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Cloning of large-conductance Ca^{2+}-activated K^+ channel α-subunits in mouse cardiomyocytes

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ABSTRACT

Large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels are widely distributed in cellular membranes of various tissues, but have not previously been found in cardiomyocytes. In this study, we cloned a gene encoding the mouse cardiac BK_{Ca} channel α-subunit (*mCardBKa*). Sequence analysis of the cDNA revealed an open reading frame encoding 1154 amino acids. Another cDNA variant, identical in amino acid sequence, was also identified by sequence analysis. The nucleotide sequences of the two *mCardBKa* cDNAs, type 1 (*mCardBKa1*) and type 2 (*mCardBKa2*), differed by three nucleotide insertions and one nucleotide substitution in the N-terminal sequence. The amino acid sequence demonstrated that mCardBKa was a unique BK_{Ca} channel α-subunit in mouse cardiomyocytes, with amino acids 41–1153 being identical to calcium-activated potassium channel SLO1 and amino acids 1–40 corresponding to BK_{Ca} channel subfamily M alpha member 1. These findings suggest that a unique BK_{Ca} channel α-subunit is expressed in mouse cardiomyocytes.

Introduction

Ca^{2+}-activated K^+ (K_{Ca}) channels participate in many physiological processes such as neuronal secretion, smooth muscle contraction, action potential shape determination, and spike frequency adaptation [1,2]. K_{Ca} channels are activated by either an increase in intracellular calcium or membrane depolarization [1,3,4]. There are three distinct classes of K_{Ca} channels, based on the primary amino acid sequence and single-channel conductance: small-conductance, intermediate-conductance, and large-conductance K_{Ca} channels [2–5].

Large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels are widely distributed in a variety of cell types, including both electrically excitable and non-excitable cells [6]. BK_{Ca} channel subtypes have distinct electrophysiological properties, including characteristic single-channel conductance, Ca^{2+}-sensitivity, and gating kinetics [3,7], and provide a physiologically important negative feedback mechanism in the regulation of membrane potential and intracellular Ca^{2+} elevation [8]. The molecular identities and electrophysiological characteristics of BK_{Ca} channels have been studied extensively over the last two decades. BK_{Ca} channel genes have been cloned from a number of organisms [9–12]. The first reported cDNAs encoding a BK_{Ca} channel α-subunit was *Drosophila slowpoke* (*Slo*) [10]. In some mammalian tissues, BK_{Ca} channels consist of two different subunits, a pore-forming α-subunit and a regulatory β-subunit [13–15].

Altered K^+ channel expression has been linked to pathophysiological conditions of cardiomyocytes, including acute myocardial infarction. For example, increased activation of ATP-sensitive K^+ (K_{ATP}) channels contributes to resistant ischemia in response to chronic hypoxia in the immature rabbit heart [16]. Both sarcolemmal and mitochondrial K_{ATP} channels mediate cardioprotection in chronically hypoxic hearts [17]. In addition to K_{ATP} channels, it has been suggested that BK_{Ca} channels also protect the heart against ischemia in adult rabbits and guinea pigs [18,19]. However, in contrast to K_{ATP} channels, the functional BK_{Ca} channels are not known to be expressed in the sarcolemma of cardiomyocytes [18]. Mitochondrial BK_{Ca} ($mitoBK_{Ca}$) channels were first found in the inner mitochondrial membranes of a glioma cell line [20]. The molecular components of $mitoBK_{Ca}$ channels have not been identified, despite their significant role in cardioprotection [18,21,22].

Several isoforms of BK_{Ca} channel α-subunits, alternatively spliced at the N- or C-terminus, have been cloned from a number

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of mammalian species, including rat (*rSlo*) [23,24], mouse (*mSlo*) [11], and human (*hSlo*) [12,25,26]. Nevertheless, none have been isolated from heart. Furthermore, although all three small-conductance K_{Ca} (SK_{Ca}) channel isoforms have been identified in mouse and rat heart [27], no functional sarcolemmal BK_{Ca} channel has been detected in cardiomyocytes.

In the present study, we describe the cloning, sequencing, and expression of the first full-length cDNA encoding a BK_{Ca} channel α-subunit of mouse cardiomyocytes.

Materials and methods

Single cell isolation. The protocols used conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the College of Medicine, Inje University. Ten-week-old male mice (23–30 g) were anesthetized with an injected mixture of pentobarbital sodium and heparin. The heart was cannulated for retrograde perfusion with an enzyme solution containing 0.01% collagenase via the aorta, on a Langendorff apparatus.

Generation of full-length cDNAs. Rapid amplification of 5′ and 3′ cDNA ends (RACE) was performed using a BD SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech, USA), following RT-PCR of mRNA extracted from mouse ventricular myocytes and of total RNA extracted from mouse heart (BD Biosciences Clontech, USA) to generate double-stranded cDNA. To isolate mRNA from total RNA preparations, a Poly(A)Purist MAG Kit (Ambion, USA) was used according to the manufacturer's recommended protocol. Both 5′- and 3′-RACE were performed using double-stranded cDNA as a template with the BK_{Ca} channel α-subunit gene-specific primers BK1263-F (5′-AAGAAATACGGGGGCTCCTA-3′) and BK2124-R (5′-CATGACAGGCCTTGCAGTAA-3′), and the adaptor primers RACE-1-F (CTCCTCCTCTTCCTCCTCGTCCTCGGTC) and RACE-1-R (GGCAGCAAA CGGTCCACAGGTACTTGAG). Additional BK_{Ca} channel α-subunit gene-specific primers, BK147-F (GGTCTTAGAATGAGCAGCAAT) and BK3711-R (TCATCTGTAAACCATTTCTTTTCT), were generated based on the sequences of progressively amplified 5′- and 3′-RACE products, to obtain full-length cDNAs. These were cloned into pGEM-T Easy vector (Promega, USA) for further studies.

Sequencing and characterization of cardiac BK_{Ca} channel α-subunit cDNAs. Additional BK_{Ca} channel α-subunit gene-specific primers generated based on the sequences of the 5′- and 3′-RACE products were used to obtain fragments for nucleotide sequencing. Sequences were initially determined using a BigDye Terminator V3.1 cycle sequencing Kit (Applied Biosystems Inc., USA), on a 3130 Genetic Analyzer (Applied Biosystems Inc., USA). All constructs were confirmed by automated sequencing using Sequence Scanner Software V1.0 (Applied Biosystems Inc., USA). For each sequencing reaction, 200–500 ng of double-stranded template and 3.2 pmol of primer were used, with a BigDye Terminator cycle sequencing ready reaction kit. Eight to 16 full-length cDNA clones were used to confirm the sequence, and each was sequenced in both directions using T7 and Sp6 primers.

RT-PCR. Total RNA (2 μg) from tissue was reverse transcribed using 200 U of SUPERSCRIPT II RT (Gibco-BRL, USA) in a total volume of 20 μl. delBK-F (5′-GACGTTCTGAGCGTGAC-3′) and -R (5′-AACTGGTGGAGCAATCATTAAC-3′) were used as primers for the expression of nucleotides 2224–2337 of *Kcnma1* (GenBank Accession No. NM010610), and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in Table 1 was used as an internal control. PCR was performed in a T Professional Thermal Cycler (Biometra, Germany) with Taq polymerase in 1.5 mM $MgCl_2$, 0.2 μM of each primer, and 20 μM of each dNTP as recommended by the supplier. The cycling profile was comprised of an initial denaturing step for 5 min at 94 °C followed by 35 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 5 min.

Table 1
Sequences of primers used.

Primer		Sequence	Product length (bp)
BK1	Forward	GACGTTCTGAGCGTGACTG	n.d.
	Reverse	TGGTGGAGCAATCATTAACAGAG	
BK2	Forward	CTCCTCCTCTTCCTCCTCGT	199
	Reverse	GCAAACGGTCCACAGGTACT	
BK3	Forward	GGCTTTCAACGTGTTCTTCCTCCT	254
	Reverse	GCAGATTCACCAGCTTGATGGAGT	
BK4	Forward	ACCTCAAGAGGGAGTGGGAAACAC	269
	Reverse	CATTCCAGGAGGTGTGAATCCTTG	
BK5	Forward	GGCTTTCAACGTGTTCTTCCTCCT	423
	Reverse	GCATAAACGTCCCCATAACCCACT	
BK6	Forward	CACTGGAATGTTTCACTGGTGTGC	490
	Reverse	CATTCCAGGAGGTGTGAATCCTTG	
GAPDH	Forward	ACTCCACTCACGGCAAATTC	370
	Reverse	CCTTCCACAATGCCAAAGTT	
Troponin-T	Forward	GCCAAAGATGCTGAAGAAGG	344
	Reverse	CTGTTCTCCTCCTCCTCACG	
Endothelin-1	Forward	CTGCTGTTCGTGACTTTCCA	316
	Reverse	GGTGAGCGCACTGACATCTA	

n.d., not detected.

Results

Cloning of cDNA encoding mouse cardiac BK_{Ca} channel α-subunit

To amplify a conserved region of the BK_{Ca} channel α-subunit in cardiomyocytes, we constructed universal primers based on an mRNA sequence with high similarity among known animal BK_{Ca} channel α-subunits (mouse, GenBank Accession Nos. NM010610; rat, NM031828; cow, NM174680; and human, NM001014797 and NM002247). The aligned amino acid sequences were 98–99% identical (mouse, GenBank Accession Nos. NP034740.1; rat, NP114016.1; cow, NP777105.1; human, NP001014797.1 and NP002238.2). The primers were designed to flank the region of interest, and care was taken to avoid sequences that could produce internal secondary structure. To prevent the formation of primer dimmers, the 3′-ends of the primers were not complementary. To avoid nonspecific primer annealing, the primers had nearly identical melting temperatures (T_m), and a hot-start PCR method was used. Gradient PCR was used to determine the best annealing temperatures. Table 1 shows the universal BK primers constructed to amplify the BK_{Ca} channel α-subunit transcripts from mouse cardiomyocyte total RNA and from commercially obtained mouse heart total RNA. As a result of RT-PCR and sequence analysis, all of the universal BK primer pair successfully amplified fragments of the BK_{Ca} channel α-subunit mRNA besides of BK1 primer set.

The purity of the isolated cardiomyocyte preparation was verified by RT-PCR using gene-specific primers in Table 1. Troponin-T, which is highly expressed in cardiomyocytes but not in endothelial cells, was used as a cardiomyocyte-specific marker. Endothelin-1 (ET-1) was used as markers for contaminating endothelial cells. GAPDH, a constitutively and ubiquitously expressed gene, was used to check the RT-PCR conditions. The purity of the samples was defined by the high expression of GAPDH, troponin-T, and BK_{Ca} channel α-subunit transcripts, and the absence of ET-1 transcripts (Supplementary Fig. 1). Furthermore, total RNA from mouse heart (Clontech, USA) and total RNA from primary cultures of rat cardiomyocytes [28] were used as sources for the isolation of the cardiac BK_{Ca} channel α-subunit gene, and gave the same results as those shown in Supplementary Fig. 1.

To determine the complete cDNA sequence of cardiac BK_{Ca} channel α-subunit, 5′- and 3′-RACE PCR were performed. The primers used for RACE PCR were constructed based in Table 1

```
   1 ccgcatagggcggcggcggcggcggcggcggaggcagcggtcttagaATGAGCAGCAATATCCACGCGAACCATCTCAGCCTAGACGCGTCCTCCTCCTC  100
 101 CTCTTCCTCCTCCTCTTCTTCTTCTTCCTCCTCCTCTTCCTCCTCGTCCTCGGTCCACGAGCCCAAGATGGATGCGCTCATCATACCGGTGACCATGGAG  200
 201 GTGCCGTGCGACAGCCGGGGCCAACGCATGTGGTGGGCTTTCTTGGCCTCCTCCATGGTGACTTTCTTCGGGGGCCTCTTCATCATCTTGCTCTGGCGGA  300
 301 CGCTCAAGTACCTGTGGACCGTTTGCTGCCACTGCGGGGGCAAGACGAAGGAGGCCCAGAAGATAAACAATGGCTCCAGCCAGGCAGATGGTACTCTCAA  400
 401 GCCAGTGGACGAAAAAGAGGAGGTGGTGGCAGCCGAGGTCGGCTGGATGACATCTGTGAAGGACTGGGCAGGGGTGATGATATCCGCCCAGACACTGACT  500
 501 GGCAGAGTCCTGGTTGTGTTAGTCTTTGCTCTCAGCATTGGTGCCCTCGTAATATACTTCATAGACTCGTCAAACCCAATAGAATCCTGCCAGAATTTCT  600
 601 ACAAAGATTTCACATTACAGATCGACATGGCTTTCAACGTGTTCTTCCTCCTCTACTTTGGCTTGCGGTTTATTGCAGCCAACGATAAGCTGTGGTTCTG  700
 701 GCTGGAAGTGAATTCAGTAGTAGATTTCTTCACAGTCCCTCCTGTGTTTGTGTCTGTGTACTTAAACAGAAGTTGGCTTGGCTTGAGATTTTTAAGAGCT  800
 801 CTCAGACTGATACAGTTTTCAGAGATTTTGCAGTTTCTGAATATCCTTAAAACAAGTAACTCCATCAAGCTGGTGAATCTGCTCTCCATATTTATCAGCA  900
 901 CGTGGCTGACTGCAGCTGGATTCATCCACTTGGTGGAGAATTCAGGGGACCCATGGGAAAATTTCCAAAACAACCAGGCACTTACGTACTGGGAATGTGT 1000
1001 CTACTTACTCATGGTCACAATGTCTACAGTGGGTTATGGGGACGTTTATGCAAAAACCACACTTGGACGCCTCTTCATGGTCTTCTTCATCCTCGGGGGA 1100
1101 CTGGCCATGTTTGCCAGCTACGTCCCTGAAATCATAGAGTTAATAGGAAACCGCAAGAAATACGGGGGCTCCTATAGCGCGGTTAGTGGAAGAAAGCACA 1200
1201 TTGTAGTCTGTGGACACATTACTCTGGAGAGTGTCTCTAACTTCCTGAAGGACTTTCTGCACAAGGACCGGGATGATGTCAACGTGGAGATTGTCTTTCT 1300
1301 TCACAACATCTCCCCTAACCTTGAGCTTGAGGCTCTGTTCAAACGGCATTTCACTCAGGTGGAGTTTTATCAGGGCTCTGTCCTCAATCCACATGATCTT 1400
1401 GCCAGAGTCAAGATAGAGTCAGCAGATGCATGCCTGATCCTTGCCAATAAGTATTGCGCTGACCCGGATGCAGAAGATGCCTCCAACATCATGAGAGTGA 1500
1501 TCTCCATCAAAAACTACCACCCAAAGATCAGGATCATCACTCAGATGCTGCAGTATCACAACAAGGCCCATCTGCTCAACATCCCCAGCTGGAACTGGAA 1600
1601 AGAGGGTGATGACGCAATATGCCTTGCAGAGCTCAAGTTGGGTTTCATAGCCCAGAGCTGTCTGGCTCAAGGCCTCTCCACAATGCTTGCCAATCTCTTC 1700
1701 TCTATGAGGTCATTCATAAAGATTGAGGAAGACACATGGCAGAAATACTACTTGGAAGGAGTCTCCAATGAAATGTACACAGAATATCTCTCCAGTGCCT 1800
1801 TCGTGGGTCTGTCCTTCCCTACTGTTTGTGAGCTGTGTTTTGTGAAGCTTAAGCTCCTGATGATAGCCATTGAGTACAAGTCTGCCAACAGAGAGAGCCG 1900
1901 AATATTAATTAACCCTGGGAACCACCTTAAGATCCAAGAAGGTACTTTAGGATTTTCATCGCAAGTGATGCCAAAGAAGTTAAAAGGGCATTTTTTTAC 2000
2001 TGCAAGGCCTGTCATGATGACGTCACAGATCCCAAAAGAATTAAAAAATGTGGCTGCAGGCGGCTTGAAGATGAGCAGCCGCCAACCCTGTCACCAAAAA 2100
2101 AAAAACAACGTAATGGGGGCATGAGGAACTCGCCCAACACCTCCCCGAAGCTGATGAGGCATGACCCCTTGTTAATTCCTGGCAATGATCAGATTGACAA 2200
2201 CATGGACTCCAATGTGAAAAAGTACGACTCCACTGGAATGTTTCACTGGTGTGCACCCAAGGAGATTGAGAAAGTCATCTTGACTCGAAGTGAAGCTGCC 2300
2301 ATGACTGTCCTGAGTGGCCATGTCGTAGTCTGCATCTTTGGGGATGTCAGCTCAGCCCTGATTGGCCTCCGGAACCTGGTGATGCCACTTCGTGCTAGCA 2400
2401 ACTTTCACTATCATGAGCTCAAACACATTGTGTTTGTGGGCTCCATTGAGTACCTCAAGAGGGAGTGGGAAACACTGCACAACTTCCCGAAAGTGTCCAT 2500
2501 ATTGCCTGGTACACCATTAAGTCGGGCTGATTTAAGGGCTGTCAACATCAACCTCTGTGACATGTGCGTTATCCTGTCAGCCAATCAGAATAATATTGAT 2600
2601 GATACTTCGCTTCAGGACAAGGAATGCATCTTGGCGTCACTCAACATCAAATCTATGCAGTTTGATGACAGCATCGGGGTCTTGCAGGCTAATTCCCAAG 2700
2701 GATTCACACCTCCTGGAATGGACAGATCATCACCCGACAACAGCCCAGTGCACGGGATGTTACGCCAGCCGTCCATCACAACTGGGGTCAACATTCCCAT 2800
2801 CATCACGGAACTCGTGAATGATACCAATGTTCAGTTTTTGGACCAAGACGATGACGATGACCCTGACACAGAGCTGTACCTCACACAGCCCTTTGCTTGT 2900
2901 GGGACAGCATTTGCCGTCAGCGTCCTGGACTCACTCATGAGCGCGACATACTTCAATGACAATATCCTCACCCTAATACGGACCCTGGTGACAGGAGGAG 3000
3001 CCACACCAGAGCTCGAGGCTCTAATAGCTGAGGAGAATGCACTTCGAGGAGGCTACAGCACTCCGCAGACATTGGCCAACAGGGACCGTTGCCGAGTGGC 3100
1201 CCAGTTAGCCCTGTTAGATGGTCCCTTTGCAGACTTAGGGGATGGTGGTTGTTATGGTGATCTGTTCTGCAAAGCTCTGAAAACATATAATATGCTTTGT 3200
3201 TTTGGAATTTACCGGCTGAGAGATGCCCACCTCAGCACCCCCAGCCAGTGTACAAAAAGGTACGTCATCACCAATCCTCCCTACGAGTTTGAGCTCGTAC 3300
3301 CAACAGACCTGATCTTCTGCCTGATGCAGTTTGACCACAACGCTGGCCAATCCCGGGCCAGTCTGTCTCATTCCTCCCACTCCTCACAGTCGTCCAGTAA 3400
3401 GAAGAGCTCCTCCGTCCACTCCATCCCGTCCACAGCAAATCGGCCGAACCGGCCCAAGTCCAGGGAGTCCCGCGACAAACAGAACAGAAAAGAAATGGTT 3500
3501 TACAGATGAaaaaaaaaaaaaaaaaaaaaaaaaa                                                                  3535
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Fig. 1. The nucleotide sequence of the BK_{Ca} α-subunit obtained from mouse ventricular myocytes and mouse heart total RNA. The large characters denote the open reading frame.

after checking the sequences of the PCR products. The cDNA contained an open reading frame encoding an 1154-amino acid mouse cardiac BK_{Ca} channel α-subunit protein (mCardBKa) (Figs. 1 and 2). A cDNA variant encoding the same 1154 amino acids was also found by sequence analysis. The two types of *mCardBKa*, type 1 (*mCardBKa1*) and type 2 (*mCardBKa2*), differed by three insertions and one nucleotide substitution in the N-terminal sequence (Fig. 3A). The amino acid sequence of mCardBKa was unique to mouse cardiomyocytes, with amino acids 41–1153 being identical to K_{Ca} channel SLO1 (GenBank Accession No. AAL69971) and amino acids 1–40 being identical to BK_{Ca} channel subfamily M alpha member 1 (GenBank Accession No. NP034740) (Fig. 3B).

In addition, we confirmed the mRNA expression pattern of *mCardBKa* in mouse cardiomyocytes by RT-PCR using gene-specific primers designed for the region of the insertion/deletion sites between *Kcnma1*, the gene coding BK_{Ca} channel subfamily M alpha member 1, and *mCardBKa1* (Fig. 4B). RT-PCR results showed that *Kcnma1* transcripts were highly expressed in mouse brain but not in cardiomyocytes (Fig. 4A). This result demonstrated that the unique BK_{Ca} channel α-subunit in cardiomyocytes was mCardBKa.

Expression of cardiac mCardBKa in H9c2 cells

Sarcolemmal BK_{Ca} channels have not been found in cardiomyocytes, although BK_{Ca} channels activity has been identified in the mitochondrial inner membrane of guinea pig ventricular myocytes [18]. The molecular component of mitoBK_{Ca} has not been identified even though their activities have been demonstrated in cardiac mitochondria. The above results suggest that the cloned BK_{Ca} channel α-subunit (*mCardBKa*) in our study may encode the mitoBK_{Ca} channel α-subunit.

Therefore, we tried to establish transiently transfected cell lines expressing mCardBKa at a high level. H9c2 was used as the target cell line in the transfection experiments; these cells, originated

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   1 MSSNIHANHLSLDASSSSSSSSSSSSSSSSSSSSSSVHEPKMDALIIPVTMEVPCDSRGQRMWWAFLASSMVTFFGGLFIILLWRTLKYLW   90
  91 TVCCHCGGKTKEAQKINNGSSQADGTLKPVDEKEEVVAAEVGWMTSVKDWAGVMISAQTLTGRVLVVLVFALSIGALVIYFIDSSNPIES  180
 181 CQNFYKDFTLQIDMAFNVFFLLYFGLRFIAANDKLWFWLEVNSVVDFFTVPPVFVSVYLNRSWLGLRFLRALRLIQFSEILQFLNILKTS  270
 271 NSIKLVNLLSIFISTWLTAAGFIHLVENSGDPWENFQNNQALTYWECVYLLMVTMSTVGYGDVYAKTTLGRLFMVFFILGGLAMFASYVP  360
 361 EIIELIGNRKKYGGSYSAVSGRKHIVVCGHITLESVSNFLKDFLHKDRDDVNVEIVFLHNISPNLELEALFKRHFTQVEFYQGSVLNPHD  450
 451 LARVKIESADACLILANKYCADPDAEDASNIMRVISIKNYHPKIRIITQMLQYHNKAHLLNIPSWNWKEGDDAICLAELKLGFIAQSCLA  540
 541 QGLSTMLANLFSMRSFIKIEEDTWQKYYLEGVSNEMYTEYLSSAFVGLSFPTVCELCFVKLKLLMIAIEYKSANRESRILINPGNHLKIQ  630
 631 EGTLGFFIASDAKEVKRAFFYCKACHDDVTDPKRIKKCGCRRLEDEQPPTLSPKKKQRNGGMRNSPNTSPKLMRHDPLLIPGNDQIDNMD  720
 721 SNVKKYDSTGMFHWCAPKEIEKVILTRSEAAMTVLSGHVVVCIFGDVSSALIGLRNLVMPLRASNFHYHELKHIVFVGSIEYLKREWETL  810
 811 HNFPKVSILPGTPLSRADLRAVNINLCDMCVILSANQNNIDDTSLQDKECILASLNIKSMQFDDSIGVLQANSQGFTPPGMDRSSPDNSP  900
 901 VHGMLRQPSITTGVNIPIITELVNDTNVQFLDQDDDDDPDTELYLTQPFACGTAFAVSVLDSLMSATYFNDNILTLIRTLVTGGATPELE  990
 991 ALIAEENALRGGYSTPQTLANRDRCRVAQLALLDGPFADLGDGGCYGDLFCKALKTYNMLCFGIYRLRDAHLSTPSQCTKRYVITNPPYE 1080
1081 FELVPTDLIFCLMQFDHNAGQSRASLSHSSHSSQSSSKKSSSVHSIPSTANRPNRPKSRESRDKQNRKEMVYR                  1153
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Fig. 2. The deduced amino acid sequence of mouse cardiac BK_{Ca} α-subunit.

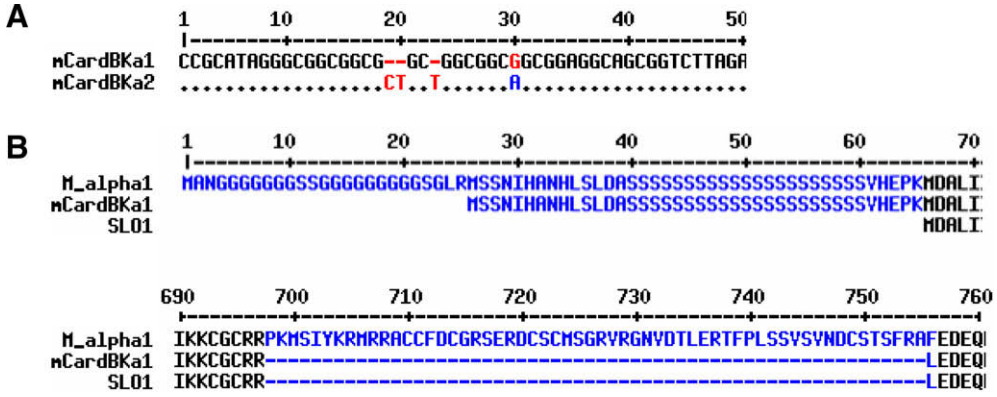

Fig. 3. (A) Alignment of the nucleotide sequences of the two BK_{Ca} α-subunit cDNA variants, *mcardBKa1* and *mcardBKa2*, from mouse cardiomyocytes. (B) Amino acid sequence alignment of the mouse cardiac BK_{Ca} α-subunit with BK_{Ca} channel subfamily M alpha member 1 (GenBank Accession No. NP034740) and calcium-activated potassium channel SLO1 (GenBank Accession No. AAL69971). M_alpha1, BK_{Ca} channel subfamily M alpha member 1; mCardBKa1, mouse cardiac BK_{Ca} α-subunit (mCardBKa) variant 1; SLO1, mouse calcium-activated potassium channel SLO1.

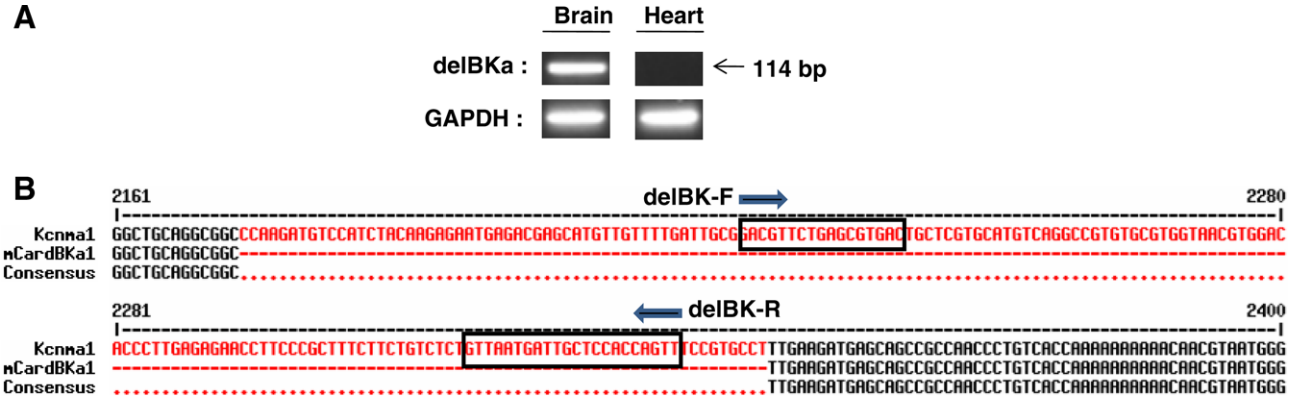

Fig. 4. (A) Expression of BK_{Ca} α-subunit transcripts (delBKa) in mouse brain and heart. For positive and negative controls, cDNA from mouse brain was used. PCR products were generated using of gene-specific primers for nucleotides 2224–2337 of *Kcnma1*, the gene coding BK_{Ca} channel subfamily M alpha member 1, and *GAPDH*. (B) Alignment of BK_{Ca} α-subunit transcripts of *Kcnma1* (GenBank Accession No. NM010610) and cardiac BK_{Ca} α-subunit (*mCardBKa*) from mouse. The box indicates the gene-specific primers used in (A).

from cardiomyocytes. The gene-specific primers BK147-F and BK3711-R were used to obtain full-length *mCardBKa* cDNA, which was cloned into pECFP-C1 for transfection into H9c2 cells. All of the clones that showed ECFP expression by confocal microscopy were confirmed by RT-PCR using gene-specific primers in Table 1 to have high *mCardBKa* mRNA expression levels. Mitochondria-specific staining of H9c2 cells revealed a remarkably regular arrangement of mitochondria, as seen in adult cardiomyocytes [29], but mCardBKa expression was not localized to the mitochondrial membrane (Supplementary Fig. 2).

Discussion

Potassium channels regulate cellular excitability. One of them, BK_{Ca} channels couple intracellular Ca^{2+} with cellular excitability, playing a critical role in linking membrane voltage to cellular calcium homeostasis. The BK_{Ca} channel is composed of a pore-forming α-subunit associated with auxiliary β-subunits [30]. Until now, sarcolemmal BK_{Ca} channels have not been found in cardiomyocytes. In this study, we cloned a gene encoding the mouse cardiac BK_{Ca} channel α-subunit (mCardBKa). The cDNAs of BK_{Ca} α-subunits, encoded by the gene *slowpoke* (*slo1*) [9–11], have been cloned from a variety of sources, and we designed primers based on the most conserved regions of these sequences (BK in Table 1). Sequence analysis showed that mCardBKa was an 1154-amino acid protein as deduced from cDNA open reading frame (Fig. 2). Based on the amino acid sequence, mCardBKa was a unique BK_{Ca} channel α-subunit in mouse cardiomyocytes; it was identical to K_{Ca} channel SLO1 in amino acids 41–1153 while amino acids 1–40 corresponded to BK_{Ca} channel subfamily M alpha member 1 (Fig. 3B). Furthermore, we confirmed the presence of cardiac BK_{Ca} channel α-subunit mRNA in the total RNA from both mouse heart and primary rat cardiomyocyte cultures. From sequence analysis, all of them contained the unique BK_{Ca} channel α-subunit (data not shown). In addition, we confirmed the mRNA expression pattern of *mCardBKa* in mouse cardiomyocytes using gene-specific primers from the region where insertion/deletion sites differ between *Kcnma1* and *mCardBKa1*. RT-PCR results showed that *Kcnma1* transcripts were highly expressed in mouse brain but not in cardiomyocytes (Fig. 4). These results indicated that the unique BK_{Ca} channel α-subunit in cardiomyocytes was mCardBKa. These findings suggest the expression of a single predominant isoform of mouse BK_{Ca} channel α-subunit in mouse cardiomyocytes.

It remains to be established whether the primary target for BK_{Ca} channel-selective agents in cardiomyocytes is the mitoBK_{Ca} channel, as sarcolemmal BK_{Ca} channels have not been cloned from ventricular myocytes. mitoBK_{Ca} channels have been detected in cardiomyocytes in a patch-clamp study performed on mitoplasts [18]; however, a proteomic analysis of the mitochondrial inner membrane did not identify the mitoK_{Ca} channel protein. Many

studies have indicated the importance of mitochondrial K_{ATP} channels in cardioprotection from ischemia–reperfusion injury [31–34]. The K^+ channels, particularly BK_{Ca} channels, have also been thought to be important in cardioprotection [22,35,36]. Xu et al. [18] suggested that the properties of mitoBK_{Ca} channels located in the inner mitochondrial membrane of guinea pig cardiomyocytes resemble those of BK_{Ca} channels, although, the molecular nature of the mitoBK_{Ca} was not identified. Thus, any BK_{Ca} channel homologs identified in cardiomyocytes may encode a physiologically functional mitoBK_{Ca} channel.

To identify the expression and localization of mCardBKa, we established a transiently transfected H9c2 cell line expressing mCardBKa. The *mCardBKa* cDNA (nucleotides 147–3711) constructs in plasmid pECFP-C1 were transfected into H9c2. The mCardBKa was expressed in H9c2 cells, and this expression was not localized to the mitochondrial membrane (Supplementary Fig. 2). It is possible that the exclusion of nucleotides 1–146 in the N-terminus and/or nucleotides beyond 3712 in the C-terminal region might have affected affect the localization of mCardBKa to the mitochondrial inner membrane. Alternatively, mCardBKa may actually be a sarcolemmal BK_{Ca} channel α-subunit. In real-time PCR experiments, *mCardBKa* mRNA expression was up-regulated by ischemic preconditioning (Supplementary Table 1), and mito-BK_{Ca} channels have been considered key effectors in cardioprotection, although the mechanism remains uncharacterized at the molecular level. Bautista et al. [37] suggested that the Ca^{2+}- and voltage-dependent K^+ (maxi-K) channel β_1-subunit is expressed in cardiomyocyte mitochondria and is regulated by sustained hypoxia. Several isoforms of BK_{Ca} channel α-subunit, alternatively spliced in the N- and C-terminus, have been cloned from a number of mammalian species, yet no isoforms corresponding to BK_{Ca} channel α-subunit have been isolated from hearts. Thus, mCardBKa may be a functional mitoBK_{Ca} channel α-subunit in heart. Alternatively, our study found that the analogs of sarcolemmal BK_{Ca} channel α-subunits in cardiomyocytes. If our cloned α-subunits are predominant expression pattern of sarcolemmal BK_{Ca} channels in cardiomyocytes, drugs targeting cardiac mitoBK_{Ca} channels should be used carefully to avoid affecting sarcolemmal BK_{Ca} channels. To clarify this issue, additional study including the channel activities and/or channel proteins should be followed in near future.

In summary, our study found that unique BK_{Ca} channel α-subunit is expressed in mouse cardiomyocytes, which could provide a better understanding of the intracellular mechanism of cardioprotection related to BK_{Ca} channels in cardiomyocytes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.08.087.

References

[1] P. Sah, Ca^{2+}-activated K^+ currents in neurones: types, physiological roles and modulation, Trends Neurosci. 19 (1996) 150–154.
[2] C. Vergara, R. Latorre, N.V. Marrion, J.P. Adelman, Calcium-activated potassium channels, Curr. Opin. Neurobiol. 8 (1998) 321–329.
[3] R. Latorre, A. Oberhauser, P. Labarca, O. Alvarez, Varieties of calcium-activated potassium channels, Annu. Rev. Physiol. 51 (1989) 385–399.
[4] W.F. Jackson, K.L. Blair, Characterization and function of Ca^{2+}-activated K^+ channels in arteriolar muscle cells, Am. J. Physiol. 274 (1998) H27–H34.
[5] A.L. Blatz, K.L. Magleby, Single apamin-blocked Ca^{2+}-activated K^+ channels of small conductance in cultured rat skeletal muscle, Nature 323 (1986) 718–720.
[6] L.Y. Jan, Y.N. Jan, Cloned potassium channels from eukaryotes and prokaryotes, Annu. Rev. Neurosci. 20 (1997) 91–123.
[7] O.B. McManus, Calcium-activated potassium channels: regulation by calcium, J. Bioenerg. Biomembr. 23 (1991) 537–560.
[8] J. Cui, H. Yang, U.S. Lee, Molecular mechanisms of BK channel activation, Cell. Mol. Life Sci. 66 (2009) 852–875.
[9] N.S. Atkinson, G.A. Robertson, B. Ganetzky, A component of calcium-activated potassium channels encoded by the *Drosophila slo* locus, Science 253 (1991) 551–555.
[10] J.P. Adelman, K.Z. Shen, M.P. Kavanaugh, R.A. Warren, Y.N. Wu, A. Lagrutta, C.T. Bond, R.A. North, Calcium-activated potassium channels expressed from cloned complementary DNAs, Neuron 9 (1992) 209–216.
[11] A. Butler, S. Tsunoda, D.P. McCobb, A. Wei, L. Salkoff, mSlo, a complex mouse gene encoding "maxi" calcium-activated potassium channels, Science 261 (1993) 221–224.
[12] J. Tseng-Crank, C.D. Foster, J.D. Krause, R. Mertz, N. Godinot, T.J. DiChiara, P.H. Reinhart, Cloning, expression, and distribution of functionally distinct Ca^{2+}-activated K^+ channel isoforms from human brain, Neuron 13 (1994) 1315–1330.
[13] H.G. Knaus, M. Garcia-Calvo, G.J. Kaczorowski, M.L. Garcia, Subunit composition of the high conductance calcium-activated potassium channel from smooth muscle, a representative of the *mSlo* and *slowpoke* family of potassium channels, J. Biol. Chem. 269 (1994) 3921–3924.
[14] J. Tseng-Crank, N. Godinot, T.E. Johansen, P.K. Ahring, D. Strøbaek, R. Mertz, C.D. Foster, S.P. Olesen, P.H. Reinhart, Cloning, expression, and distribution of a Ca^{2+}-activated K^+ channel β-subunit from human brain, Proc. Natl. Acad. Sci. USA 93 (1996) 9200–9205.
[15] C.P. Chang, S.I. Dworetzky, J. Wang, M.E. Goldstein, Differential expression of the alpha and beta subunits of the large-conductance calcium-activated potassium channel: implication for channel diversity, Brain Res. Mol. Brain Res. 45 (1997) 33–40.
[16] J.E. Baker, S.J. Contney, G.J. Gross, Z.J. Bosnjak, K_{ATP} channel activation in a rabbit model of chronic myocardial hypoxia, J. Mol. Cell. Cardiol. 29 (1997) 845–848.
[17] X. Kong, J.S. Tweddell, G.J. Gross, J.E. Baker, Sarcolemmal and mitochondrial K_{ATP} channels mediate cardioprotection in chronically hypoxic hearts, J. Mol. Cell. Cardiol. 33 (2001) 1041–1045.
[18] W. Xu, Y. Liu, S. Wang, T. McDonal, J.E. Van Eyk, A. Sidor, B. O'Rourke, Cytoprotective role of Ca^{2+}-activated K^+ channels in the cardiac inner mitochondrial membrane, Science 298 (2002) 1029–1033.
[19] D.F. Stowe, M. Aldakkak, A.K. Camara, M.L. Riess, A. Heinen, S.G. Varadarajan, M.T. Jiang, Cardiac mitochondrial preconditioning by big Ca^{2+}-sensitive K^+ channel opening requires superoxide radical generation, Am. J. Physiol. 290 (2006) H434–H440.
[20] D. Siemen, C. Loupatatzis, J. Borecky, E. Gulbins, F. Lang, Ca^{2+}-activated K^+ channel of the BK-type in the inner mitochondrial membrane of a human glioma cell line, Biochem. Biophys. Res. Commun. 257 (1999) 549–554.
[21] T. Sato, T. Saito, N. Saegusa, H. Nakaya, Mitochondrial Ca^{2+}-activated K^+ channels in cardiac myocytes. A mechanism of the cardioprotective effect and modulation by proteinase A, Circulation 111 (2005) 198–203.
[22] S.H. Kang, W.S. Park, N.R. Kim, J.B. Youm, M. Warda, J.H. Ko, E.A. Ko, J. Han, Mitochondrial Ca^{2+}-activated K^+ channels are more efficient at reducing mitochondrial Ca^{2+} overload in rat ventricular myocytes, Am. J. Physiol. 293 (2007) H307–H313.
[23] M.M. Zarei, N. Zhu, A. Alioua, M. Eghbali, E. Stefani, L. Toro, A novel MaxiK splice variant exhibits dominant-negative properties for surface expression, J. Biol. Chem. 276 (2001) 16232–16239.
[24] D.L. Price, J.W. Ludwig, H. Mi, T.L. Schwarz, M.H. Ellisman, Distribution of *rSlo* Ca^{2+}-activated K^+ channels in rat astrocyte perivascular endfeet, Brain Res. 956 (2002) 183–193.
[25] S.I. Dworetzky, J.T. Trojnacki, V.K. Gribkoff, Cloning and expression of a human large-conductance calcium-activated potassium channel, Mol. Brain Res. 27 (1994) 189–193.
[26] L. Pallanck, B. Ganetzky, Cloning and characterization of human and mouse homologs of the *Drosophila* calcium-activated potassium channel gene, *slowpoke*, Hum. Mol. Genet. 3 (1994) 1239–1243.
[27] D. Tuteja, D. Xu, V. Timofeyev, L. Lu, D. Sharma, Z. Zhang, Y. Xu, L. Nie, A.E. Vázquez, J.N. Young, K.A. Glatter, N. Chiamvimonvat, Differential expression of small-conductance Ca^{2+}-activated K^+ channels SK1, SK2, and SK3 in mouse atrial and ventricular myocytes, Am. J. Physiol. 289 (2005) H2714–H2723.
[28] J.R. González-Juanatey, R. Piñeiro, M.J. Iglesias, O. Gualillo, P.A. Kelly, C. Diéguez, F. Lago, GH prevents apoptosis in cardiomyocytes cultured in vitro through a calcineurin-dependent mechanism, J. Endocrinol. 180 (2004) 325–335.
[29] N. Beraud, S. Pelloux, Y. Usson, A.V. Kuznetsov, X. Ronot, Y. Tourneur, V. Saks, Mitochondrial dynamics in heart cells: very low amplitude high frequency fluctuations in adult cardiomyocytes and flow motion in non beating Hl-1 cells, J. Bioenerg. Biomembr. 41 (2009) 195–214.

[30] J.P. Ding, Z.W. Li, C.J. Lingle, Inactivation BK channels in rat chromaffin cells may arise from heteromultimeric assembly of distinct inactivation-competent and noninactivating subunits, Biophys. J. 74 (1998) 268–289.
[31] Y. Liu, T. Sato, B. O'Rourke, E. Marbán, Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? Circulation 97 (1998) 2463–2469.
[32] T. Sato, N. Sasaki, J. Seharaseyon, B. O'Rourke, E. Marbán, Selective pharmacological agents implicate mitochondrial but not sarcolemmal K_{ATP} channels in ischemic cardioprotection, Circulation 23 (2000) 2418–2423.
[33] K.D. Garlid, S.P. Dos, Z.J. Xie, A.D. Costa, P. Paucek, Mitochondrial potassium transport: the role of the mitochondrial ATP-sensitive K^+ channel in cardiac function and cardioprotection, Biochim. Biophys. Acta 1606 (2003) 1–21.
[34] X. Wang, P.W. Fisher, L. Xi, R.C. Kukreja, Essential role of mitochondrial Ca^{2+}-activated and ATP-sensitive K^+ channels in sildenafil-induced late cardioprotection, J. Mol. Cell. Cardiol. 44 (2008) 105–113.
[35] C.M. Cao, Q. Xia, Q. Gao, M. Chen, T.M. Wong, Calcium-activated potassium channel triggers cardioprotection of ischemic preconditioning, J. Pharmacol. Exp. Ther. 312 (2005) 644–650.
[36] B.H. Bentzen, O. Osadchii, T. Jespersen, R.S. Hansen, S.P. Olesen, M. Grunnet, Activation of big conductance Ca^{2+}-activated K^+ channels (BK) protects the heart against ischemia–reperfusion injury, Eur. J. Physiol. 457 (2009) 979–988.
[37] L. Bautista, M.J. Castro, J. López-Barneo, A. Castellano, Hypoxia inducible factor-2α stabilization and maxi-K^+ channel β_1-subunit gene repression by hypoxia in cardiac myocytes, Circ. Res. 104 (2009) 1364–1372.