Molecular Variants of KCNQ Channels Expressed in Murine Portal Vein Myocytes
A Role in Delayed Rectifier Current

Susumu Ohya, Gerard P. Sergeant, Iain A. Greenwood, Burton Horowitz

Abstract—We have analyzed the expression of KCNQ genes in murine portal vein myocytes and determined that of the 5 known KCNQ channels, only KCNQ1 was expressed. In addition to the full-length KCNQ1 transcript, a novel spliced form (termed KCNQ1b) was detected that had a 63 amino acid truncation at the C-terminus. KCNQ1b was not detected in heart or brain but represented approximately half the KCNQ1 transcripts expressed in PV. Antibodies specific for KCNQ1a stained cell membranes from portal vein myocytes and HEK cells expressing the channel. However, because the antibodies were generated against an epitope in the deleted, C-terminal portion of the protein, these antibodies did not stain HEK cells expressing KCNQ1b. In murine portal vein myocytes, in the presence of 5 mmol/L 4-aminopyridine, an outwardly rectifying K⁺ current was recorded that was sensitive to linopirdine, a specific blocker of KCNQ channels. Currents produced by the heterologous expression of KCNQ1a or KCNQ1b were inhibited by similar concentrations of linopirdine, and linopirdine prolonged the time-course of the action potential in isolated portal vein myocytes. Our data suggest that these two KCNQ1 splice forms are expressed in murine portal vein and contribute to the delayed rectifier current in these myocytes. (Circ Res. 2003;92:1016-1023.)

Key Words: smooth muscle ■ voltage-dependent potassium channels ■ linopirdine

Potassium channels encoded by KCNQ genes regulate electrical excitability of neurons and cardiac myocytes.¹,² To date, five members of KCNQ (1 to 5) have been identified in different mammalian cell types, and the expression products serve a number of functions. KCNQ1 contributes to the slow component of delayed rectifier K⁺ currents, Iₛ, in cardiac muscles.³ KCNQ2 and KCNQ3 contribute to M-currents in neurons.⁴,⁵ KCNQ4 and KCNQ5 are predominantly expressed in the inner ear and brain and sympathetic neurons,⁶ respectively. The electrophysiological properties of KCNQ channels differ between individual isoforms and are also modulated by coexpression with regulatory subunits encoded by KCNE genes. For example, the complex of KCNQ1 and KCNE1 constitutes the channel underlying native Iₛ, in cardiac muscles, and the complex of KCNQ1 and KCNE3 forms a basolateral K⁺ channel in tracheal and colonic epithelial cells.⁷ Mutations in several members of KCNQ and KCNE underlie inherited diseases such as cardiac long QT syndrome, epilepsy, and deafness.

In comparison to the data on KCNQ expression in neuronal and cardiac cells, there is no information as to whether these channels are expressed in vascular smooth muscle cells where K⁺ channels play significant roles in the control of vascular reactivity. The present study was to determine if KCNQ isoforms were expressed in smooth muscle cells isolated from murine portal vein (PV). Although we have shown recently that murine PV myocytes express ERG K⁺ channels,⁸ the overall data on murine K⁺ currents are sparse. In rabbit PV myocytes, a large component of the delayed rectifier current is 4-aminopyridine (4-AP) sensitive and virtually all the voltage-dependent current can be blocked by a combination of 4-AP and tetraethylammonium (TEA).¹⁰,¹¹ The molecular identification of the 4-AP–sensitive component of this current is encoded primarily by Kv1.2/Kv1.5 heterotetramer;¹² however, the calcium independent, TEA-sensitive component has not been identified as yet. The present study examined the possible contribution of KCNQ isoforms to delayed rectifier K⁺ currents in murine PV myocytes by comparing the pharmacological and biophysical properties of native currents in murine PVC with currents produced by the heterologous expression of KCNQ isoforms.

Materials and Methods
Dissection of Smooth Muscles and Smooth Muscle Cell Preparations
BALB/c mice (>30 days old) were sedated by exposure to isoflurane (Baxter Laboratories) and euthanized by cervical dislocation in accordance with Institutional Animal Care and Use Committee

Original received October 24, 2002; resubmission received March 4, 2003; accepted March 24, 2003.
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Circulation Research is available at http://www.circresaha.org
DOI: 10.1161/01.RES.0000070880.20955.F4
protocols. After incisions along the abdomen, individual PVs were dissected out as previously reported.13

**Total RNA Extraction and RT-PCR**

As previously reported,13 total RNA was extracted from tissues and isolated myocytes with the use of a TRIZOL (Life Technology Inc) and a SNAP total RNA isolation kit (Invitrogen), respectively. Total RNA was also isolated from brain and heart tissues.

**Quantitative PCR**

Real-time quantitative PCR was performed with the use of Syber Green chemistry on an ABI 5700 sequence detector (Perkin Elmer Inc), as previously reported.13

**Immunocytochemistry**

Immunocytochemistry was performed as previously reported.9 Briefly, isolated murine PV myocytes or HEK cells were seeded onto glass-bottom dishes. After fix with paraformaldehyde and permeabilization with Triton X-100, nonspecific binding sites were blocked with PBS containing 0.2% Triton X-100 and 1% normal goat serum. Cells were then exposed to anti-KCNQ1 polyclonal antibody (1:800 dilution, Chemicon and Santa Cruz Biotechnology) for 12 to 16 hours at 4°C. After removing excess primary antibody by repeated washing with PBS, the cells were exposed to Alexa Fluor 488 goat anti-rabbit (for Chemicon) or Alexa Fluor 488 donkey anti-goat (for Santa Cruz Biotechnology) IgG antibody (1:200 dilution, Molecular Probes). Excess secondary antibody was also removed by repeated washing with PBS. Digital images were viewed on a scanning confocal microscope (MRC600, BioRad). As a negative control, cells were preincubated with excess antigen before the addition of primary antibody.

**Isolated-Cell Preparations**

Enzymatically dispersed smooth muscle cells were placed in an experimental chamber under an inverted microscope as we had performed previously.9,14-16 Cells that were elongated and spindle-shaped (length 80 to 200 μm; diameter 5 to 6 μm) were selected. This process was performed until 10 to 60 smooth muscle cells were obtained. The cells were expelled from the micropipette into an RNase-free 0.5 mL tube. The micropipette was washed with Ca2+ free Hanks solution to remove any cells that might have adhered to the glass. The desired cells were snap frozen in liquid nitrogen and stored at −70°C.

**Cell Culture and Transformation**

Membrane currents were recorded from COS and HEK cells transiently transfected with KCNQ1 and the novel KCNQ1 splice variant, respectively. The full lengths of murine KCNQ1a and KCNQ1b were ligated into mammalian expression vectors, pcDNA3.1(+) and pTracer-CMV2 (Invitrogen) using T4 DNA ligase, respectively. The resulting constructs were confirmed by DNA sequencing, then transiently transfected into the COS and HEK cells, respectively, by the calcium phosphate coprecipitation technique. Electrophysiological experiments were performed after 48 to 72 hours.

**Mammalian Two-Hybrid Assay**

The protein-protein interaction between KCNQ1a, KCNQ1b, and KCNE3 significantly expressed in murine portal vein was analyzed by the mammalian two-hybrid system (Mammalian MATCH-MAKER Two-Hybrid Assay Kit; Clontech) as previously reported.17 Both pM and pVP16 constructs were transfected into HEK293 cells by calcium phosphate method, together with chloramphenicol acetyl transferase (CAT) reporter gene (pG5CAT). Forty-eight hours after transfection, CAT activity was measured. In the transactivation assays, only the pM and pG5CAT vectors were transfected. Analysis of CAT concentration was performed 48 hours after transfection on the CAT enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Interactions between the two gene products trigger CAT activity in pG5CAT. All transfections were performed in duplicate, and mean CAT concentrations were calculated. The CAT results were normalized in respect to total protein concentration. All interaction experiments were confirmed 3 times.

**Electrophysiological Experiments**

Current-voltage data from all cells were generated by stepping to test potentials between −80 mV and +60 mV from a holding potential of −60 mV and for durations of either 200 or 400 ms in 20-mV increments at 10-second intervals. The effect of different pharmacological agents was investigated after an initial equilibrium period. Cells were depolarized to +60 mV every 20 seconds and pharmacological agents were only applied if the control currents were stable for a period of 2 minutes. The concentration-effect relationships of TEA and linopirdine were determined by applying increasing concentrations of each agent with an exposure time of 2 minutes per concentration. The peak amplitude recorded in each concentration was normalized to the peak control amplitude recorded in the absence of either TEA or linopirdine. The data from each cell were fitted by a sigmoid dose-response (variable slope) function using GraphPad Prism 3.0 software.

**Results**

**Molecular Identification of KCNQ Subtypes Expressed in Murine PV**

We examined the expression levels of the KCNQ transcripts (KCNQ1 to 5) in murine PV by use of qualitative RT-PCR. Murine brain and heart-derived cDNAs were used as positive and/or negative controls for KCNQ primers to test their ability to produce correctly amplified mRNA transcripts. β-Actin primers were used to confirm that the products generated were representative of RNA (498-bp) and not contaminated with genomic DNA (which would produce an intron containing 708-bp band) because these primers were designed to amplify a segment that spanned an intron as well as two exons.

As shown in Figure 1A, KCNQ1 and KCNQ3 signals were easily detected in PV, whereas weak or no signals for KCNQ2, 4, and 5 were detected. As positive controls, all signals for KCNQ1 to 5 were easily detected in brain. Heart expressed KCNQ1, 4, and 5 (Figure 1A). Our previous study showed that KCNE3 but not KCNE1, KCNE1L, KCNE2, or KCNE4 was significantly expressed in murine PV.8 To avoid contamination from nonmyocytes, cell-based RT-PCR analyses were performed on freshly isolated murine PV smooth muscle cells (PVCs). Consistent with the results from Figure 1A, KCNQ1 signals were easily detected in PVC, whereas KCNQ2 to 5 ones were very weak or undetectable (Figure 1B).

**Quantitative Determination of KCNQ Transcripts in Murine PV**

We performed quantitative determination of KCNQ transcripts in murine PV. The ABI 5700 genetic analyzer (PE
Biosystems) was used for accurate quantification of steady-state transcript levels by RT-PCR. Total RNA from murine PV was reverse transcribed to cDNA, and steady-state transcripts were determined relative to an endogenous control housekeeping gene (β-actin). Therefore, the data are expressed as ratios of KCNQ1 to 5 to β-actin, respectively, and the relative transcriptional expressions of KCNQ1 to 5 are shown in Figure 1C. All expression data are expressed as mean±SE.

KCNQ1 expression relative to β-actin (arbitrary units) was 0.015±0.0018 in PV (n=5), whereas KCNQ2 to 5 expressions were less than 0.002 (Figure 1C). As positive controls, KCNQ1 to 5 expression was 0.019±0.00082 (KCNQ1, heart), 0.069±0.0042 (KCNQ2, brain), 0.024±0.0058 (KCNQ3, brain), 0.0069±0.00092 (KCNQ4, brain), and 0.040±0.0036 (KCNQ5, brain), respectively (n=5 for each).

Molecular Cloning of an Alternatively Spliced Isoform of KCNQ1 in Murine PV

Paulsen et al18 have shown that KCNQ1 is on mouse chromosome 11p15.5 and is comprised of 16 exons. Moreover, exon 1 of murine KCNQ has 5 different alternatively spliced isoforms (exon 1a-e). On the basis of genomic sequence information of murine KCNQ1 (GenBank accession No. AJ251835), we determined the isoforms specifically expressed in PV by RT-PCR. Of 5 different isoforms, only exon 1a was detected in PV.

We designed specific PCR primers to isolate full-length KCNQ1 from murine PV (primers are described in the online data supplement). As shown in Figure 2A, one single band of approximately 1.9-kb was detected in heart, whereas two different sized fragments were obtained in PV: approximately 1.9 and 1.6 kb. DNA sequencing determined that the larger fragment was original KCNQ1 (we term this KCNQ1a) and the shorter fragment was a novel alternatively spliced isoform of KCNQ1 (KCNQ1b). Analyses of the mouse heart and PV mRNA with RT-PCR and primers designed to span exons showed that KCNQ1b was missing exons 12 to 15 (Figures 2B and 2C). To identify the relative expression levels of KCNQ1b to KCNQ1a in PV, we performed quantitative RT-PCR analyses using specific primers for KCNQ1a but not

Figure 1. Expression of KCNQ channels in murine PV. A, RT-PCR detection of KCNQ channels (KCNQ1 to 5) in murine PV (PV). A 100-bp molecular weight marker was used to estimate the size of the amplicon, and the migration is shown on the right. Primers were tested on murine brain (Br) and heart (He) and sequenced to confirm their identity. B, Cell-based RT-PCR analysis in murine PV myocytes for 45 cycles. C, Quantitative RT-PCR for KCNQ1 to 5 expressions relative to β-actin in murine PV. Values are shown for steady-state transcripts relative to β-actin in the same preparation. Results are expressed as mean±SEM (n=5).

Figure 2. Expression of an alternatively spliced isoform of KCNQ1 and KCNQ1b in murine PV. A, RT-PCR detection of alternatively spliced isoforms of KCNQ1 (KCNQ1a and KCNQ1b) in murine PV. Primers were also tested on murine heart and sequenced to confirm their identity. A 1-kbp molecular weight marker was used to estimate the size of the amplicon. B, RT-PCR detection of KCNQ1 isoforms in murine PV. PCR products were generated through the use of gene-specific primers for the conserved region between KCNQ1a and 1b in the cytoplasmic C-terminal cytoplasmic domain (625 bp for KCNQ1a; 345 bp for KCNQ1b). C, Cell-based RT-PCR analysis in murine PV myocytes for 45 cycles. D, Quantitative RT-PCR for KCNQ1a expression relative to β-actin in murine heart and PV. Values are shown for steady state transcripts relative to β-actin in the same preparation.
KCNQ1b. KCNQ1a expression relative to β-actin (arbitrary units) in PV was 0.0084±0.00055 (n=5), KCNQ1a and KCNQ1b expression in PV was 0.015±0.0018, whereas KCNQ1a expression was 0.016±0.0022 in heart (n=5) (Figure 2D). Because primers designed to amplify both KCNQ1a and KCNQ1b detected no statistically significant difference with those designed to amplify KCNQ1a alone in murine heart, we conclude that KCNQ1b is not expressed (confirming the qualitative RT-PCR). However, in murine PV, the expression of KCNQ1a and KCNQ1b was approximately twice that of KCNQ1a alone, indicating that there is approximately equal expression of the 2 spliced transcripts. Each PCR product was confirmed by DNA sequencing (not shown). These results suggest that KCNQ1b transcripts may be significantly expressed in PV at similar expression levels to KCNQ1a.

The DNA sequence of KCNQ1a and KCNQ1b in the alternatively spliced region is shown in Figure 3. By deleting exons 12 to 15 the resulting transcript of KCNQ1b introduces a premature stop codon in exon 16 that truncates the encoded protein at amino acid 440 (Figure 3A). Comparison of the exon structure and C-terminal amino acids between the 2 splice variants are shown in Figures 3B and 3C.

**Cellular Distribution of KCNQ1 Proteins in Murine PV Myocytes**

To confirm that the identified KCNQ1 transcripts are translated into KCNQ1 proteins and expressed on the surface membranes of murine PV myocytes, the cellular localization of KCNQ1 proteins was examined by immunocytochemistry. However, all anti-KCNQ1 antibodies obtained from 2 biochemical companies were raised against a peptide mapping at the C-terminus of KCNQ1, showing that commercially available anti-KCNQ1 antibodies are specific for KCNQ1a but not KCNQ1b. Freshly isolated myocytes from murine PV were stained with two different anti-KCNQ1a antibodies and the local distribution of immunoreactivity was visualized by laser scanning confocal microscopy. By use of anti-KCNQ1a antibody from Santa Cruz Biotechnology Inc, the strong staining patterns of KCNQ1a proteins were localized along cell membrane in PV myocytes (Figure 4A), and KCNQ1a signals disappeared by preincubation with excess antigen (Figure 4B). We further stained HEK cells expressing either KCNQ1a or KCNQ1b that were used for functional expression. Figures 4C and 4D demonstrate that KCNQ1a-expressing cells show membrane staining with KCNQ1 antibody. However, KCNQ1b-expressing cells, in which the protein is truncated before the epitope encoding sequence, are not stained.

**Physical Interactions Between KCNQ1a, KCNQ1b, and KCNE3**

We used mammalian two-hybrid experiments to test whether KCNQ1b and KCNE3 physically interact. Figure 5 shows that when KCNQ1a and KCNQ1b or KCNQ1b and KCNE3 are expressed together does CAT activities increase. None of the negative control transfections increased CAT activity.

**Characteristics of Currents Produced by Expression of KCNQ1a and KCNQ1b**

Expression of KCNQ1a and KCNQ1b yielded voltage-dependent, outwardly rectifying K⁺ currents that were not observed in nontransfected cells (Figure 6). The peak amplitude of currents elicited from cells expressing KCNQ1a and KCNQ1b were not significantly different [mean amplitude at +40 mV was 563±71 pA (n=5) and 618±79 pA (n=18), respectively]. However, the mean time constant (τ) of activation for KCNQ1a measured at +60 mV was 126±7.8 ms, (n=16) versus 11±2 (n=8) for KCNQ1b. As the expression product of KCNE3 affects markedly the kinetics of KCNQ1 currents and mammalian-two hybrid experiments showed a physical interaction between KCNQ1b and KCNE3 expressing products, we determined whether KCNQ1b currents contained KCNE3.
were modified by coexpression with KCNE3. Figure 6Ci shows that currents generated by the coexpression of KCNQ1b and KCNE3 were qualitatively similar to currents due to KCNQ1b expression alone (mean \( \tau \) for activation at +60 mV was 9±1.8 ms, \( n=4 \)).

Figure 5. Interaction between KCNQ1a, KCNQ1b, and KCNE proteins. Two-hybrid analysis shows the interaction between KCNQ1a, KCNQ1b, and KCNE3. CAT activity is increased only when KCNQ1a, KCNQ1b, and KCNE3 are transfected in the same HEK cells.

Currents generated by the expression of mPV KCNQ1 isoforms were characterized further by comparing the relative sensitivities to linopirdine, which is considered to be a relatively selective inhibitor of KCNQ channels.1 Linopirdine produced a concentration-dependent inhibition of currents...
**Figure 7.** KCNQ1 currents contribute to delayed rectifier current in murine portal vein myocytes. A, Representative current traces evoked in a mPV myocyte by stepping to a test potential of +40 mV from −60 mV in the absence and presence of 5 mM 4-AP. B, Representative currents, evoked using the protocol in A, in the absence and presence of 100 μmol/L linopirdine. 4-AP (5 mM) decreased conductance levels by ~33%, whereas the addition of 100 μmol/L linopirdine decreased conductance levels by ~70%. C, Mean current-voltage relationships for outward currents in mPV myocytes in the absence and presence of 4-AP (5 mM) and linopirdine (100 μmol/L). Each point is the mean of 6 to 9 cells ±SEM. D, Mean concentration-effect curve for the inhibitory action of linopirdine. Each point is the mean of 6 cells ±SEM, and the solid line is a sigmoidal fit. E and F, Comparison of linopirdine-sensitive outward currents in mPV myocytes and HEK cells expressing KCNQ1b, respectively. Figures were generated by subtracting digitally the latter from control currents and are representative of at least 5 similar experiments.

**KCNQ Channels Expressed in Murine PV**

Outward currents in mPV myocytes were inhibited by 4-AP and linopirdine (Figures 7A and 7B). 4-AP (5 mM) reduced the amplitude of peak current at +40 mV from 466±40 to 309±36 pA, whereas a combination of 100 μmol/L linopirdine and 5 mM 4-AP 4-AP reduced the mean outward current further to 161±19 pA (n=6, Figure 7C). These figures show the inhibitory effect on the current-voltage (I-V) relationship of total outward current produced by 5 mM 4-AP alone and 4-AP plus 100 μmol/L linopirdine. The effects of linopirdine were concentration-dependent (Figure 7D, mean IC50 was 48±15 μmol/L, n=5) and were readily reversible within 2 minutes of washout. The mean IC50 values for inhibition of the native outward current by TEA and linopirdine were similar to those determined for currents generated by the expression of KCNQ1a and KCNQ1b (see previous section). The linopirdine-sensitive K+ currents in mPV cells (Figure 7E) activated with a time-course similar to that of KCNQ1b currents (mean τ for activation of the native current at +60 mV was 12±0.5, n=6). However, in comparison to linopirdine-sensitive KCNQ1b currents (Figure 7F), the native current declined during maintained depolarizations (mean decay τ at +40 mV was 3.9±0.25 seconds, n=5; Figure 7E). These data show that in murine portal vein cells a rapidly activating outward current is present that is relatively resistant to 4-AP and shares some pharmacological and kinetic characteristics with currents generated by the expression of KCNQ1a isoforms.

The physiological importance of KCNQ1 currents in mPV myocytes was assessed by examining the effect of linopirdine on action potentials elicited by the injection of depolarizing current in current clamp mode. Figure 8A shows that application of 100 μmol/L linopirdine markedly altered evoked depolarizations in mPV myocytes. Linopirdine augmented the amplitude of the evoked depolarization from 57±4 to 85±4 mV (n=5) and prolonged the time-course of the action potential (mean duration at 20% of peak amplitude was increased significantly from 68±17 to 123±25 ms, n=5, Figures 8B and 8C).

generated by expression of both isoforms of KCNQ1 cloned from mPV (Figures 6Ai and 6Bi) as well as KCNQ1b coexpressed with KCNE3 (Figure 6Ci) that was readily reversible on washout. The mean IC50 values for the linopirdine-dependent inhibition of K+ currents generated by the expression of KCNQ1a, KCNQ1b, and KCNQ1b and KCNE3 determined from the concentration effect curves shown in Figure 6 were 42±4 (n=11), 35±2.7 (n=6), and 40±4 μmol/L (n=4), respectively. These values were not significantly different to each other (P>0.05). The classical K+ channel blocker, TEA, inhibited both KCNQ1a and KCNQ1b currents with mean IC50 values of 2.1±0.4 (n=6) and 2.4±3.2 mmol/L (n=5), respectively. These values were similar to those for KCNQ channels described previously.19
formation of functional assembly. It has been shown that the assembly of the cytoplasmic C-terminal domain of KCNQ1 is essential for the assembly of $\alpha$-subunit, and deletions and mutations in the C-terminus of KCNQ1 cause nonfunctional channels. In addition, Nakamura et al have isolated 11 isoforms of murine KCNQ2 with different C-terminal cytoplasmic regions, resulting in the diverse M-current properties in neuronal cells. The novel splice variant recovered from this preparation differs in the carboxyl terminus from the established heart form. In the human form of this channel, the C-terminal portion is important in assembly of functional channels. Indeed, a mutation that eliminates a small domain between amino acids 589 and 620 renders the resulting channels nonfunctional. The mouse and human forms are identical in 25 of 31 residues in this domain, but the alternatively spliced KCNQ1b is deleted for this entire region. No information has been reported concerning the functional importance of this portion of the protein in mouse KCNQ1. However, KCNQ1b alone elicits functional channels when expressed in HEK cells, and there are only minor differences in the properties of the KCNQ1a and KCNQ1b channels. It is not clear which of the splice variants is the predominant channel in murine PV myocytes and more studies concerning blockers and electrophysiological properties will be necessary to determine differences in the two splice forms to make a distinction in native mixed current records. In addition, there is the distinct possibility that the two splice forms of the channel form heteromultimers with somewhat different properties than either form alone. Because KCNE3 is also expressed in this preparation, the role of auxiliary subunits must also be considered. In human colonic crypt cells and the human forms of these proteins in heterologous expression systems, KCNE3 combines with KCNQ1 to alter the activation kinetics and results in a constitutively open $K^+$ channel with linear voltage dependence. These properties were not observed for the linopirdine-sensitive current in murine PV myocytes that exhibited an outwardly rectifying current-voltage relationship, although interactions between these subunits were detected by mammalian two-hybrid analysis.

Significantly less of the delayed rectifier $K^+$ current in mouse PV myocytes is sensitive to block by 4-AP than that of rabbit or guinea pig PV myocytes. However, it has not been determined whether rabbit or rat PV cells express KCNQ channels. Because such a large portion of the delayed rectifier $K^+$ current in rabbit and rat PV myocytes is sensitive to 4-AP, much of the effort to identify molecular components for this current centered on 4-AP–sensitive channel genes. This is not the case for murine PV myocytes, and the identification of KCNQ1 in these cells may be the cause of this difference.

The human LQT1 locus is responsible for the most common forms of inherited cardiac arrhythmias (Romano-Ward syndrome and Jervell and Lange-Neilsen syndrome). In a multimeric arrangement, KCNQ1 and KCNE1 encode the slowly activating component of delayed rectifier current ($I_{ca}$) in cardiac myocytes. The elimination of this current through mutations in either subunit causes the arrhythmias. The results of the present study show that KCNQ genes are also expressed in vascular smooth muscle cells, and as a consequence, hereditary mutations may contribute to vascular perturbations. It is interesting that murine portal vein myo-
cytes also express ERG genes\(^\text{10}\) that are also the focus of hereditary mutations leading to cardiac arrhythmias. At the moment, it is not clear how KCNQ channels interact with other K\(^+\) channels to control vascular excitability. However, analysis of a murine KCNQ1 knock-out positioned in smooth muscles or studying vascular myocytes from human subjects with hereditary arrhythmias may answer these functional questions.

In conclusion, this study has shown for the first time the existence of a novel splice variant of KCNQ1 that is expressed in murine portal vein smooth muscle cells. This isoform, possibly in concert with accessory subunits contributes to a part of the native outward current in these cells.

Acknowledgments

This work was supported by DK41315. The nucleotide sequence data reported in this article (murine KCNQ1b) has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession No. AB170963. We thank Lisa Miller for technical support for molecular biology and Heather Beck and Nancy Horowitz for technical support with cell culture.

References

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_Circ Res._ 2003;92:1016-1023; originally published online April 10, 2003; doi: 10.1161/01.RES.0000070880.20955.F4

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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MATERIALS AND METHODS

PCR primers for KCNE subtypes


Dissection of smooth muscles and smooth muscle cell preparations

BALB/c mice (>30 days old) were sedated by exposure to isoflurane (Baxter Laboratories) and sacrificed by cervical dislocation in accordance with Institutional Animal Care and Use Committee protocols. After incisions along the abdomen, individual PVs were dissected out. Thin strips of PV muscle were placed in PSS (composition below) containing 50 μM Ca²⁺ for 10 min at 36 °C, then in 50 μM Ca²⁺ PSS containing 3 mg ml⁻¹ collagenase (type 1A, Sigma), 2 mg ml⁻¹ trypsin inhibitor (Sigma), 2 mg ml⁻¹ bovine serum albumin (BSA, Sigma) and 0.1 mg ml⁻¹ protease (type XIV,
Sigma) for 10 min at 36 °C. Then freshly dispersed smooth muscle cells were used for
electrophysiological experiments and RNA preparations.

All electrophysiological experiments were performed at room temperature using
the whole-cell configuration and an Axopatch 200B amplifier. Voltage clamp protocols
were generated and analyzed using pCLAMP 8 software (Axon Instruments) and Origin
5 software. For RNA preparation, single cells were collected through applied suction by
aspirating them into a wide-bore borosilicate pipette and the pipette contents were ejected
into a sterile 1.5 ml tubes. Their characteristic spindle shaped morphology differentiated
smooth muscle cells. Approximately 50 smooth muscle cells were collected, flash-frozen
in liquid nitrogen, and stored at -80°C until use.

**Total RNA extraction and RT-PCR**

Total RNA was extracted from tissues and isolated myocytes with the use of a
TRIZOL (Life Technology Inc.) and a SNAP total RNA isolation kit (Invitrogen),
respectively. Total RNA was also isolated from brain and heart tissues. cDNA was
prepared from the RNA preparations by using the SUPERSCRIPT™ II RNase H⁻ (Life
Technology Inc.) and 200 µg/ml of random hexamer were used to reverse transcribe the
RNA sample. The resulting cDNA product was amplified with gene-specific primers by
PCR and AmpliTaq-Gold Taq DNA polymerase (Applied Biosystems). The
amplification profile for these primer pairs were follows: a 10 sec denaturation step at 95
° C, a 10 sec annealing step at 60 ° C, and a 30 sec primer extension step at 72 ° C. In the
tissue- and cell-based RT-PCR, the amplification was performed for 35 and 45 cycles,
respectively. The amplified products (6 µl) were separated by electrophoresis on a 2 %
agarose/1x TAE (Tris, acetic acid, EDTA) gel, and the DNA bands were visualized by ethidium bromide staining. For the RNA control, a cDNA reaction was used as a template for which the reverse transcriptase was not added, controlling for genomic DNA contamination in the source RNA. The no-template control was a PCR amplification for which the template was not added, controlling for non-specific amplification and spurious primer-dimer fragments. These negative controls were subjected to a second round of amplification to assure specificity of the reaction and the quality of the reagents. Each amplified product was sequenced by the chain termination method with an ABI PRIZM (model 310, Perkin Elmer Inc.).

**Quantitative PCR**

Real time quantitative PCR performed with the use of Syber Green chemistry on an ABI 5700 sequence detector (Perkin Elmer Inc.) as previously reported. Regression analysis of the mean values of four multiplex RT-PCRs for the log_{10} diluted cDNA was used to generate standard curves. Unknown quantities relative to the standard curve for a particular set of primers were calculated, yielding transcriptional quantification of KCNQ gene products relative to the endogenous standard (β-actin).

**Immunocytochemistry**

Immunocytochemistry was performed as previously reported. Briefly, isolated smooth muscle cells of the murine PV or HEK cells were seeded onto glass-bottom dishes. After fix with paraformaldehyde and permeabilization with Triton X-100, non-specific binding sites were blocked with PBS containing 0.2 % Triton X-100 and 1 %
normal goat serum. Cells were then exposed to anti-KCNQ1 polyclonal antibody (1:80 dilution, Chemicon & Santa Cruz Biotechnology) for 12-16 hr at 4 °C. After removing excess primary antibody by repeated washing with PBS, the cells were exposed to Alexa Fluor 488 goat anti-rabbit (for Chemicon) or Alexa Fluor 488 donkey anti-goat (for Santa Cruz Biotechnology) IgG antibody (1:200 dilution, Molecular Probe). Excess secondary antibody was also removed by repeating washing with PBS. Digital images were viewed on a scanning confocal microscope (MRC600, BioRad). As a negative control, cells were preincubated with excess antigen before the addition of primary antibody.

**Isolated-Cell Preparations**

Enzymatically dispersed smooth muscle cells were placed in an experimental chamber under an inverted microscope as we had performed previously (Nikon, Japan). The isolated cells were allowed to settle to the bottom of the chamber for approximately 5 minutes. A micromanipulator (World Precision Instruments Inc., Sarasota, FL) containing a large diameter micropipette tip was positioned at the bottom of the chamber in which the cells had attached. Capillary pipettes were made from borosilicate glass capillaries (Sutter Instrument Co., Navato, CA), pulled on a micropipette puller, then flame polished to obtain a diameter of ≈500µm. Through applied suction, smooth muscle cells were aspirated into the micropipette. The cells selected were based on the same criteria as those used for electrophysiological examination. Cells that were elongated and spindle-shaped (length 200-300µm; diameter 5-6µm) were selected. This process was carried out until 10-60 smooth muscle cells were obtained. The cells were expunged from the micropipette into an RNase free 0.5ml tube. The micropipette was washed with Ca²⁺ -
free Hanks solution to remove any cells that might have adhered to the glass. The desired cells were snap frozen in liquid nitrogen and stored at -70°C.

**Cell culture and transformation**

Membrane currents were recorded from COS and HEK cells transiently transformed with KCNQ1 and the novel KCNQ1 splice variant respectively. The full lengths of murine KCNQ1a and KCNQ1b were ligated into mammalian expression vectors, pcDNA3.1(+) and pTracer-CMV2 (Invitrogen) using T4 DNA ligase (New England BioLabs, Beverly MA), respectively. The resulting constructs were confirmed by DNA sequencing, then transiently transformed into the COS and HEK cells respectively by the calcium phosphate co-precipitation technique. Electrophysiological experiments were performed after 48-72 hours.

**Mammalian two-hybrid assay**

The protein-protein interaction between KCNQ1b and KCNE3 significantly expressed in murine portal vein was analyzed by the mammalian two-hybrid system (Mammalian MATCHMAKER Two-Hybrid Assay Kit; Clontech) as previously reported. Both pM and pVP16 constructs were transfected into HEK293 cells by calcium phosphate method, together with chloramphenicol acetyl transferase (CAT) reporter gene (pG5-CAT). 48 hours after transfection, chloramphenicol acetyltransferase (CAT) activity was measured to evaluate the association between KCNQ1b and KCNE3. In the transactivation assays, only the pM and pG5-CAT vectors were transfected. Analysis of CAT concentration was performed 48 hours post-transfection on the CAT enzyme-linked
immunosorbent assay kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. All transfections were performed in duplicate, and mean CAT concentrations were calculated. The CAT results were normalized in respect to total protein concentration. All interaction experiments were confirmed three times.

**Electrophysiological experiments**

Current-voltage data from all cells were generated by stepping to test potentials between –100 mV and +60 mV from a holding potential of -60 mV and for durations of either 200 or 400 ms in 20 mV increments at 10 s intervals. The effect of different pharmacological agents was investigated after an initial equilibrium period. Cells were depolarized to +60 mV every 20 s and pharmacological agents were only applied if the control currents were stable for a period of 2 min. The concentration-effect relationships of TEA and linopirdine were determined by applying increasing concentrations of each agent with an exposure time of 2 min per concentration. The peak amplitude recorded in each concentration was normalized to the peak control amplitude recorded in the absence of either TEA or linopirdine. The data from each cell were fitted by a sigmoid dose-response (variable slope) function using Prism 3 GraphPad Software.

**Solutions**

The composition for the PSS was as follows (mM): NaCl (135), KCl (5), HEPES (10), glucose (10), CaCl₂ (2.0), MgCl₂ (1.2), the pH was set to 7.4 with TRIS (1M.) The basic external solution used for all electrophysiological experiments had the following composition (mM): NaCl (126), KCl (5), HEPES (10), glucose (20), CaCl₂ (0.1), MgCl₂
(1.0) pH was set to 7.3 with 10 M KOH. All experiments on murine PV smooth muscle cells were also conducted in the presence of 5 mM 4-AP to reduce contamination from endogenous Kv channels. The pH of these solutions was set to 7.3 by the addition of 10 N HCl. The internal solution for these experiments had the following composition (mM): K-aspartate (100), KCl (30), HEPES (10), EGTA (5), ATP-\(\text{Na}_2\) (5), GTP (0.1), MgCl\(_2\) (1), pH 7.2. Activation of Ca\(^{2+}\)-dependent Cl\(^-\) currents, common in murine PV smooth muscle cells\(^2\) was precluded by the inclusion of a high concentration of the Ca\(^{2+}\)-chelator EGTA. Recording of ATP-sensitive K\(^+\) currents was similarly inhibited by the inclusion of 1 mM ATP in the pipette solution. Drugs were added by means of a close perfusion delivery system placed ~ 300 uM away via a tip diameter of ~ 200 uM. The dead-space time for switch of solutions is less than 5 secs.

**Statistics**

All data are the mean of at least 5 cells ± SEM and in experiments using murine PV smooth muscle cells experiments data was taken from at least 3 different animals. For quantitative RT-PCR the reproducibility of the assay was tested by analysis of variance (ANOVA) comparing repeat runs of samples, and mean values generated at individual time points were compared by Student’s \(t\)-test.

**Figure 1. Expression of KCNE subtypes in HEK293 and COS-7 cells.** A: RT-PCR detection of five KCNE subtypes (KCNE1-4 & 1L) in HEK293 and COS-7 cells. PCR products were generated through the use of gene-specific primers for KCNE1-4, KCNE1L, and GAPDH in 35 cycle PCR. Amplified products were separated on 2.5 % agarose gels and were identified by ethidium bromide staining. A 100-bp molecular
weight marker was used to estimate the size of the amplicon and the migration is shown on the right.

Figure 2. Expression of KCNQ1 isoforms and KCNE3 in HEK293 transfectants. A: RT-PCR detection of KCNQ1 isoforms (upper panel: 625 bp for KCNQ1a; 345 bp for KCNQ1b) and KCNE3 (lower panel) in HEK293 transfectants: HEK-C, the vector DNA, pcDNA3.1(+)/Neo expressed-HEK293 cells; HEK-Q1a, KCNQ1a-expressed HEK293 cells; HEK-Q1b, KCNQ1b-expressed HEK293 cells; HEK-Q1a+Q1b, both KCNQ1a and KCNQ1b-expressed HEK293 cells; HEK-Q1b+E3, both KCNQ1b and KCNE3-expressed HEK293 cells. PCR products were generated through the use of gene-specific primers for KCNQ1a/b and KCNE3 in 30 and 35 cycle PCR, respectively. Amplified products were separated on 2.0 % agarose gels and were identified by ethidium bromide staining. A 100-bp molecular weight marker was used to estimate the size of the amplicon and the migration is shown on the right. B: Quantitative RT-PCR for KCNE3 expressions relative to GAPDH in HEK293 transfectants. Values are shown for steady state transcripts relative to GAPDH in the same preparation. Results are expressed as means ± SEM (n=4).

Reference List


Figure 1 supplement

HEK293

COS-7
Figure 2 supplement

A

HEK-C  HEK-Q1a  HEK-Q1b  HEK-Q1a+Q1b  HEK-Q1b+E3

KCNQ1a/b

KCNE3

bp

200  100

B

ratio to β-actin

HEK-C  HEK-Q1a  HEK-Q1b  HEK-Q1a+1b  HEK-Q1b+E3  HEK-E3