones [6], the physiological relevance of $K_V\beta$-mediated catalysis remains unclear. However, it has been shown previously that $K_V\beta$ subunits participate in the regulation of membrane excitability via distinct modulation of K_V channel gating [7,8] and membrane trafficking [9,10].

Shaker-type K_V1 potassium channels play a critical role in the regulation of vascular smooth muscle membrane potential and contractility, and thereby control resistance artery diameter and tissue perfusion. In pulmonary resistance arteries, reduced current through oxygen-sensitive K_V channels contributes to hypoxia-elicited vasoconstriction via a mechanism proposed to involve associated $K_V\beta$ subunits [11,12]. Conversely, small diameter arteries and arterioles of the coronary circulation exhibit vasodilation in response to low oxygen to maintain myocardial oxygen availability [13,14]. Channels that are sensitive to inhibition by 4-aminopyridine and consisting of $K_V1.5$ α subunits have been implicated as essential components of coronary vasodilation in response to myocardial oxygen demand [15,16]. While association

* Corresponding author. Diabetes and Obesity Center, University of Louisville, Delia Baxter Bldg., 580 S. Preston St., Rm 421C, Louisville, KY 40202, United States.
E-mail address: matthew.nystoriak@louisville.edu (M.A. Nystoriak).

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with oxygen-sensing auxiliary subunits may impact $K_V1.5$ function under altered metabolic demand, the molecular identity of $K_V\beta$ subunits interacting with $K_V1.5$ channels in coronary arterial myocytes is not known.

In this study, we examined basal mRNA expression and relative protein abundance of $K_V\beta$ subunit isoforms in murine left anterior descending coronary arteries by qRT-PCR and Western blot, respectively. In situ proximity ligation assays performed in isolated coronary arterial myocytes revealed that $K_V1.5$ channels in coronary arterial myocytes are primarily associated with $K_V\beta1$ and $K_V\beta2$ subunits in coronary arterial myocytes. Consistent with the composition of $K_V\beta$ complexes found in other excitable cell types, our data suggest that these subunits may be arranged as hetero-multimers in native K_V channels within this vascular bed. Furthermore, cellular imaging and subcellular fractionation analyses of arterial myocytes from wild type and Kvβ2-null mice suggest that expression of $K_V\beta2$ subunits promotes surface localization of $K_V1.5$ channels at the sarcolemma of arterial myocytes. Based on these findings, we propose a potential functional role for $K_V\beta$ proteins in K_V1-mediated regulation of coronary vascular tone.

2. Materials and methods

2.1. Animals and tissue/cell isolation

This study was performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and in strict accordance with protocols approved by the Animal Care and Use Committee of the University of Louisville. Male mice, 16–20 weeks of age, were used. Wild type (C57Bl/6J, 129SvEv), $K_V\beta2^{-/-}$ (129SvEv background) [17] and $K_V\beta1.1^{-/-}$ (C57Bl/6NJ background) [18] were euthanized by intraperitoneal injection of sodium pentobarbital (200 mg/kg) and thoracotomy. First- and second-order left anterior descending coronary arteries were immediately dissected in ice-cold buffer containing (in mM): 134 NaCl, 6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 7 glucose, pH 7.4. Coronary arterial myocytes were isolated using enzymatic digestion procedures similar to those described previously [19]. Briefly, vessels were incubated in digestion buffer containing (in mM): 140 NaCl, 5 KCl, 2 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4 for 1 min at 37 °C, before incubation in digestion buffer containing 1 mg/mL papain (Worthington) and 1 mg/mL dithiothreitol (37 °C, 5 min with gentle agitation) and then in digestion buffer containing 1.25 mg/mL collagenase type H (Sigma) and 1 mg/mL trypsin inhibitor (Sigma) (37 °C, 5 min with gentle agitation). Following incubation in enzyme solutions, the cells were washed three times with ice-cold digestion buffer and triturated with a flame-polished Pasteur pipette. Cells were kept on ice until use (≤1 h).

2.2. Quantitative real time polymerase chain reaction

Total RNA was isolated from approximately 8 pooled coronary arteries per sample (n = 3) with Trizol (Invitrogen) and RNeasy MiniKit (QIAGEN) according to manufacturer's instructions. RNase-free DNase (QIAGEN) was used to remove any contaminating DNA. RNA was eluted from RNeasy minicolumns into 30 μL of RNase-free water and the concentration was measured using NANODROP 2000C (Thermo Scientific). Total cDNA was synthesized using iScript cDNA synthesis Kit (BIO-RAD) according to manufacturer's instructions. Briefly, 15 μL denatured RNA (1 μg), 4 μL 5X iScript reaction mix and 1 μL of iScript reverse transcriptase were mixed (total volume, 20 μL) and cDNA synthesis was carried out in a Bio-Rad MyCycler thermocycler using the following conditions: 42 ° C for 60 min and 94 ° C for 5 min. Total cDNA was diluted 5-fold using RNase-free water before use. Real-time quantitative PCR was performed using iTaq Universal SYBR green supermix (BIO-RAD) according to manufacturer's instructions. The following were added to each well of a 384 well plate sequentially: 5.0 μL iTaq 2X Universal SYBR green supermix, 2 μL diluted cDNA and 3 μL of mouse K_V channel primers (see Table 1 for primer sequences; mouse Kcna3 primer set was purchased from Qiagen-cat. no. PPM0407A) or primers for mouse GAPDH (for a total of 10 μL final volume) and subjected to qRT-PCR using standard protocols on an Applied Biosystems 7900 HT Real-Time PCR system. For each RNA sample, the cDNAs were run in triplicate for each primer set in the same plate with GAPDH as an internal control.

2.3. Western blot analysis

Whole tissue lysates were obtained from coronary arteries and cerebral cortex by sonication (10 min in ice bath) in lysis buffer containing (in mM): 150 NaCl, 10 Na_2HPO_4, 1 EDTA with 1% deoxycholic acid, 0.1% sodium dodecyl sulfate and protease inhibitors (Complete Mini protease inhibitor cocktail, Roche). For a set of experiments, crude membrane fractions were obtained as described previously [20]. Briefly, mesenteric arteries were pooled from two mice and were homogenized in a glass micro tissue grinder (Wheaton) with 10 mM Tris-HCl (pH 7.4) with 1.4 mM 2-mercaptoethanol containing protease inhibitors and centrifuged at 8000×*g* for 45 min. The pellets were discarded and the supernatants were centrifuged at 105,000×*g* for 1 h to obtain the membrane pellets, which were then solubilized in 75 μl of lysis buffer containing protease inhibitors. The solubilized membrane fractions were then sonicated in an ice bath for 30 min, incubated for 4 h at 4 °C with occasional shaking, and centrifuged at 48,000×*g* for 1 h. Supernatants were boiled in Laemmli sample buffer for 10 min and run on a 10% acrylamide gel (Bio-Rad) and subjected to SDS-PAGE. Following transfer to a polyvinylidene fluoride (PVDF) membrane, non-specific binding was blocked with 5% dry milk in Tris-buffered saline (TBS). Membranes were incubated overnight at 4 °C in primary antibodies against $K_V1.5$ (Alomone), $K_V\beta1$, $K_V\beta2$, $K_V\beta3$ or cadherin (Abcam) in TBS containing 0.1% Tween-20 (TBS-t). After five washes (10 min each, room temp) in TBS-t, membranes were incubated in TBS-t containing 1% BSA and horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling;

Table 1
qRT-PCR primer sequences used for K_V transcript detection in mouse coronary artery samples.

Gene	Primer sequences
$K_V1.1$	Forward: 5′ - AGATCGTGGGCTCCTTGTGT - 3′ Reverse: 5′ - ACGGGCAGGGCAATTGT - 3′
$K_V1.2$	Forward: 5′ - AGCTGATGAGAGAGATTCCCAGTT - 3′ Reverse: 5′ - GACTGCCCACCAGAAAGCA - 3′
$K_V1.4$	Forward: 5′ - TTTTGCAGAGGCGGATGAA - 3′ Reverse: 5′ - CAAAACGCATCTGGAATG CTT - 3′
$K_V1.5$	Forward: 5′ - TCAGGATCACTCCATCACCA - 3′ Reverse: 5′ - CTCAAGGGATCGAAGTAGCG - 3′
$K_V1.6$	Forward: 5′ - GAGTCCGTTTCTTTGACCCCT - 3′ Reverse: 5′ - GGCGACCTCCAGATTGATAGTA - 3′
$K_V2.1$	Forward: 5′ - CGTCATCGCCATCTCTCATG - 3′ Reverse: 5′ - CAGCCCACTCTCTCACTAGCAA - 3′
$K_V2.2$	Forward: 5′ - CACCTGGCTTGAACAGAAAG - 3′ Reverse: 5′ - TTGCTTCGGATAATGTCCAC - 3′
$K_V\beta1$	Forward: 5′ - GGAAGCCTACTCTGTCGCAC - 3′ Reverse: 5′ - CATGTCATTGCACCAACTCC - 3′
$K_V\beta2$	Forward: 5′ - GGGCAATAAACCCTACAGCA - 3′ Reverse: 5′ - ACTTTGGAGGTCGATTCCCT - 3′
$K_V\beta3$	Forward: 5′ - GGAAGGCAAGAAGCAACAAG - 3′ Reverse: 5′ - TGTAGGGAGCCCAGATGTTC - 3′
GAPDH	Forward: 5′ - AGGTCATCCCAGAGCTGAACG - 3′ Reverse: 5′ - GGAGTTGCTGTTGAAGTCGCA - 3′

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