Cardioprotective effects of estradiol include the activation of large-conductance Ca\(^{2+}\)-activated K\(^+\) channels in cardiac mitochondria

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Ohya, Susumu, Yukiko Kuwata, Kazuho Sakamoto, Katsuhiko Muraki, and Yuji Imaizumi. Cardioprotective effects of estradiol include the activation of large-conductance Ca\(^{2+}\)-activated K\(^+\) channels in cardiac mitochondria. Am J Physiol Heart Circ Physiol 289: H1635–H1642, 2005; doi:10.1152/ajpheart.00016.2005.—The molecular components of the large-conductance Ca\(^{2+}\)-activated K\(^+\) channels that are functionally expressed in mitochondria (mitoK\(_{Ca}\)) in cardiac myocytes have not been identified. Our experimental results show that the transcript corresponding to the large-conductance Ca\(^{2+}\)-activated K\(^+\) channel \(\beta_1\)-subunit (BK-\(\beta_1\)) is substantially expressed in mammalian heart. A yeast two-hybrid assay showed the BK-\(\beta_1\) protein can interact with a mitochondrial protein, cytochrome \(c\) oxidase subunit I (Cco1). Results from immunocytochemical experiments also demonstrated that BK-\(\beta_1\) interacted with Cco1 and localized in rat cardiac mitochondria. Furthermore, 17\(\beta\)-estradiol, which enhances the activity of the BK channel \(\alpha\)-subunit only in the presence of the \(\beta_1\)-subunit, significantly increased flavoprotein oxidation in rat ventricle myocytes and decreased the rate of cell death under simulated ischemia. Single-channel recordings from mitochondrial inner membrane indicated that the activity of mitoK\(_{Ca}\), which had a conductance of \(~270\ pS\), was enhanced by 17\(\beta\)-estradiol and blocked by paxilline. In combination, the present study revealed a new mechanism for the cardioprotective effects of 17\(\beta\)-estradiol, which include the activation of mitoK\(_{Ca}\) via the interaction with BK-\(\beta_1\). BK-\(\beta_1\) may be an important molecular component that functionally couples with both Cco1 and mitoK\(_{Ca}\) pore-forming \(\alpha\)-subunit.

BK channel \(\beta_1\)-subunit; \(\beta_1\)-subunit; cytochrome \(c\) oxidase; cardioprotection

LARGE-CONDUCTANCE Ca\(^{2+}\)-activated K\(^+\) (BK) channels are ubiquitously expressed in both excitable and nonexcitable cells and contribute to diverse physiological processes. The pore-forming \(\alpha\)-subunit of BK channel (BK-\(\alpha\)) is encoded by the Slowpoke-1 (Slo-1), and its RNA splicing at different sites can result in multiple distinct biophysical properties (2, 25). BK-\(\alpha\) has a unique NH\(_2\)-terminal segment (S0), and this region is important for the association with auxiliary \(\beta\)-subunits (BK-\(\beta\)) (1, 7). BK-\(\beta\), which have two membrane-spanning domains, localize in the plasma membrane and strongly modulate Ca\(^{2+}\) sensitivity, kinetics of activation and inactivation, and toxin affinity. The tissue-specific distribution of four BK-\(\beta\) isoforms (BK-\(\beta_1\)–4) is the major determinant for diverse characteristics of BK channels in various tissues.

Recent studies have shown that ion channels on the mitochondrial inner membrane can influence cellular events that lead to tissue damage in diverse pathological situations, including cardiac and brain ischemia (5, 9). The opening of mitochondrial ATP-sensitive K\(^+\) channel (mitoK\(_{ATP}\)) is cardioprotective in the setting of ischemia-reperfusion injury; therefore, specific mitoK\(_{ATP}\) openers are a major focus (12). Xu et al. (24) have shown that one type of Ca\(^{2+}\)-activated K\(^+\) channel (mitoK\(_{Ca}\)) is expressed in the mitochondrial inner membrane of ventricular cells. BK channel openers can also protect mammalian hearts against ischemic injury (20, 24). In contrast to ATP-sensitive K\(^+\) channels, the BK channels are not functionally expressed in the sarcolemma. Studies of the pharmacological and biochemical profiles have suggested that mitoK\(_{Ca}\) belongs to the Slo-1 family (21, 24); however, the molecular components of mitoK\(_{Ca}\) have not been identified.

In a preliminary study aimed at identifying the molecular components of mitoK\(_{Ca}\), we found only weak expression of BK-\(\alpha\) transcripts. However, more substantial expression of BK-\(\beta_1\) was observed in RT-PCR analyses using whole heart. BK-\(\beta_1\) is known to be a target of estrogen. Estrogen can activate BK channels, and this mechanism contributes to the gender-specific lower incidence of systemic hypertension, by reducing tonus of arterial smooth muscles in women before menopause (23). The present study was undertaken to examine the possibility that BK-\(\beta_1\) is one of the molecular components of mitoK\(_{Ca}\) and to clarify molecular mechanisms underlying the estrogen-induced protection of cardiac myocytes from ischemia-reperfusion injury.

MATERIALS AND METHODS

RNA extraction and real-time PCR. Total RNA extraction and RT-PCR were performed as previously reported (15). Single myocytes were isolated from rat cardiac ventricle as previously reported (13), and ~50 ventricular myocytes were collected through applied suction by aspirating them into a wide-bore borosilicate pipette under phase-contrast microscope, flash-frozen in liquid nitrogen, and stored at ~80°C until use. The primers utilized were as follows: BK-\(\alpha\) primers were 505–609 (GenBank accession number, AY040849, human), 408–509 (NM_010610, mouse), and 516–617 (U55995, rat); BK-\(\beta_1\) primers were 412–559 (NM_001437, human), 346–447 (NM_031169, mouse), and 283–384 (NM_091283, rat); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were 4370–4473 (J04038, human), 377–484 (XM_354601, mouse), and 1533–1637 (AF106860, rat). Real-time quantitative PCR was performed using SyberGreen chemistry on an ABI 7000 sequence detector system (Applied Biosystems, Foster City, CA) as previously reported (14). Each amplified product was sequenced with an ABI PRIZM 3100 genetic analyzer (Applied Biosystems). All experiments were carried out in accordance with the guiding principles for the care and use of laboratory animals (the

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Science and International Affairs Bureau of the Japanese Ministry of Education, Science, Sports and Culture) and also with the approval of the Ethics Committee in Nagoya City University.

Yeasts two-hybrid assay for protein-protein interaction. The yeast two-hybrid Matchmaker 3 system (BD Biosciences, San Jose, CA) was used to screen for proteins that interact with human BK-β1 (hBK-β1). A human heart cDNA library was screened according to the manufacturer’s instructions. To construct the bait plasmid, the coding region of the hBK-β1 was subcloned into pGBK7T7. To reduce false-positive interactions, the cotransformants were plated on the most stringent selection media (SD/-Leu/-Trp/-Ade/-His) containing X-a-gal.

**Plasmids constructs and transfection.** To visualize the distribution and detect protein-protein interactions between hBK-β1 and human cytochrome c oxidase subunit I (hCc1), the full-length of hBK-β1 and hCc1 were subcloned into pECPF-C1, pEYFP-C1, pCMV-Myc, and pCMV-HA (Clontech Laboratories). The primers were as follows: hBK-β1, 401–976 (NM_001437); hCc1, 4–1545 (NM_173704). All constructs were verified by DNA sequencing. HEK-293 cells were transiently transfected with pECPF-hBK-β1, pEYFP-hCc1, pCMV-Myc-hBK-β1, and/or pCMV-HA-hCc1 using a Ca3(PO4)2 coprecipitation method; 48–72 h after transfection, cells were used in these assays.

**Immunostaining.** Cell homogenates were subjected to SDS-PAGE. The blots were incubated with the anti-ha, the anti-c-Myc (Sigma, St. Louis, MO), and the anti-BK-β1 antibodies [N-15 and Y-16, Santa Cruz Biotechnology (SCB), Santa Cruz, CA] and then incubated with anti-horseradish peroxidase-conjugated IgG (Chemicon, Temecula, CA). Resulting images were analyzed with a LAS-1000 device (Fuji Film, Tokyo). To examine the interaction of hBK-β1 and hCc1, HEK-293 cells were transiently transfected with pCMV-Myc-hBK-β1 and/or pCMV-HA-hCc1. Immunoprecipitation was performed as previously reported (15). Immunoprecipitates were analyzed by immunoblotting with anti-c-Myc antibody or anti-BK-β1 antibody. Mitochondria were isolated from ventricles of adult male rats, and single mitoplasts were prepared as previously reported (24). For these immunocytochemical experiments, goat anti-BK-β1 antibodies, mouse anti-Cco1 antibody, Alexa Fluor 488 donkey anti-goat IgG antibody, and Alexa Fluor 546 goat anti-mouse IgG antibody were purchased from SCB and Molecular Probes (Eugene, OR).

**Electrophysiological experiments.** The inside-out patch clamp was applied to mitoplasts using EPC-7 amplifier (List, Darmstadt, Germany). The resistance of the pipette was 15–25 MΩ when filled with the pipette solutions. Single-channel current analyses were performed using the software, PAT V7.0C, developed at the University of Strathclyde (Dr. J. Dempster). The open probability (Po) was measured from the event histogram plotted against current amplitude. Pipette solution composition was (in mM) 29.5 KCl, 110.5 K-glutamate, 10 HEPES, 1 EGTA, 0.76 CaCl2. Bathing solution composition was (in mM) 140 KC1, 1 EGTA, 0.76 CaCl2. In both solutions, pCa and pH were adjusted to 6.3 and 7.2, respectively.

**Ventricular myocyte isolation and flavoprotein fluorescence measurement.** Male Wistar-ST rats (200–250 g) were anesthetized with an intravenous injection of pentobarbital sodium, and then hearts were rapidly mounted on a Langendorff apparatus. Ventricular myocytes were isolated as described previously (13). Flavoprotein fluorescence was measured as an index of the mitochondrial redox state as previously reported (19). Endogenous flavoprotein fluorescence was excited using a xenon arc lamp at 480 nm. Emitted fluorescence was recorded at 520 nm and monitored with a CCD digital camera cited using a xenon arc lamp at 480 nm. Endogenous flavoprotein fluorescence was previously reported (19). Endogenous flavoprotein fluorescence was measured as an index of the mitochondrial redox state as previously described (13). In brief, isolated myocytes were suspended in incubation buffer, and after centrifugation, supernatant was removed and mineral oil was layered on the top of the cell pellet to keep out air communication. After 30 and 120 min the cell pellet was mixed with hypotonic staining solution. Cells permeable to trypan blue were counted as dead and expressed as a percentage of the total cells counted (>300 for each sample).

**RESULTS**

**Molecular identification of BK channel subunits in mammalian hearts.** We first examined the expression levels of the transcripts of BK channel subunits in human, mouse, and rat cardiac muscles by use of conventional RT-PCR (Fig. 1). To prevent RNA contamination from coronary arterial smooth muscles, which abundantly express BK-α and BK-β1 subunits, RNA was extracted from endocardium of cardiac ventricle in mice and rats. As shown in Fig. 1, in cardiac muscles of human (Fig. 1A), mouse (Fig. 1B), and rat (Fig. 1C), weak signals for BK-β1 were detected, whereas no signals for BK-α were detected. As controls, BK-α signals were easily detected in both brain and aorta, whereas BK-β1 signals were detected in aorta alone in all species examined. GAPDH was used as an endogenous standard. When multicell RT-PCR was performed, freshly isolated rat ventricle myocytes (RVM) were collected using glass pipettes under the microscope (Fig. 1D) (14). BK-β1 signals were detected in RVM. The negative controls were run by addition of water in place of template cDNAs, resulting in no detectable signals in all species examined (not shown). Similar results were obtained from at least four separate experiments, and the specificity of each PCR product was confirmed by DNA sequence analysis.

**Simulated ischemia.** To determine effects of 17β-estradiol on cell injury, we examined simulated ischemia as previously described (10). In brief, isolated myocytes were suspended in incubation buffer, and when filled with Ca2+−activated K+ channel α-subunit (BK-α) and BK-β1 transcripts in mammalian hearts. RT-PCR detection of BK-α and BK-β1 in hearts of humans (A), mice (B), and rats (C) and isolated ventricular myocytes of the rat (D) A–C: 35 cycles. D: 45 cycles. PCR products were generated through the use of gene-specific primers for BK-α, BK-β1, and GAPDH. A 100-bp molecular weight marker was used to estimate the size of the amplicon. As positive/negative controls, cDNAs from brain and aorta were used. NTC, no template control.
We further examined expression levels of the transcripts corresponding to BK-α and BK-β1 in cardiac muscles and brain from human, mouse, and rat using real-time RT-PCR (Fig. 2A). Steady-state transcripts were determined relative to an endogenous control, GAPDH. Therefore, the data are expressed as ratios of BK subtypes to GAPDH, respectively, and the relative transcriptional expressions of BK-α and BK-β1 subunits are shown in Fig. 2A. All expression data are expressed as means ± SE. As positive controls, the expression levels of BK-α and BK-β1 subunits were also determined in brain and aorta. In human heart, the expression relative to GAPDH was 0.009 and 0.0037 for hBK-α and hBK-β1, respectively (n = 6 each, Fig. 2Aa). Similarly, in mouse (m) and rat (r) cardiac muscles, the expression was 0.002 ± 0.001, 0.006 ± 0.001, 0.0013 ± 0.0004, and 0.0042 ± 0.0004 for mBK-α, mBK-β1, rBK-α, and rBK-β1, respectively (n = 6 for each, Fig. 2Ab and 2Ac). In contrast, BK-β2, -β3, and -β4 expression levels in hearts were negligible in all species examined (not shown).

Based on these findings, BK-β1 was cloned from rat heart. This yielded two novel spliced isoforms, named BK-β1b (coding sequences (cds) = 441bp, 146 amino acids, GenBank accession number, AB010963) and BK-β1c (cds = 381 bp, 126 amino acids, AB050745) (Fig. 2B). The expression levels of these two isoforms, as determined by RT-PCR, were less than one tenth of original BK-β1 signals in rat heart. Sequence alignments and hydrophobicity plots showed that both isoforms lacked the second transmembrane domain (TM2) (Fig. 2C).

Screening for proteins that interact with BK-β1 in human heart with a yeast two-hybrid system. To identify proteins interacting with the BK-β1, a human heart cDNA library was screened by yeast two-hybrid system using human BK-β1 (hBK-β1) as a molecular bait. Based on the results of blue/white selection on SD/-Leu/-Trp/-Ade/-His media containing X-α-gal for the most stringent selection, positive prey clones (blue colonies, 111 clones) were rescued and retransformed into fresh yeast cells with hBK-β1 or control baits. On this basis, three classes of complementary DNAs were isolated. These encoded SNAP-25-binding protein (Snapin) (29 colonies of 111), Cco1 (22/111), and calcium-modulating cyclophilin ligand (CAML) (7/111). These findings suggested that BK-β1 may interact with Cco1 in mitochondrial inner membrane and form mitoKCa with yet unidentified α-subunit.

Confocal images of BK-β1 expression in mitochondria. The yeast two-hybrid system can detect a low-affinity interaction that may not occur either in vivo and in vitro. To determine whether the interaction between hBK-β1 and HcCo1 was functional in mammalian cells, the mitochondrial localization of hBK-β1 in HEK-293 cells transfected with CFP-tagged hBK-β1 (CFP-hBK-β1) was examined using laser-scanning confocal microscopy. Mitochondria were detected using spe-

![Fig. 2. Expression of BK-α and BK-β1 transcripts in mammalian hearts and cloning of novel BK-β1 spliced isoforms from rat hearts. A: quantitative PCR demonstrate BK-α and BK-β1 expression levels relative to GAPDH in heart, brain, and/or aorta of human (Ac), mice (Ab), and rats (Ac). Results are expressed as means ± SE (n = 1 for human and n = 4 for mice and rats). B: alignment of the amino acid sequences corresponding to 3 alternatively spliced isoforms of rat BK-β1 (rBK-β1a, rBK-β1b, and rBK-β1c). The number of the first residue of each line is given on the left. Asterisks (*) indicate sequences identical to rBK-β1a. Dashes (–) indicate gaps introduced into the sequence to improve alignment. TM, transmembrane domain. C: hydrophobicity plots of rBK-β1 isoforms. Hydrophobicity values were calculated based on the Kyte and Doolittle parameter using Genetyx-Win software (version 4.0): rBK-β1a (a), rBK-β1b (b), rBK-β1c (c).](image-url)
specific fluorescent dye, tetramethylrhodamine ethyl ester (TMRE). This analysis showed that CFP-hBK-β1 signals (green, Fig. 3Aa) almost completely overlapped with TMRE signals (red, Fig. 3Ab) (yellow, Fig. 3Ac) in CFP-hBK-β1-expressed HEK-293 cells. Next, we examined the colocalization of hBK-β1 and hCco1 in HEK-293 cells transfected with both CFP-hBK-β1 and YFP-hCco1 (YFP-hCco1). Full length of hCco1 was ligated into pEYFP-C1. CFP-hBK-β1 (green, Ba) and YFP-hCco1 signals (red, Fig. 3Bb) consistently overlapped (yellow, Fig. 3Bc) in coexpressed HEK-293 cells. Subunits of cytochrome c oxidase are known as a mitochondrial inner membrane marker, and staining patterns of YFP-Cco1 and Mito Tracker Green were also overlapped in this study (not shown). Similar results were obtained from at least 15 other cells for each type of experimental test. The fusion proteins of CFP-hBK-β1 and YFP-hCco1 can be stuck in the endoplasmic reticulum in the overexpression experiments with a heterologous expression system. To confirm the distribution of both CFP-hBK-β1 and YFP-hCco1 in mitochondria, we isolated mitochondria from CFP-hBK- and YFP-hCco1-cotransfected HEK-293 cells. It was found that the signals of both CFP-hBK-β1 and YFP-hCco1 were observed in mitochondria (diameter < 0.1 μm) (not shown).

The mitochondrial localization of BK-β1 was examined in more detail by preparing giant mitoplasts (diameter ~2 μm) from rat ventricle (see MATERIALS AND METHODS). Double-positive fluorescence signals for anti-BK-β1 (Fig. 4Aa) and anti-Cco1 (Fig. 4Ab) antibodies were detected in >95% of these cardiac ventricular mitoplasts. Two different anti-BK-β1 antibodies showed similar immunostaining patterns. Alexa Fluor 488 and Alexa Fluor 546 signals were strongly suppressed by preincubation with the excess BK-β1 and Cco1 antigens, respectively (not shown).

Immunoblot identification of hBK-β1 in purified mitochondria from rat heart. We then confirmed selective and direct interaction between BK-β1 and Cco1 using in vitro binding assay with recombinant proteins. Immunoblots of hBK-β1 were first performed with purified mitochondria from rat ventricular myocytes were costained by anti-BK-β1 and anti-Cco1 antibodies. BK-β1 and Cco1 signals are shown as green (Aa) and red (Ab). The bar denotes 2 μm. B: immunoblotting of BK-1 was performed with purified mitochondria from rat ventricle using the whole myocyte and the mitochondrial lysates.
HEK-293 cells transfected with c-Myc-tagged hBK-β1 (c-Myc-hBK-β1). A band with a molecular mass of ~25 kDa that reacted with the anti-c-Myc antibody was observed in the mitochondrial lysate (Fig. 3C), and c-Myc signals disappeared by preincubation with the excess antigen. Bands that were stained with anti-BK-β1 antibody were also detected at the similar molecular weight in the mitochondrial lysate.

To confirm the interaction between hBK-β1 and hCc1 in mammalian cells, expression vectors of hBK-β1 tagged with the c-Myc and hCc1 tagged with HA were cotransfected into HEK-293 cells. When immunoprecipitated proteins with anti-HA antibody were stained with anti-c-Myc antibody in Western blotting, a band of molecular mass 25 kDa for BK-β1 was readily detected (Fig. 3Da). Similarly, when immunoprecipitated proteins with anti-c-Myc antibody were stained with anti-HA antibody, a band of molecular mass 55 kDa for Cc1 was readily detected (Fig. 3Db). These findings suggest that hBK-β1 strongly interacts with hCc1 in mammalian cells. In addition, BK-β1 protein was also detected in both the heart whole lysate and the cardiac mitochondrial lysate, and BK-β1 signals disappeared by preincubation with the excess antigen (Fig. 4B). Densitometric analysis showed ~3 times stronger signals of hBK-β1 protein in cardiac mitochondria preparation than whole lysate.

**17β-Estradiol enhanced activity of single mitoKCa channels in mitoplasts from the rat ventricular myocytes.** Single-channel currents were recorded at +40 mV in a patch from cardiac mitoplast under symmetrical 140 mM K⁺ conditions under the inside-out patch clamp configuration (Fig. 5). In most of the patch membranes examined, several types of channels were recorded. We often observed large-conductance channels that presumably corresponded to mitoKCa (O1, O2 in Fig. 5) but more often several types of channels with smaller conductance (typically shown as Ox in Fig. 5). In Fig. 5Aa, the unitary current amplitude (UC) and the P₀ of O1 were 11.9 pA and 0.0087, respectively, at pCa 6.3 in the bath solution. The relation of single UC between 0 and +40 mV was fitted to a linear line, and the single-channel conductance was determined from the slope (not shown). In 9/43 patch membranes, we observed ~270-pS channel currents, which were very similar to the conductances of guinea-pig cardiac mitoKCa channel (24). The mitoKCa activity was markedly reduced by addition of 10 mM EGTA (not shown). The application of 3 μM paxilline, a BK channel blocker, completely suppressed mitoKCa currents (Fig. 5Ab). P₀ of Ox (UC = 3.6 pA) was not affected by the application of paxilline (~0.03).

Figure 5B showed the effects of 17β-estradiol on mitoKCa channel currents (UC ~12 pA), UC and P₀ of O1 were 11.7 pA and 0.0952, respectively in control (Fig. 5Ba), and the application of 30 μM 17β-estradiol enhanced the events of mitoKCa currents (P₀ = 0.3611 and 0.6065 in O1 and O2, respectively) (Fig. 5Bb). The activities of some other channels appeared to be somewhat reduced by 17β-estradiol. Although this finding made the exact analyses of changes in mitoKCa activity by 17β-estradiol difficult, the marked increase in P₀ of mitoKCa by 30 μM 17β-estradiol was consistently observed in three separate patches.

**17β-Estradiol affects autofluorescence of mitochondrial flavoproteins and survival rate during simulated ischemia in rat ventricular myocytes.** 17β-Estradiol activates BK-α-mediated channel activity only when BK-β1 is also present (23). The opening of K⁺ channels in the mitochondrial inner membrane can be inferred from increases in native autofluorescence of mitochondrial flavoproteins after their oxidation (19). With this method, effects of 17β-estradiol on rat mitochondria were examined. After a control period, each myocyte was exposed to 30 μM 17β-estradiol followed by 3 μM paxilline, a specific blocker of BK channel. At the end of each experiment, myocytes were treated with 3 mM sodium cyanide (CN) to fully reduce the matrix and, then, with 100 μM 2,4-dinitrophenol to achieve full oxidation. These procedures minimized or maximized the fluorescence, respectively (Fig. 6A). 17β-Estradiol significantly increased the mitochondrial matrix signal (P < 0.01 vs. control, n = 7), and 3 μM paxilline inhibited this redox effect (P < 0.01 vs. 17β-estradiol, n = 7).

Possible cardioprotective effects of 17β-estradiol mediated by model of simulated ischemia (SI) were examined using myocytes (10). Myocyte viability was reduced after exposure to SI for 120 min (Fig. 6Ba). The percentage of the viable cells at the beginning of experiments was 41 ± 2% (n = 21). In the controls, simulated ischemia for 30 min resulted in 62 ± 5% (n = 7) of cell lethality. The inclusion of 30 μM 17β-estradiol significantly decreased cell death during simulated ischemia (23 ± 5%, P < 0.01 vs. control), and the cardioprotection by 17β-estradiol was completely blocked by 3 μM paxilline (57 ± 8%, P < 0.01 vs. 17β-estradiol) (Fig. 6Bb).

**DISCUSSION**

Our results demonstrate that BK-β1 transcripts are expressed in mammalian cardiac mitochondria and that BK-β1 protein interacts with Cc1 in cardiac mitochondria. Activation of BK-β1 by 17β-estradiol results in a significant enhancement of flavoprotein oxidation and marked increase in survival rate of myocytes subjected to simulated ischemia. Both of these effects are inhibited by a specific BK channel blocker. In combination these findings reveal a novel, functionally important role of BK-β1 in the regulation of cell respiration in cardiac myocytes.

Although BK channel currents have never been recorded from sarcolemma of cardiac myocytes, mitoKCa activity has been demonstrated in cardiac mitochondria (24). Xu et al. (24) have suggested that one molecular component of mitoKCa has sequence similarity in COOH terminus to plasma membrane BK α-subunit (120 kDa). They reported a molecular mass of ~55 kDa based on Western blot analysis (24). Although several alternatively spliced isoforms of BK-α in the NH₃ and COOH termini cloned from mammalian tissues (11, 22) have been identified, no isoforms corresponding to BK-α have been isolated from hearts. We have performed a BLAST search of GenBank with the sequences of BK-α (including the epitopes of anti-BK-α antibody). No hit was obtained from this database.

Results from our yeast two-hybrid assay suggested that BK-β1 may interact with Snapin, Cco1, and/or CAML. A possible reason why the mitoKCa α-subunit cannot be isolated using this assay is that the corresponding protein is much less abundantly expressed than Snapin, Cco1, and CAML in cardiac myocytes. In this study, the interaction between BK-β1 and Cco1 was chosen for more detailed study. Mitochondria play a role in intracellular Ca²⁺ homeostasis, and they also...
generate reactive oxygen species (ROS), which play crucial roles in the onset of cell injuries (26). K⁺ influx causes alkalinization in the matrix of the mitochondrial membrane and increases in ROS production (5). The final oxidase reaction in cellular respiration, Cco is also involved in the regulation of ROS signaling during hypoxia in heart and the proton movement from the mitochondria matrix toward the cytoplasm (4). K⁺ uptake through mitochondrial K⁺ channels may compensate for this charge transfer (proton pumping) and also enable myocytes to maintain their pH gradient and transmembrane potential (3). By analysis with these functional roles of mitoKATP, mitochondrial K⁺ influxes through mitoKCa are thought to maintain K⁺ homeostasis within mitochondria, control mitochondrial volume, and affect cytochrome c release and caspase activation after myocyte injury (24). Accordingly, K⁺ uptake through mitoKCa may be related to Cco activity, and functional analysis of the mitoKCa-Cco1 complex could provide new insights into the molecular basis for preconditioning.

Fig. 5. Effects of 17β-estradiol on single mitoKCa channel in rat ventricular mitoplasts. A, top: single-channel currents were recorded by an inside-out patch-clamp technique at +40 mV in a patch from mitoplast under symmetrical 140 mM K⁺ conditions. The pCa in the bath solution was adjusted to 6.3. The original current traces were obtained before (Aa) and after (Ab) the application of 3 μM paxilline (PAX), a BK channel blocker. A, bottom: amplitude histograms in the control (Aa) and in the presence of 3 μM paxilline (Ab) calculated from the recording shown at top. B, top: original current traces were obtained before (Ba) and after (Bb) the application of 30 μM 17β-estradiol (EST). B, bottom: amplitude histograms in the control (Ba) and in the presence of 30 μM 17β-estradiol (Bb) calculated from the recording shown at top. mitoKCa, large-conductance Ca²⁺-activated K⁺ channels that are functionally expressed in mitochondria; C, level of channel-closed state; Ox, channel-open state of contaminated small-conductance channel; O1 and O2, mean channel-open state of mitoKCa channels.

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is involved in some of the cardioprotective effects of estrogen, including the reduction of infarct size (8). In the present study, 17β-estradiol significantly enhanced the oxidation of flavoproteins in myocytes and reduced the myocyte lethality caused by simulated ischemia. Both of these effects were blocked by 3 μM paxilline, suggesting that 17β-estradiol could act on mitoKCa and that BK1 functions as a component of mitoKCa molecules. Moreover, single-channel recordings from cardiac mitoplast in the present study indicate that there are mitoKCa, a kind of Ca2+-activated K+ channel that has a large conductance of ~270 pS and susceptibility to paxilline, as has been reported (24). The activity of mitoKCa channels was markedly enhanced by 17β-estradiol. This finding supports the functional interaction of BK-β1 to mitoKCa α-subunit, if it is also the case here that estrogen activates BK channels by binding to β-subunit (23). Although many lines of indirect evidence supporting the cardioprotective effects of mitoKATP opening have been accumulated (16, 18), the shortening of action potential duration after KATP channel activation has been suggested to be also arrhythmogenic (6). In contrast, BK channels are not functionally expressed in mammalian cardiac sarcolemma. Consequently, it is unlikely that BK channel openers will directly change electrophysiological parameters of the ventricle. As a consequence, our findings may provide a novel approach and a possible therapeutic target for ischemic injury.

BK-β1 interacts with S0 and COOH-terminal domains of mammalian BK-α subunits (11), and this interaction increases the apparent Ca2+ sensitivity of BK channels as well as reducing its voltage dependency (2). Further studies aimed at molecular characterization of the site of BK-β1 that interacts with Cco1 molecule will provide additional information about functional integration of the complex BK-α and BK-β1 subunits with Cco1.

Fig. 6. Effects of 17β-estradiol on autofluorescence of flavoprotein (Fp) under control conditions (A) and during simulated ischemia (B) in rat ventricular myocytes. A: autofluorescence of mitochondrial flavoproteins was measured. These images are shown in pseudocolors from blue (low) to red (high) in the bar (Aa). Left (Aa1): a transmitted light image. The number of images corresponds to those in the time course (Ab). After a control period, cells were exposed to 30 μM 17β-estradiol followed by 3 μM paxilline. At the end of each experiment, the flavoprotein autofluorescence was normalized using minimal and maximal fluorescence. In this procedure myocyte respiration was first blocked using 3 mM sodium cyanide (CN) to fully reduce the matrix and minimize fluorescence, then uncoupled using 100 μM 2,4-dinitrophenol (DNP), which results in maximal oxidation and fluorescence. Ac: summarized data. **P < 0.01 vs. 17β-estradiol (n = 7 cells for each). Ba: cell viability was measured before and during simulated ischemia applied for 30 or 120 min in the absence (control) and presence of 10 μM 17β-estradiol or plus 3 μM paxilline; 300 –350 myocytes were utilized for each trial. Bb: cell viability after 30- or 120 min-simulated ischemia was evaluated by percentages of dead myocytes compared with total viable myocytes before ischemia. **P < 0.01 vs. 17β-estradiol (n = 7 rats for each).
In conclusion, our results provide the first identification of one of the molecular components of mitoK<sub>Ca</sub> in mammalian heart. The findings suggest that BK-β1 may be involved in oxygen metabolism, proton transfer, and cytochrome c release in ventricular mitochondria. The putative molecular mechanism interacts directly with not only BK-α subunits but also mitochondrial cytochrome c oxidase. This functional complex may play important roles in pathophysiological settings including cardiac ischemic injury and preconditioning. Moreover, the novel regulation of mitochondrial BK-β1 function by estrogen is likely to be one of the mechanisms of estrogen-induced cardioprotection in women. These findings provide new therapeutic strategies for human ischemic diseases, including increased incidence of heart attack in postmenopausal women and a basis for a new target of drug development when cardioprotection is being addressed.

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