Cloning and Functional Expression of a Novel Cardiac Two-Pore Background K⁺ Channel (cTBAK-1)

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Abstract—We have cloned from a mouse heart cDNA library a novel K⁺ channel subunit that has two pore-forming domains and four transmembrane regions. Its amino acid sequence shares 25% identity with mouse TWIK-1, 22% with mouse TREK-1, and 33% with a putative K⁺ channel of C. elegans (C40C9). Strikingly abundant mRNA for this clone was expressed in the heart. The mRNA was also detected in kidney, brain, skin, testis, lung, skeletal muscle, small intestine, and stomach but not in liver, thymus, or spleen. Reverse transcription–polymerase chain reaction analyses of single cells showed that the mRNA of the clone was expressed in both atrial and ventricular myocytes per se. Xenopus oocytes injected with the cRNA of the clone expressed a Ba²⁺-selective K⁺-selective current with an almost linear steady-state current-voltage relationship. In cell-attached patches, the expressed channel exhibited short-lasting openings with a mean open time of ≈2 milliseconds and a unitary conductance of ≈16 pS (150 mmol/L [K⁺]). The K⁺ current was insensitive to intracellular Na⁺ (50 mmol/L), Ca²⁺ (0.1 mmol/L), H⁺ (pH 6.4), and arachidonic acid (10 μmol/L) in inside-out patches. Thus, the current flowing through the channel may contribute to the cardiac cellular electrical activity as a linear background K⁺ conductance. Therefore, we designated the clone cTBAK (cardiac two-pore background K⁺ channel). (Circ Res. 1998;82:513-518.)

Key Words: two-pore-domain K⁺ channel • molecular cloning • heart • cardiac myocyte • functional expression

Since the cloning of the Shaker K⁺ channel,¹ many types of K⁺ channels have been identified by expression and homology cloning and by searching in the GenBank for conserved K⁺ channel pore-domain sequences. K⁺ channels can now be structurally classified into three major groups containing two, four, and six putative TM regions. The K⁺ channels with six TM regions include voltage-dependent Kv channels, such as Shaker and HERG, and large- and small-conductance Ca²⁺-activated K⁺ channels, those with two TM regions are the inwardly rectifying Kir channels composed of six subfamilies (Kir 1.x to Kir 6.x).² ¹

Recently, TWIK-1, a weak inwardly rectifying K⁺ channel with two pore domains and four putative TM regions, was identified from a human kidney cDNA library.³ TWIK-1 mRNA was expressed abundantly in heart and brain and, to a much lesser extent, in other tissues such as lung, kidney, placenta, and liver. In C. elegans, 23 putative two pore-domain K⁺ channel subunits with four TM regions have been identified.² In functional expression experiments, TWIK-1 exhibited weak inwardly rectifying K⁺ channel properties.³ On the other hand, another two pore-domain K⁺ channel, TREK-1, showed outwardly rectifying properties.⁴ Thus, these two pore-domain K⁺ channels may serve as background K⁺ currents in various cells. However, in mammals, the molecular diversity of the two pore-domain K⁺ channels compared with voltage-dependent Kv and inwardly rectifying Kir channels is still poorly understood.

To elucidate the diversity of the mammalian two pore-domain K⁺ channel subunits, we have screened the mouse heart cDNA library using a partial DNA fragment of TWIK-1 cDNA. Subsequently, we have isolated a novel two pore-domain K⁺ channel subunit cDNA. Its amino acid sequence shares ≈20% to 30% homology with other two pore-domain K⁺ channels. The RNA transcript of the new clone was found to be expressed abundantly in the heart. Its expression was further detected in isolated cardiac myocytes by single-cell RT-PCR analyses. The novel K⁺ channel subunit was able to form a functional K⁺ channel when heterologously expressed in Xenopus oocytes or in HEK293T cells. The expressed channels exhibited weak rectification and short-lasting openings. Because the channel was constitutively active at various potentials, it may contribute as a linear background K⁺ current to the formation of cardiac action potential. Thus, we designated the clone cTBAK (cardiac two-pore background K⁺ channel). The physiological function and native equivalent of this K⁺ channel in cardiac myocytes remain to be determined.

Materials and Methods

Library Screening

Total RNAs prepared from whole rat atria were reverse-transcribed using an oligo(dT) primer.⁷ A rat TWIK-1 cDNA fragment of 630 bp...
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Selected Abbreviations and Acronyms

cTBAK = cardiac two-pore background K+ channel
M (with number) = TM region
Np_o = open probability
P (with number) = pore domain
PCR = polymerase chain reaction
RT = reverse transcription
TM = transmembrane

was obtained by RT-PCR using total RNAs from rat atria. Primers for PCR reaction were located in nucleotides 343 to 363 and nucleotides 971 to 990 of human TWIK-1 cDNA. A mouse heart cDNA library (Clontech Laboratories, Inc) in λgt10 was screened with a rat TWIK-1 cDNA fragment as a probe under a mild-stringency condition. Hybridization was carried out in 20% formamide, 5× SSC, 5× Denhardt’s solution, 0.5% SDS, and 0.2 mg/ml denatured salmon sperm DNA at 42°C. Filters were washed twice with the solution containing 0.2× SSC and 0.1% SDS for 15 minutes each at 42°C. Twenty positive clones were isolated. One positive clone (16e) contained a partial coding region of a new K+ channel subunit that has some similarities with TWIK-1. Thus, we screened the same heart library with the Kpnl-EorI fragment (1 kb) of 16e in a high-stringency condition. Filters were washed twice with solution containing 0.1× SSC and 0.1% SDS for 20 minutes each at 65°C. Two positive clones were isolated. One clone (10e) contained a cDNA with the full open reading frame. Sequencing was carried out by the dye-odeoxy chain termination method using DNA sequencers (A-310 and A-381, Perkin-Elmer Corp).

Northern Blotting

Nitrocellulose filters (purchased from Origene Technologies Inc) containing 20 μg total RNA in each lane from 12 different mouse tissues were hybridized with a P32-labeled Kpnl-EorI DNA fragment, and the filters were washed at high-stringency condition as described above. The filters were then exposed to Fuji x-ray film (Fuji Photo Film Co) and developed 5 hours later.

PCR Amplification of the Novel K+ Channel cDNA Fragment From Single Cardiac Myocytes

Atrial and ventricular myocytes were dissociated by perfusion of rat heart with collagenase as described previously. Total RNAs of a dissociated single cardiac myocyte, which was aspirated and transferred into a microcentrifuge tube using a glass tip such as used for patch-clamp experiments under a microscope, were extracted, and cDNAs were synthesized as described by Sucher and Deitcher. To eliminate contamination of genomic DNA, RNA samples were treated with DNase (Takara Shuzo Corp) before cDNA synthesis. Primers for PCR reaction were located in nucleotides 958 to 980 and nucleotides 1194 to 1216 of mouse cTBAK-1 cDNA. PCR amplification was performed using AmpliTaq Gold DNA polymerase (Perkin-Elmer Corp) as follows: initial denaturation at 94°C for 12 minutes, 30 cycles at 94°C for 1 minute and 60°C for 1 minute, and a final extension step at 60°C for 8 minutes. Because the products from a single cell were not enough to be visualized, second PCR reactions (50 μL) using the same primers and amplification conditions were carried out using 5 μL of the first PCR products. The products were electrophoresed on a 2% agarose gel. The amplified PCR products were confirmed to be rat cTBAK-1 cDNA fragments by their nucleotide sequences. The nucleotide sequence of the amplified PCR products was performed using the dye-primer method and DNA sequencer after TA cloning (Invitrogen).

Functional Expression of cTBAK-1 in X enopus Oocytes or in HEK293T Cells

The coding region of mouse cTBAK-1 was subcloned into pgEMHE11 and pcDNA3 (Invitrogen) for the expression in X enopus oocyte and HEK293T cells, respectively. For the heterologous ex-
end (Ser-Ser-Val), and both of two Ser residues of the motif may be phosphorylated by protein kinase A. 14–16

Search in the GenBank indicated that the amino acid sequence of the 10e clone was most similar to sequences of the putative K channel with two pore domains identified in C elegans C40C9,2 TWIK-1,5,17 and TREK-1.6 Amino acid sequences of C40C9, TWIK-1, and TREK-1 showed 33%, 25%, and 22% identities with that of 10e, respectively. We named the 10e clone cTBAK-1, based on the abundant expression in the cardiac myocyte and electrophysiological properties of the expressed current as shown later. The sequence of cTBAK-1 was compared with sequences of C40C9, mouse TWIK-1, and mouse TREK-1 in the TM regions (M1 to M4) and the pore domains (P1 and P2). Although the homology of cTBAK-1 with those of other two pore-domain K channels was low (20% to 30%), the M1, M2, P1, and P2 regions of cTBAK-1 exhibited higher identities (30% to 70%) (Fig 1C). The sequences of M3 and M4 did not show a significant similarity among these four K channel subunits.

Tissue Distribution of Mouse cTBAK-1 mRNA
We examined the expression of cTBAK-1 mRNA in various mouse tissues. cTBAK-1 mRNA (5 kb) was strikingly abundant in the heart compared with other tissues (Fig 2A). The mRNA was also detected in kidney, brain, skin, testis, lung, skeletal muscle, small intestine, and stomach but not in liver, thymus, or spleen. In the heart, two larger mRNAs of ≈7.5 and 10 kb were present.

To examine whether cardiac myocytes per se express cTBAK-1 mRNA, we performed RT-PCR analyses using a single cell of rat atrial and ventricular myocytes. Because mouse heart was difficult to perfuse with collagenase, we used rat heart for this experiment. As shown in Fig 2B, cDNA fragments of ≈260 bp were amplified from the samples of single atrial and single ventricular cells. Because nucleotide sequences of the amplified rat DNA fragments from single cardiac myocytes showed 99.6% identity with the sequence of mouse cTBAK-1 cDNA, amplified DNA fragments were identified as rat cTBAK-1 cDNA (data not shown). These data indicate that both rat atrial and ventricular myocytes per se actually express cTBAK-1 mRNA.

Functional Expression of Mouse cTBAK-1
Fig 3A, left, illustrates an example of cTBAK-1 currents obtained from one Xenopus oocyte injected with cTBAK-1 cRNA 48 to 96 hours before the recording. Voltage steps of 1.2-second duration were applied from a holding potential of 0 mV to various potentials ranging from −120 to +60 mV in 20-mV increments. Large inward and outward currents were observed in the cRNA-injected, but not water-injected, oocytes (a and c, respectively, in Fig 3A, left), producing an almost linear steady-state current-voltage relationship under this condition (Fig 3A, right). Application of 3 mmol/L Ba2+...
to the extracellular medium blocked the inward current by >80% and the outward current by <20%. Similar results were obtained in three other experiments. To examine the ion selectivity of the expressed channel in oocytes, [K\(^{+}\)], was changed from 90 mmol/L to 10 mmol/L, 30 mmol/L, or 50 mmol/L, and the reversal potential of the expressed current was determined for each concentration of extracellular K\(^{+}\). The reversal potential was -57 mV at 10 mmol/L [K\(^{+}\)], -24 mV at 30 mmol/L [K\(^{+}\)], and -20 mV at 50 mmol/L [K\(^{+}\)], respectively (Fig 3B), producing a 57-mV shift for a 10-fold change in [K\(^{+}\)]. (n=3), which was in agreement with the Nernst equation for the K\(^{+}\)-selective ion channel.

Single-channel properties were studied in HEK293T cells transfected with cTBAK-1 cDNA. In cell-attached patches of the cells, briefly opening single channels were present at both negative and positive membrane potentials (−100 to +60 mV). Fig 4A shows an example of current recordings at various potentials from a cell-attached patch. The expressed channel opened in bursts of short duration at different membrane potentials with 150 mmol/L KCl in the pipette solution. No such channels were observed in untransfected control cells (n=10).

Fig 4B shows an example of the amplitude histogram of the current openings at −60 mV. The most frequently observed unitary open level was at ~1.1 pA (indicated by arrows in Fig 4B). There was another smaller peak of ~0.6 pA between the closed and the main open level, which might be regarded as the sublevel of this channel (asterisks, Fig 4B). When the amplitude of the main open level is plotted against membrane potentials, a weak inwardly rectifying current-voltage relationship was obtained (Fig 4C). The unitary conductance of the channel estimated from the current-voltage relationship at negative potentials was ~16 pS. The mean open time was ~2 milliseconds at −100 mV (not shown). N_Po of this channel at the main opening levels was estimated from amplitude histograms at potentials between +60 and −100 mV (Fig 4D). The channel N_Po remained almost constant at these potentials. Similar results were obtained in three other patches.

The channels remained active in the inside-out patch condition, indicating that soluble cytoplasmic molecules were not involved in channel activation (n=5, data not shown). In Xenopus oocytes injected with cTBAK-1 cRNA, the channels that exhibited the same properties as those in HEK293T cells were observed (n=5, data not shown). Although a variety of K\(^{+}\) channels have been reported to be affected by intracellular Na\(^{+}\), Ca\(^{2+}\), H\(^{+}\), and arachidonic acid, application of Na\(^{+}\) (50 mmol/L), Ca\(^{2+}\) (0.1 mmol/L), H\(^{+}\) (pH 6.4), and arachidonic acid (10 μmol/L) to the intracellular side of inside-out patches had no significant effect on the channel amplitude or N_Po (data not shown, n=3 for each).
Discussion

In the present study, we have isolated a mouse heart cDNA that encoded a novel isoform of K⁺ channel subunits having two pore domains and four TM regions. So far, three representative two pore-domain K⁺ channel subunits with four TM regions have been reported in mammals, i.e., human TWIK-1,5 mouse TWIK-1,16 and mouse TREK-1.6 Because the voltage-dependent Kv channels and the inwardly rectifying Kir channels are composed of a tetrameric assembly of subunits possessing one pore domain, the two pore-domain K⁺ channel subunits may form dimers. It is speculated that TWIK-1 subunits can self-assemble to form a dimer through the formation of an interchain disulfide bridge between the subunits.24 Cys69, which is localized on the extracellular M1-P1 linker loop of TWIK-1, is implicated in the formation of the disulfide bond. Although reducing agents (dithiothreitol or 2,3-butanedione monoxime) applied to the extracellular side did not reduce TWIK-1 currents, TWIK-1/CS in which Cys69 has been replaced by Ser had no function. These results suggest that the disulfide bond is not necessary for forming a dimer of subunits but is necessary for the stable expression in the membrane. On the other hand, cTBAK-1 does not possess a Cys residue at the corresponding segment (see Fig 1A) but still forms a functional K⁺ channel when expressed in both Xenopus oocytes and HEK293T cells. Therefore, the mechanism for the formation of dimers may differ between TWIK-1 and cTBAK-1.

Functional expression studies revealed that cTBAK-1 has an almost linear current-voltage relationship. The outward-going currents through cTBAK-1 were not affected by 2 mmol/L Mg²⁺ or 100 µmol/L spermine added to the intracellular side of patches (n = 3, not shown). In the inwardly rectifying Kir channels, the subunits of which have two TM regions and one pore-forming domain, the negatively charged amino acid residues, such as Asp or Glu in the second TM region and/or the hydrophilic C-terminal region, are responsible for Mg²⁺ and polyamine-induced block of the outward-going K⁺ current flowing through these channels.25 Consistent with the lack of the effect of Mg²⁺ and spermine, cTBAK-1 does not possess negatively charged amino acids at the corresponding sites.

The sequence of the cTBAK-1 C-terminal end is -Arg-Arg-Ser-Ser-Val. This sequence possesses two functionally important motifs. One is the motif sequence for the interaction with PSD-95, an anchoring protein.16,16 It is reported that PSD-95 family proteins regulate the subcellular localization of N-methyl-D-aspartate receptor channels,26 voltage-dependent Kv channels,27 and inwardly rectifying Kir channels.15,28 The other motif is a functional K⁺ channel when expressed in both X. oocytes and H. EK293T cells. Therefore, the mechanism for the formation of dimers may differ between TWIK-1 and cTBAK-1.
phosphorylation site. The third Ser residue from the C-terminal end can be phosphorylated by protein kinase A. Similarly, Kir2.3/IRK3 has the PSD-95 binding and protein kinase A phosphorylation site at its C-terminal end (-Arg-Arg-Glu-Ser-Arg-Ile). Cohen et al. showed that Kir2.3 could bind to PSD-95 through its C-terminal end and that phosphorylation of the C-terminal end by protein kinase A inhibited the binding to PSD-95. Because the interaction of PSD-95 family proteins with Kir channels enhanced the channel activity, it is possible that the subcellular localization and/or the function of cTBAK-1 are dynamically regulated by the interaction of PSD-95 family anchoring proteins and the protein kinase A-mediated phosphorylation. Further studies are needed to elucidate the regulation of cTBAK-1 by the anchoring proteins and the phosphorylation.

The functional K⁺ channels composed of cTBAK-1 subunits expressed in Xenopus oocytes and HEK293T cells showed short-lasing openings (mean open time, \( \sim 2 \) milliseconds) with the unitary conductance of \( \sim 16 \) pS. The channel current exhibited a weak inward rectification (Fig 4B). So far, there is no report on K⁺ channels corresponding to cTBAK-1 in native cardiac myocytes, although cTBAK-1 mRNA is expressed predominantly in atrial and ventricular myocytes per se (Fig 2). It is possible that we might have missed so far this small conductance background K⁺ current in native cardiac myocytes. Because a number of cardiac K⁺ channels, such as muscarinic K⁺ channel and slowly activating delayed rectifying K⁺ channel, proteins are composed of a heteromeric assembly of various K⁺ channel subunits (ie, Kir3.1 GIRK1+Kir3.4 GIRK4 for the muscarinic K⁺ channel and K⁺ LQT1+minK/IšK for the slowly activating delayed rectifying K⁺ channel, respectively), it is also possible that the cTBAK-1 subunit might assemble with the subunits in other K⁺ channel families or with unidentified members of the same family to form an active channel in native cells. Further studies using myocytes in the heart may be necessary to determine the native counterpart of the cloned K⁺ channel.

Acknowledgments
This study was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan, "Research for the Future" Program of the Japan Society for the Promotion of Science (96L00302), and the Human Frontier Science Program (RG0158/1995–1997). The authors cordially thank Prof. Lutz Pott (Bochum, Germany) for his critical reading of this manuscript, Kiyomi O Kuto for her technical assistance, and Keiko Tsudo for her secretarial support. The nucleotide sequence data reported in this article have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following accession number: AB008537.
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*Circ Res.* 1998;82:513-518
doi: 10.1161/01.RES.82.4.513

*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

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