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M. T. Jiang, Y. Nakae, M. Ljubkovic, W.-M. Kwok, D. F. Stowe and Z. J. Bosnjak

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## Characterization of human cardiac mitochondrial ATP-sensitive potassium channel and its regulation by phorbol ester in vitro

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**Jiang, Ming Tao, Marko Ljubkovic, Yuri Nakae, Yang Shi, Wai-Meng Kwok, David F. Stowe, and Zeljko J. Bosnjak.** Characterization of human cardiac mitochondrial ATP-sensitive potassium channel and its regulation by phorbol ester in vitro. *Am J Physiol Heart Circ Physiol* 290: H1770–H1776, 2006. First published December 16, 2005; doi:10.1152/ajpheart.01084.2005.—Activation of the mitochondrial ATP-sensitive K<sup>+</sup> channel (mitoK<sub>ATP</sub>) and its regulation by PKC are critical events in preconditioning induced by ischemia or pharmaceutical agents in animals and humans. The properties of the human cardiac mitoK<sub>ATP</sub> channel are unknown. Furthermore, there is no evidence that cytosolic PKC can directly regulate the mitoK<sub>ATP</sub> channel located in the inner mitochondrial membrane (IMM) due to the physical barrier of the outer mitochondrial membrane. In the present study, we characterized the human cardiac mitoK<sub>ATP</sub> channel and its potential regulation by PKC associated with the IMM. IMM fractions isolated from human left ventricles were fused into lipid bilayers in symmetrical potassium glutamate (150 mM). The conductance of native mitoK<sub>ATP</sub> channels was usually below 80 pS (~70%), which was reduced by ATP and 5-hydroxydecanoic acid (5-HD) in a dose- and time-dependent manner. The native mitoK<sub>ATP</sub> channel is activated by diazoxide and inhibited by ATP and 5-HD. The PKC activator phorbol 12-myristate 13-acetate (2 μM) increased the cumulative open probability of the mitoK<sub>ATP</sub> channel previously inhibited by ATP (*P* < 0.05), but its inactive analog 4α-phorbol 12,13-didecanoate had no effect. Western blot analysis detected an inward rectifying K<sup>+</sup> channel (Kir6.2) immunoreactive protein at 56 kDa and PKC-δ in the IMM. These data provide the first characterization of the human cardiac mitoK<sub>ATP</sub> channel and its regulation by PKC(s) in IMM. This local PKC control mechanism may represent an alternative pathway to that proposed previously for cytosolic PKC during ischemic/pharmacological preconditioning.

bilayers; mitochondria; protein kinase C; ischemia; preconditioning

THE MITOCHONDRIAL ATP-sensitive K<sup>+</sup> (mitoK<sub>ATP</sub>) channel was first discovered in the inner membrane (IMM) of rat liver mitochondria (21). Subsequent studies have confirmed its presence by patch-clamp studies of the mitoplasts (11, 13) or IMM reconstituted in lipid bilayers (1, 5, 27, 28, 31, 45). However, the unitary conductance of the putative mitoK<sub>ATP</sub> channel reported in these studies differed significantly, varying between ~10 and 100 pS. Equally controversial is the proposed molecular composition of the mitoK<sub>ATP</sub> channel (1, 27). Several groups have even questioned the very existence of mitoK<sub>ATP</sub>, based on the measurement of mitochondrial K<sup>+</sup> fluxes or volume change (7, 12). In humans, the mitoK<sub>ATP</sub>

channel was detected in a T-lymphocyte cell line (11), but the existence and properties of human cardiac mitoK<sub>ATP</sub> channel have not been reported.

Numerous pharmacological studies have shown that the mitoK<sub>ATP</sub> channel plays a critical role in myocardial protection, induced by ischemic (15) and pharmacological preconditioning (14, 39, 44). Ischemic preconditioning (IPC) triggers the translocation of PKC isoforms, such as δ or ε, from the cytosolic to the particulate fraction containing the mitochondria (33, 42, 43). The translocated PKC has been proposed to regulate the mitoK<sub>ATP</sub> channel, and blockade of the PKC transfer prevents IPC in both animals and humans (20, 38, 42, 43). Activation of the mitoK<sub>ATP</sub> channel by phorbol 12-myristate 13-acetate (PMA), a PKC activator, was found to protect the integrity of isolated mitochondria by preventing the opening of permeability transition pore and cytochrome-*c* release to simulated ischemia (24). Furthermore, there is evidence that PKC activation by PMA potentiates the effect of diazoxide on flavoprotein oxidation, an indirect indicator of mitoK<sub>ATP</sub> channel activity (35). Although these studies suggest a role of PKC in preconditioning, there is no evidence that the mitoK<sub>ATP</sub> channel is directly regulated by cytosolic PKC or other protein kinases due to the physical barrier of outer mitochondrial membrane (OMM). Costa et al. (9) proposed that phosphorylation of a target protein on OMM by protein kinase G (PKG) transmits the cardioprotective signals to PKC-ε located in the intermembrane space during preconditioning. On the other hand, it is possible that the mitoK<sub>ATP</sub> is regulated by local protein kinase(s) associated with IMM or intermembrane space.

The purpose of our study was to characterize human cardiac mitoK<sub>ATP</sub> channel and to investigate its potential regulation by a local PKC control mechanism. Reconstitution of the human mitoK<sub>ATP</sub> channel in lipid bilayers, away from the cytosolic environment, allowed us to examine whether the mitoK<sub>ATP</sub> channel is directly regulated by PKC located within the IMM.

### METHODS

**Mitochondrial isolation.** The Institutional Review Board for Clinical Studies at the Medical College of Wisconsin approved this study. The investigation conforms to the principles outlined in the Declaration of Helsinki. Human ventricular muscles were obtained from two nonfailing donor hearts from brain-dead patients. Human atria were obtained during cardiac surgery with informed consent. Cardiac mitochondria were isolated according to the procedure of Solem and

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Wallace (37), with modifications as described previously (28). Briefly, tissues from the left ventricles or atria were cut into small pieces and homogenized in an ice-cold isolation buffer containing (in mM) 30 MOPS (pH 7.2), 200 mannitol, 50 sucrose, 5 KH<sub>2</sub>PO<sub>4</sub>, and 1 EGTA and 0.1% BSA in the presence of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). After centrifugation at 3,000 *g* for 20 min, the supernatant was centrifuged at 8,000 *g* to obtain mitochondria (the pellet). Mitochondria were resuspended in 3% Percoll in 10% sucrose solution, layered over a 6% Percoll in 20% sucrose solution, and centrifuged at 20,000 *g* for 30 min. The pellet was resuspended with the isolation medium without EGTA and then stored on ice for preparation of IMM.

**Preparation of IMM.** The submitochondrial fraction enriched with IMM was prepared as reported (6, 29). The mitochondrial pellet was osmotically shocked by incubation in 10 mM phosphate buffer (pH 7.4) for 20 min and then in 20% sucrose for another 15 min. Membranes were sonicated (Dual Horn for model 550, Fisher Scientific, Hanover Park, IL) three times for 30 s and centrifuged at 8,000 *g* for 10 min. The supernatant, containing submitochondrial particles, was fractionated by using a continuous sucrose gradient (30% to 60%) and then centrifuged at 80,000 *g* overnight. The heavy fraction was resuspended with the isolation medium without EGTA and centrifuged at 380,000 *g* for 30 min. The final pellet, enriched in IMM, was resuspended in the isolation medium without EGTA and BSA and then stored at  $-80^{\circ}\text{C}$  in small aliquots until use.

**Reconstitution of mito<sub>K</sub>ATP channels into lipid bilayers.** The vesicles of IMM were reconstituted into lipid bilayers as reported previously (28). Briefly, the IMM were added to the *cis* chamber and fused into the lipid bilayers in a symmetrical solution containing (in mM) 30 MOPS (pH 7.4), 150 potassium glutamate, 1 EGTA, 1.03 CaCl<sub>2</sub> (free Ca<sup>2+</sup>, 10  $\mu\text{M}$ ), 0.05 K<sub>2</sub>ATP, and 0.5 MgCl<sub>2</sub>. Ag/AgCl electrodes were placed into each chamber via agar salt (0.5 M KCl) bridges, and the *trans* chamber was connected to the head stage of a bilayer clamp amplifier (BC-525C, Warner Instrument, Hamden, CT). The *cis* chamber was held at virtual ground. Experiments were performed at room temperature. After incorporation of cation channel(s), single channel currents at a holding potential of +30 or +40 mV (*trans/cis*,  $-30$  or  $-40$  mV by convention) or as indicated were collected by using an Axon Digidata 1332 AD/DA interface (Axon Instruments, Union City, CA) with pClamp software (version 8.01, Axon Instruments). The currents were filtered at 0.5 kHz with an eight-pole Bessel filter and digitized at 2.5 kHz. The channel activity, accumulated over 2 to 4 min, was expressed as cumulative channel open probability ( $NP_o$ ), where  $N$  is the apparent number of channels, and  $P_o$  is the mean open-state probability.  $NP_o$  was determined from amplitude histograms after multiple Gaussian curve fitting (Origin 6.0, Microcal Software, Northampton, MA), as described previously (28).

**Western blot analysis.** Western blot analysis assays for sarcolemmal K<sub>ATP</sub> (sarcoK<sub>ATP</sub>) channel subunits in mitochondria were conducted as described previously (22) using a 4–20% gradient Criterion Precast Gel (Bio-Rad), and that for PKC isoforms were done by using 7.5% Criterion Precast Gel (Bio-Rad) as described elsewhere (36). The antibodies were obtained from Santa Cruz Biotechnology, including inward rectifying K<sup>+</sup> channel (Kir)6.2 (G-16), Kir6.1 (C-16), sulfonylurea receptor (SUR)2 (C-15), SUR1 (C-16), PKC- $\epsilon$ , PKC- $\delta$ , and PKC- $\beta$ . The secondary antibodies were conjugated with horseradish peroxidase. Detected proteins were visualized by using chemiluminescence. To ensure detection specificity, negative controls were done by using primary antibodies preabsorbed with immunogen peptides in a one-to-five ratio.

**Characterization of mito<sub>K</sub>ATP channel reconstituted in lipid bilayers.** Mito<sub>K</sub>ATP channels were identified by their inhibition with ATP and 5-hydroxydecanoic acid (5-HD) and their activation by diazoxide, a mito<sub>K</sub>ATP channel opener. All modulators were added to the *cis* chamber and stirred for 30 s.

**Effect of PMA on mito<sub>K</sub>ATP channel opening.** After the appearance of K<sup>+</sup>-conducting current in lipid bilayers, 0.5 mM ATP was used to

screen ATP-sensitive channels. PMA (2  $\mu\text{M}$ ) was added to the *cis* chamber while being stirred. The inactive phorbol ester 4 $\alpha$ -phorbol 12,13-didecanoate (PDD) was used as the negative control. Channel activities were monitored for up to 15 min, and the identity of mito<sub>K</sub>ATP channels was confirmed at the conclusion of the experiment by their inhibition with 5-HD.

**Chemicals.** The following drugs and chemicals were used: protease inhibitor cocktail, *n*-decane, MOPS, diazoxide, 5-HD, and ATP (Sigma-Aldrich); BSA (Serologicals, Milwaukee, WI); L- $\alpha$ -phosphatidylethanolamine and L- $\alpha$ -phosphatidyl-serine (Avanti Polar-Lipid, Alabaster, AL); and PMA and PDD (Calbiochem). PMA, PDD, and diazoxide were dissolved in DMSO before adding to the experimental solution. The final concentration of DMSO (<0.1%) alone did not exhibit any effect on channel current.

**Statistical analysis.** Because of significant variance in  $NP_o$  between groups, data were analyzed after square root transformation and presented as means  $\pm$  SE. Multiple comparisons between groups were analyzed by analysis of variance, followed by Duncan's range tests. A value of  $P \leq 0.05$  was considered significant.

## RESULTS

**Identification of human cardiac mito<sub>K</sub>ATP channel.** The human IMM vesicles were fused into the lipid bilayers, and K<sub>ATP</sub> channels were identified based on their inhibition by ATP and/or 5-HD (15, 16, 32), as well as on their activation by diazoxide, a putative agonist selective for the mito<sub>K</sub>ATP channel (15, 18, 19).

In the presence of 150 mM symmetrical potassium glutamate and at the holding potential of +40 mV, recorded chord conductance of single mito<sub>K</sub>ATP channels was mostly below 80 pS ( $n$  = number of observations):  $\sim 20$  pS ( $n$  = 8),  $\sim 40$  pS ( $n$  = 8),  $\sim 60$  pS ( $n$  = 7), although the channel conductance  $>80$  pS was also recorded ( $n$  = 9). Figure 1A shows a K<sup>+</sup> channel with a chord conductance of 65 pS at a holding potential of +40 mV. The channel activity was abolished by 5-HD, confirming its identity as a mito<sub>K</sub>ATP. Figure 1B shows original traces of another mito<sub>K</sub>ATP recorded at varying holding potentials, and Fig. 1C illustrates its corresponding current-voltage relationship (*I-V*). Linear regression analysis revealed a slope conductance of 57 pS, similar to that observed for mito<sub>K</sub>ATP from bovine (45) or rat hearts (28). Figure 2A shows original traces of a mito<sub>K</sub>ATP channel with a small conductance at various holding potentials. Its *I-V* curve is presented in Fig. 2B, and calculated slope conductance is 24 pS. We did not observe significant rectification within the range of voltages used, which probably can be better evaluated at higher voltages closer to a physiological range of matrix potential ( $\sim 180$  mV). However, we were limited by the poor stability of artificial bilayers beyond  $-60$  or  $+60$  mV, especially in patches with multiple channels.

The single channel conductance from patches with multiple mito<sub>K</sub>ATP channels was often better appreciated in the presence of mito<sub>K</sub>ATP inhibitors ATP or 5-HD. As shown in Fig. 3, at baseline (control, Fig. 3A), the observed peak current was  $\sim 6$  pA at +30 mV, with a small conductance channel opening at or above 0.75 pA (25 pS). An addition of 0.5 mM ATP (Fig. 3B) inhibited most of the larger flickering channels, but the small channel remained mostly open. Increasing ATP to 1 mM (Fig. 3C) gradually inhibited this channel until complete closure. Fig. 3C, *inset*, shows the segment of recordings with transition of the small conductance mito<sub>K</sub>ATP from opening (at two levels, O<sub>1</sub> and O<sub>2</sub>) to closing on an expanded scale. The

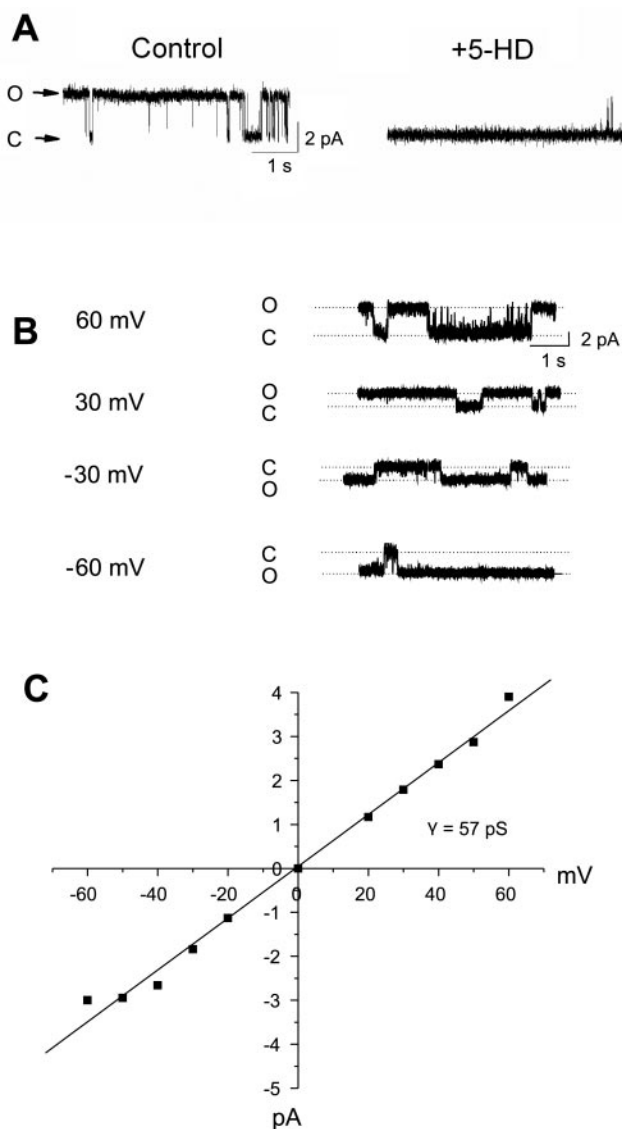


Fig. 1. Characteristics of single human cardiac mitochondrial ATP-sensitive  $\text{K}^+$  (mito $\text{K}_{\text{ATP}}$ ) channel at  $\sim 60 \text{ pS}$ . *A*: 60-pS (cord conductance) mito $\text{K}_{\text{ATP}}$  channel during control [mean open-state probability ( $P_o$ ) = 0.9] and its blockade by 5-hydroxydecanoate (5-HD, 200  $\mu\text{M}$ ,  $P_o$  = 0.03). *C*, closed state; *O*, open state. *B*: voltage-dependent current amplitudes of another mito $\text{K}_{\text{ATP}}$  channel reconstituted in lipid bilayers. *C*: current-voltage relationship in symmetrical 150 mM potassium glutamate and 0.5 mM  $\text{Mg}^{2+}$ . A slope conductance ( $\gamma$ ) of 57 pS was obtained from the linear portion of the curve. A slight rectification toward the *trans* chamber was observed. Data represent 6 observations.

corresponding amplitude histogram clearly shows a single channel conductance of 25 and 75 pS, with the latter likely representing simultaneous opening of three channels of the 25 pS conductance. These data show that ATP induced both concentration- and time-dependent inhibition of mito $\text{K}_{\text{ATP}}$  channels, which was frequently observed.

This transition can also be observed in the presence of another blocker, 5-HD. Figure 4 shows regulation of mito $\text{K}_{\text{ATP}}$  channels by ATP, GTP, and 5-HD from a different patch of IMM fused in bilayers. At baseline (Fig. 4A), mito $\text{K}_{\text{ATP}}$  channel openings were active; an addition of 0.5 mM ATP (Fig. 4B) reduced the peak current by  $\sim 75\%$  to give a smaller conduc-

tance of  $\sim 20 \text{ pS}$ . An addition of 0.5 mM GTP (Fig. 4C), a mito $\text{K}_{\text{ATP}}$  channel agonist (32), markedly increased the peak current that exceeded that seen at baseline. 5-HD (200  $\mu\text{M}$ , Fig. 4D) turned the sustained opening into a burst, with steplike conductance levels of  $\sim 40$ , 80, and 120 pS, as shown on an expanded scale (Fig. 4D, *inset*). This was followed by complete closure (Fig. 4E). Thus similar to ATP, 5-HD also caused a steplike closure of the channels, revealing multiple conductance states. These smaller conductance levels could arise from clusters of smaller channels, or they could represent subconductance states of the larger channels. Similar steplike behavior was also reported for sarc $\text{K}_{\text{ATP}}$  inhibited by glibenclamide (23).

We also tested the effect of the mito $\text{K}_{\text{ATP}}$  channel opener diazoxide on some mito $\text{K}_{\text{ATP}}$  channels with low initial activities ( $NP_o < 0.1$ ). Data from multiple experiments are summarized in Fig. 5. An addition of diazoxide (either 50 or 100  $\mu\text{M}$ ) significantly increased  $NP_o$  from the control levels ( $P < 0.05$ ),

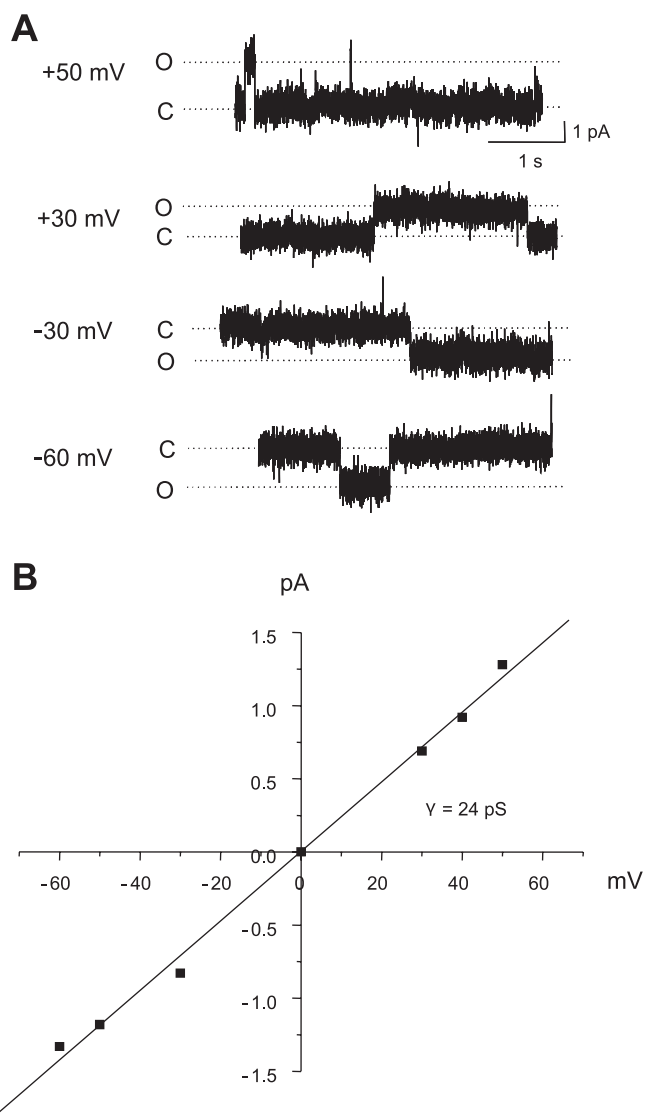


Fig. 2. Characteristics of a single human cardiac mito $\text{K}_{\text{ATP}}$  channel with a slope conductance of 24 pS. *A*: voltage-dependent current amplitudes of a mito $\text{K}_{\text{ATP}}$  channel reconstituted in lipid bilayers. *B*: current-voltage relationship in symmetrical 150 mM potassium glutamate and 0.2 mM  $\text{Mg}^{2+}$ . A slope conductance of 24 pS was obtained. Data represent 8 observations.

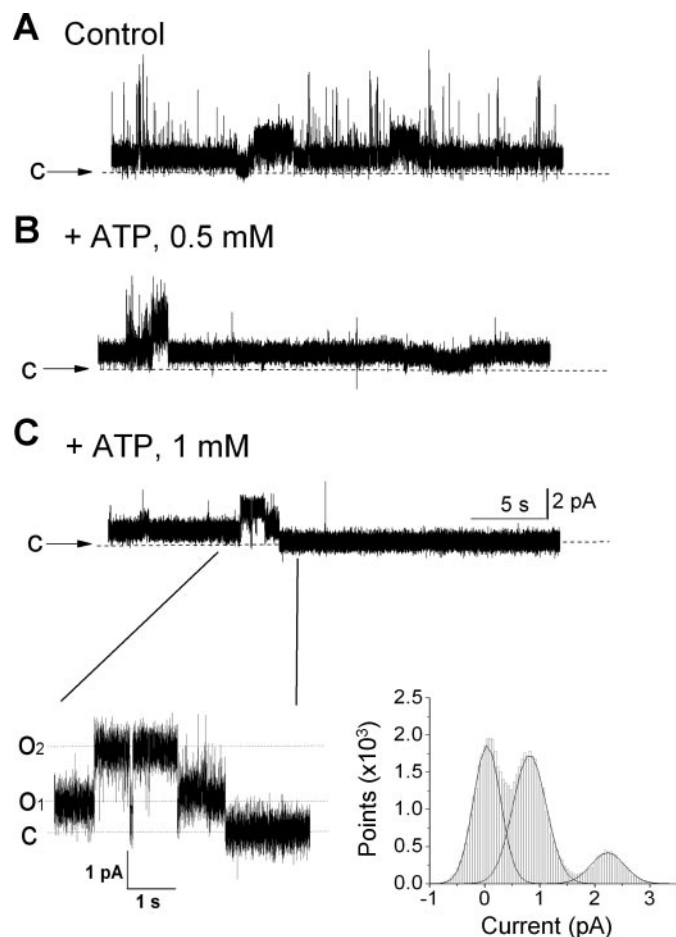


Fig. 3. ATP-induced steplike inhibition of mito $K_{ATP}$  conductance. At control (A), a cluster of channels was seen open at and above 0.75 pA (25 pS), with peak current of  $\sim 6$  pA due to the spikes of openings. Subsequent addition of ATP (0.5 mM) into *cis* (B) inhibited most of the spiking channels, with sustained opening at 0.75 pA. When ATP was raised to 1 mM (C), channels underwent steplike transition to smaller conductance states before complete closure (C); this is clearly shown on an expanded scale (C, left inset) ( $O_2$  and  $O_1$ , open states). Histogram analysis (C, right inset) showed a transition from 75 pS (i.e.,  $O_2$ ) to 25 pS (i.e.,  $O_1$ ) before closure (C).

and this current was subsequently blocked by 5-HD (200  $\mu$ M,  $P < 0.05$  vs. diazoxide). These results confirmed the presence of mito $K_{ATP}$  channels in native human IMM. The effects of diazoxide and 5-HD were similar to those reported in reconstituted bovine or rat cardiac  $K_{ATP}$  channels (28, 45). These concentrations were chosen because they produced consistent activation (diazoxide) or inhibition (5-HD) in preliminary studies. Similar concentrations were found to be effective for bovine (45) or rat mito $K_{ATP}$  (28) channels.

Because the exact molecular composition of the mito $K_{ATP}$  channel is not known, we explored its potential similarity to the sarc $K_{ATP}$  channel. We probed the isolated IMM with antibodies targeting the subunits of sarc $K_{ATP}$ : the SUR (SUR1 and SUR2) and the Kir channel (Kir6.1 and Kir6.2). As shown in Fig. 6 (left), Kir6.2 antibody detected a 56-kDa peptide in human ventricular IMM and in two atrial mitochondrial preparations (Fig. 6, M). The specificity was confirmed by preincubation of the antibody with its antigen peptide (Fig. 6, right). Interestingly, this 56-kDa peptide is similar to the ethanol-extracted 55-kDa mitochondrial protein reported by Miranova

et al. (27), which was proposed to be the mitochondrial inward rectifier subunit. However, we were not able to detect Kir6.1, as reported by Lacza et al. (25), or SUR subunits with specificity (not shown).

**Regulation of human cardiac mito $K_{ATP}$  by local PKC.** To explore whether human mito $K_{ATP}$  channels are regulated by PKC associated with the IMM, we tested the effect of PKC activator PMA on mito $K_{ATP}$  channels reconstituted into the lipid bilayers. Figure 7A shows the original recording of mito $K_{ATP}$  openings at baseline (control), followed by inhibition with 0.5 mM ATP. An addition of PMA (2  $\mu$ M) in the *cis* chamber reactivated the channels that were eventually inhibited by 5-HD (400  $\mu$ M). In contrast to PMA, its inactive analog PDD had no effect (Fig. 7B). Cumulative data from six separate observations are summarized in Fig. 7C. These results confirmed that PMA significantly increases the  $NP_o$  of mito $K_{ATP}$  channels previously inhibited by ATP ( $P < 0.05$ ). The effects of PMA on mito $K_{ATP}$  obtained at the single-channel level are in agreement with the effect of PMA on the mito $K_{ATP}$  activity in studies (9, 24) done on isolated mitochondria. Because the mito $K_{ATP}$  channels reconstituted in lipid bilayers are in cytosol-free environment, no cytosolic PKC

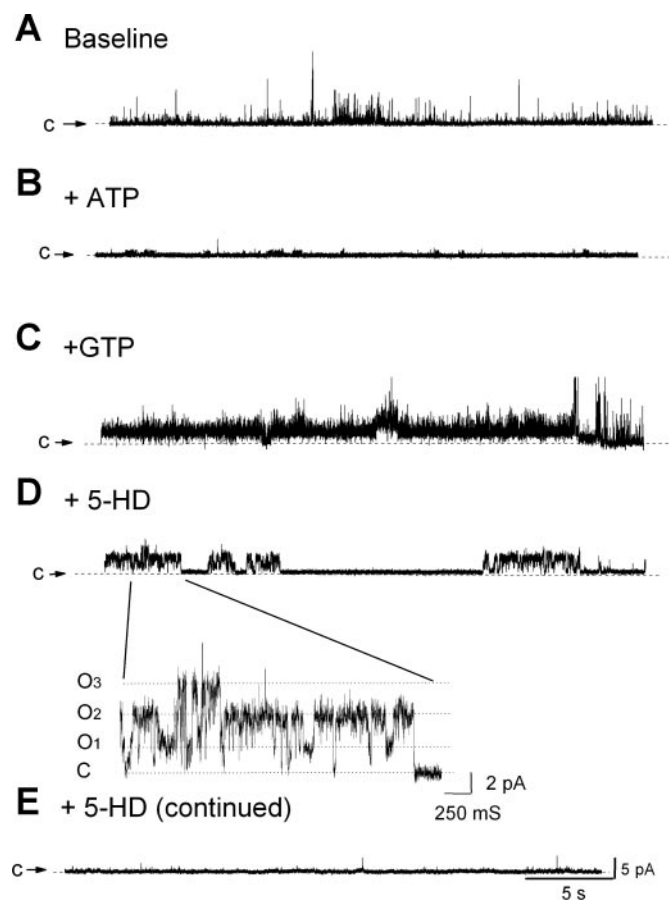


Fig. 4. Effects of ATP, GTP, and 5-HD on mito $K_{ATP}$  channels reconstituted in lipid bilayers. Original recordings of mito $K_{ATP}$  channel activities are shown, and upward deflection represents channel opening at +30 mV (*trans/cis*). A cluster of mito $K_{ATP}$  channels was active at baseline (control, A), inhibited by 0.5 mM ATP (B), and then reactivated by 0.5 mM GTP (C). Addition of 5-HD inhibited channels, which initially appeared in bursts (D), with conductance levels seen at  $\sim 40$  ( $O_1$ ), 80 ( $O_2$ ), and 120 ( $O_3$ ) pS (D, inset) before complete closure (E). Data represent 5 observations.

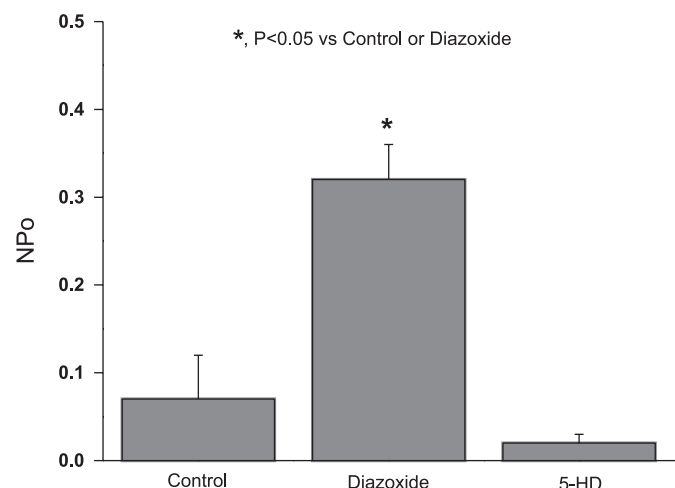


Fig. 5. Effect of diazoxide and 5-HD on human mito<sub>K</sub>ATP channels reconstituted in lipid bilayers. Control refers to activities observed under control conditions. Addition of diazoxide (50 or 100 μM) increased cumulative open-state probability (NP<sub>o</sub>) and subsequent addition of 5-HD (200 μM) blocked diazoxide-induced activation. \*P < 0.05 vs. control or 5-HD; n = 6 observations.

translocation was thus possible; the effect of PMA was likely mediated by PKC associated with the IMM. To confirm this hypothesis, we screened the IMM for PKC isozymes. As shown in Fig. 7C, inset, we were able to detect PKC-δ but not PKC-ε (or β, not shown) in human IMM.

DISCUSSION

To our knowledge, this is the first study characterizing the human cardiac mito<sub>K</sub>ATP channel and its modulation by PMA. Our results demonstrate that the mito<sub>K</sub>ATP channels are present in human cardiac mitochondria, activated by diazoxide, and inhibited by ATP and 5-HD. Moreover, the mito<sub>K</sub>ATP channels reconstituted in lipid bilayers can be directly stimulated by the PKC activator PMA without translocation of cytosolic kinases. Western blot analysis demonstrated that Kir6.2 immunoreactive protein and PKC-δ are present in human IMM. These data suggest that the mito<sub>K</sub>ATP channel is regulated locally by PKC associated with the IMM. This local control mechanism may serve as an alternative signal transduction pathway for the mito<sub>K</sub>ATP channel activation during preconditioning to that proposed for PKC translocated from the cytosol to the mitochondria (33, 34, 41, 42).

The existence of the mito<sub>K</sub>ATP channel was first reported by Inoue et al. (21) in a patch-clamp study on liver mitoplasts. In the present study, we observed multiple conductances of the mito<sub>K</sub>ATP channels (mostly below 80 pS), similar to previous

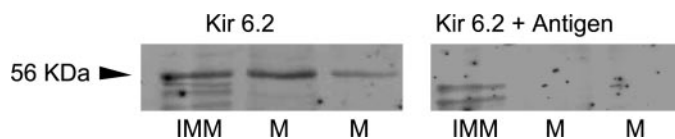


Fig. 6. Detection of inward rectifying K<sup>+</sup> channel (Kir)6.2 in mitochondria. Left: 56-kDa peptide was detected by anti-Kir6.2 antibody in human inner mitochondrial membrane (IMM) and atrial mitochondria (M, two separate samples, lanes 2 and 3). Right: same blot was stripped and then incubated with anti-Kir6.2 antibody preabsorbed with antigen peptide. Anti-Kir6.2 antibody failed to redetect the 56-kDa peptide.

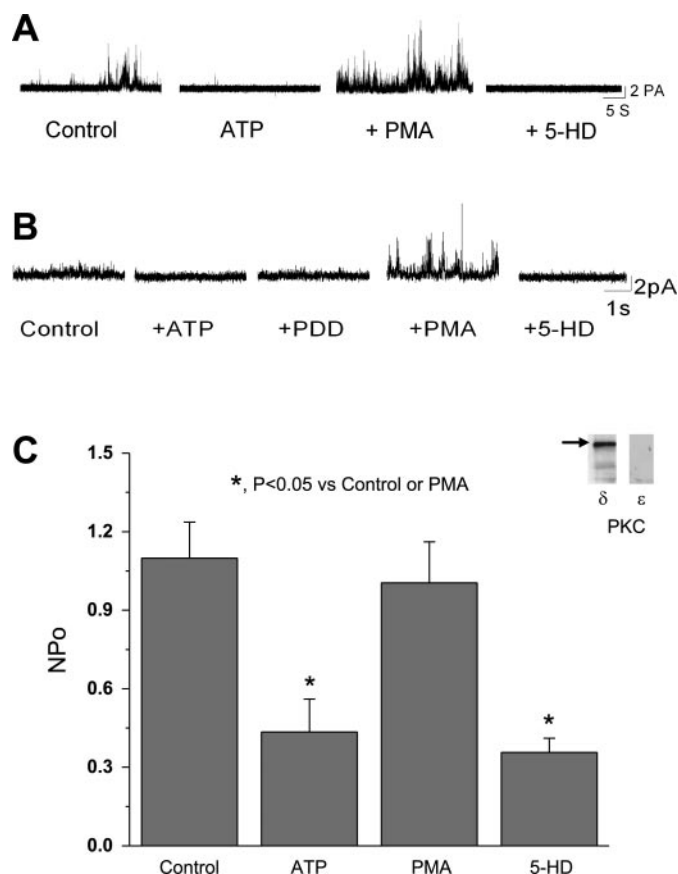


Fig. 7. Activation of human mito<sub>K</sub>ATP channels by phorbol 12-myristate 13-acetate (PMA) in lipid bilayers. A: original recordings of mito<sub>K</sub>ATP channels in lipid bilayers. ATP at 0.5 mM markedly inhibited channel activities seen at baseline (control); addition of PMA (2 μM) reactivated the channels after ~6 min, which were then completely blocked by 5-HD (200 or 400 μM). B: inactive analog of PMA, 4α-phorbol 12,13-didecanoate (PDD, 2 μM), failed to activate mito<sub>K</sub>ATP channels inhibited by ATP (0.5 mM), but subsequent addition of PMA reactivated the mito<sub>K</sub>ATP activity (representative of 3 observations). C: summarized data on mito<sub>K</sub>ATP channel activation by PMA. This PKC activator significantly increased mito<sub>K</sub>ATP NP<sub>o</sub> that was previously inhibited by ATP. \*P < 0.05 vs. control or PMA; n = 6 observations. C, inset: Western blot analysis showing detection of ~80-kDa PKC-δ, but not PKC-ε, in human IMM. Data represent 2 determinations.

observations in the rat heart (28). The conductance levels of these channels, however, are modulated by 5-HD as well as ATP. In mitochondria isolated from human lymphocyte cell line, Dahlem et al. (11) observed an outwardly rectifying K<sup>+</sup> channel with a slope conductance of 82 pS at positive test potentials and 15 pS at negative test potentials. This channel, however, responded poorly to ATP inhibition: it was only partially inhibited by 12.5 mM ATP, whereas a higher ATP concentration had no effect. In our experiments, 0.5 mM or 1 mM ATP was effective in blocking most of the mito<sub>K</sub>ATP channels.

A variety of mito<sub>K</sub>ATP channel conductances have been reported since its initial discovery. In mitochondria from bovine hearts reconstituted in lipid bilayers, recorded mito<sub>K</sub>ATP conductance was 30 pS (31) and 56 pS (45). In mitoplasts prepared from rat cardiomyocytes, Er et al. (13) observed a 13-pS channel that was voltage dependent with no rectification. Ardehali et al. (1) observed a mito<sub>K</sub>ATP channel at 200 pS in 500 mM K<sup>+</sup> and <100 pS in 100 mM K<sup>+</sup>. Szewczyk and

coworkers (4, 5) recorded the mito<sub>K</sub><sub>ATP</sub> channel with 103-pS conductance that was reduced by over 50% in the presence of 1 mM Mg<sup>2+</sup>. Different observations may result from differences in preparations, concentrations of charge carriers, as well as the presence of modulators such as Mg<sup>2+</sup> and ATP. They also reflect the complexity of studying the mito<sub>K</sub><sub>ATP</sub> channels and raise the possibility that there may be more than one type of channel that respond to known activators and inhibitors of mito<sub>K</sub><sub>ATP</sub> channels. Although we detected mito<sub>K</sub><sub>ATP</sub> channels at ~20 pS, channels with smaller conductance at ~10 pS could not be resolved under holding voltages used in our observations. Nevertheless, the results from our study revealed multiple conductance states of the human mito<sub>K</sub><sub>ATP</sub> channel. Whether they represent clusters of mito<sub>K</sub><sub>ATP</sub> channels or multiple-conducting states of a single channel has yet to be determined.

Mito<sub>K</sub><sub>ATP</sub> channel has not yet been identified at the molecular level. Several studies (10, 25) that attempted to reveal its molecular structure relied on probing the mitochondria with antibodies against sarcK<sub>ATP</sub> subunits. However, this approach is often criticized due to lack of proper negative controls with antigen peptide competition, which may show false positive findings (7). In the present study, anti-Kir6.2 antibody detected a 56-kDa protein in human cardiac IMM, but we failed to detect Kir6.1 and SUR subunits in the same sample with specificity. Garlid and coworkers (3, 27) have extracted the putative mitochondrial KIR and mitochondrial SUR from mitochondria and proposed a molecular structure similar to sarcK<sub>ATP</sub> (3, 27). An alternative molecular complex, which consists of five mitochondrial proteins within the IMM [the ATP binding cassette protein, a phosphate carrier, adenosine nucleotide translocator (ANT), ATP synthase, and succinate dehydrogenase], has been recently proposed (1). Thus the definition of the mito<sub>K</sub><sub>ATP</sub> channel molecular nature remains far from resolved. Moreover, the very existence of the mito<sub>K</sub><sub>ATP</sub> channel has been challenged, based on measurements of mitochondrial volume change, K<sup>+</sup> fluxes, and respiration in the presence of diazoxide or 5-HD (7, 12).

Activation of the mito<sub>K</sub><sub>ATP</sub> channel by PKC has been considered a crucial event in cardiac preconditioning (17, 30). Although it has been reported that PKC-ε translocates to the particulate fraction during IPC (33, 42, 43), there is no evidence that cytosolic PKC isozymes can permeate the OMM and interact directly with the mito<sub>K</sub><sub>ATP</sub> in the IMM. The role of mitochondrial PKC in the mito<sub>K</sub><sub>ATP</sub> channel regulation was suggested in the study (24) on isolated mitochondria, which are devoid of cytosolic components. The authors of that study observed that PKC activation by PMA mimics the effect of diazoxide in preventing permeability pore transition, which was abolished by the mito<sub>K</sub><sub>ATP</sub> inhibitor 5-HD. Costa et al. (9) proposed that PKG may phosphorylate some target protein on the OMM and transmit the cardioprotective signals from cytosol to IMM via PKC-ε located in the intermembrane space. The evidence for this proposal, however, was based only on pharmacological studies in isolated mitochondria, using changes in mitochondrial volume and respiration as indirect indexes of the mito<sub>K</sub><sub>ATP</sub> channel activity.

In our experiments, PMA-activated mito<sub>K</sub><sub>ATP</sub> channels reconstituted in lipid bilayers, and we were able to detect PKC-δ in IMM. These data suggest that PKC is associated with the IMM where it can directly activate mito<sub>K</sub><sub>ATP</sub> channels, prob-

ably by forming a signaling module. Further study is needed to investigate whether Kir6.2 is physically bound with PKC-δ in IMM. PKC was previously shown to phosphorylate the Kir subunits in sarcK<sub>ATP</sub> (26), and a similar mechanism may apply to mito<sub>K</sub><sub>ATP</sub>. This phosphorylation likely reduced the sensitivity of mito<sub>K</sub><sub>ATP</sub> to ATP inhibition, as well as 5-HD inhibition (Fig. 7).

In the mitochondria, the role of PKC is not limited to regulating the mito<sub>K</sub><sub>ATP</sub> channels. PKC-δ was also shown recently to bind specifically with pyruvate dehydrogenase and to prevent its reactivation during reperfusion (8), although the source (cytosolic vs. mitochondrial) of the PKC remains elusive. PKC-ε, although not detected in the present study with human IMM, has been shown to form a functional module with components of the permeability transition pore from mouse mitochondria, including the voltage-dependent anion channel (VDAC) located on the OMM and the ANT located on the IMM (2, 40). The VDAC, located on the OMM, is obviously a better target for cytosolic kinases, such as PKCs, than the mito<sub>K</sub><sub>ATP</sub> located in the IMM. On the other hand, ANT may also form a part of the proposed mito<sub>K</sub><sub>ATP</sub> complex (1); its interaction with PKC-ε may also regulate mito<sub>K</sub><sub>ATP</sub> activity.

PKC-ε was also shown recently to interact with cytochrome-*c* oxidase (19) and with a scaffold protein cypher and ANT on the IMM (40). This evidence suggests that PKC (and probably other kinases), located within the cardiac mitochondria, may play an important role in regulating respiration as well as ionic homeostasis. In the intact cell, a soluble second messenger, such as the diacylglycerol or reactive oxygen species, may activate mitochondrial PKC during IPC.

In summary, we have provided the first characterization of the human cardiac mito<sub>K</sub><sub>ATP</sub> channel and its regulation by PMA in vitro. Our data suggest that the mito<sub>K</sub><sub>ATP</sub> channel is activated by PKC associated with the IMM. This local control mechanism may represent an alternative regulatory pathway to that proposed for cytosolic PKC translocated to mitochondria during preconditioning. Further studies are needed to validate this local control mechanism.

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