

Detection of Mitochondrial ATP-Sensitive Potassium Channels in Rat Cardiomyocytes

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Mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channels play a role in early and late ischemic preconditioning. Nevertheless, the subunit composition of mitoK_{ATP} channels remains unclear. In this study, we investigated the subunit composition of mitoK_{ATP} channels in mitochondria isolated from rat cardiac myocytes. Mitochondria were visualized using the red fluorescence probe, Mitrotracker Red, while mitoK_{ATP} channels were visualized using the green fluorescence probe, glibenclamide-BODIPY. The immunofluorescence confocal microscopy revealed the presence of Kir6.1, Kir6.2 and SUR2 present in the cardiac mitochondria. Western blot analysis was carried to further investigate the nature of mitoK_{ATP} channels. For SUR proteins, a 140-kDa immunoreactive band that corresponded to SUR2, but no SUR1 was detected. For Kir6.2, three bands (~44, ~46, and ~30 kDa) were detected, and a specific ~46-kDa immunoreactive band corresponding to Kir6.1 was also observed. These observations suggest that the subunits of mitoK_{ATP} channels in rat myocytes include Kir6.1, Kir6.2, and a SUR2-related sulfonylurea-binding protein.

Key Words: Mitochondrial ATP-sensitive K⁺ channel, Ischemic preconditioning

INTRODUCTION

A sufficiently prolonged reduction in coronary blood flow can result in severe damage to the myocardium and this causes cellular injury, eventually leading to cell death due to apoptosis and/or necrosis. Ischemic preconditioning (IPC), which comprises a brief episode of ischemia then reperfusion of a coronary artery, can blunt subsequent lethal injury of the heart by following a long-term occlusion (Murry et al, 1986). Numerous physiological stimuli and pharmacological agents can mimic IPC. Recently, both sarcolemmal (sarc-) and mitochondrial (mito-) ATP-sensitive K⁺ (K_{ATP}) channels were suggested to play an important role as the end effectors in IPC in the heart (Dos Santos et al, 2002; Peart et al, 2002). The activation of K_{ATP} channels may improve the recovery of regional contractile function of stunned myocardium by shortening the duration of action potentials and attenuating membrane depolarization, thus decreasing contractility and preserving energy during ischemia (Gross, 2002; Ichinose et al, 2003). Typical K_{ATP} channels are composed of a pore-forming inwardly rectifying K⁺ (Kir) and sulfonylurea receptor (SUR), and the channels are located within plasma membrane. Cardiac sarcK_{ATP} channels have been characterized at the molecular

level, but the components of mitoK_{ATP} channels are largely unknown. Few studies have examined putative mitoK_{ATP} channel subunits (Bajga et al, 2001; Lacza et al, 2003). Therefore, in the present study, we used immunofluorescent probes and Western blotting to elucidate the K_{ATP} channel subunits in isolated cardiac mitochondria. The results suggest that the pore-forming subunits of mitoK_{ATP} channels may include Kir6.1 and Kir6.2, while the sulfonylurea receptor subunit may be a SUR2-related protein.

METHODS

Isolation of cardiomyocytes

Cardiomyocytes were isolated as described previously (Han et al, 2002; Han et al, 2003). Briefly, rats were anesthetized (10 mg/ml pentobarbital, 1 ml/kg body weight, i.p.) and injected with heparin (300 IU/ml). The heart was removed rapidly via thoracotomy, and the aorta was cannulated. The dissected heart was then mounted on a Langendorff apparatus, and was perfused retrogradely with oxygenated normal Tyrode solution (5~6 min), then with normal Ca⁺⁺-free Tyrode solution (5~6 min), followed by a 15~25 min perfusion with Ca⁺⁺-free Tyrode solution containing 0.01% collagenase. Thereafter, the heart was

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ABBREVIATIONS: SarcK_{ATP}, sarcolemmal ATP-sensitive K⁺ channels; MitoK_{ATP}, mitochondrial ATP-sensitive K⁺ channels; IPC, ischemic preconditioning; Kir, inward rectifier K⁺ channel subunit; SUR, sulfonylurea receptor; BSA, bovine serum albumin.

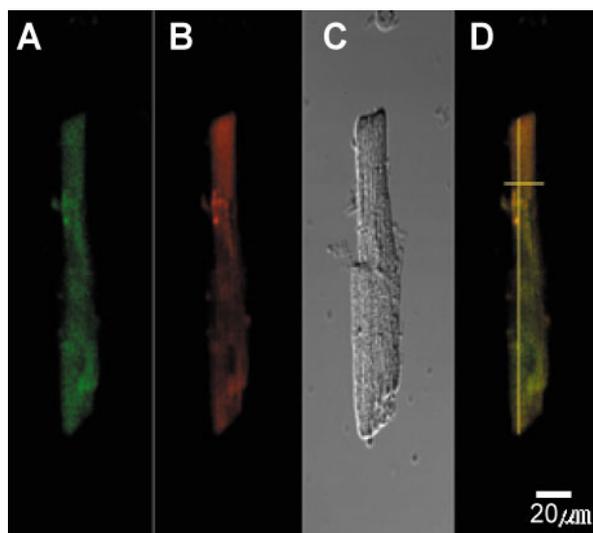


Fig. 1. Fluorescence detection of sulphonylurea receptors in isolated rat cardiomyocytes. (A) Sulphonylurea receptors (SUR) stained with glibenclamide-BODIPY. (B) Mitochondria within isolated cardiomyocytes stained with the mitochondrion-specific fluorescent probe, MitoTracker Red CMXRos. (C) Combination of transmitted light with differential interference contrast optics. (D) An overlay of the individual fluorescent staining in A and B. The overlay revealed that the SUR protein was localized to mitochondria, as indicated by the orange color (corresponding to co-localization of MitoTracker Red CMXRos and glibenclamide-BODIPY staining). All images were captured at 400× magnification.

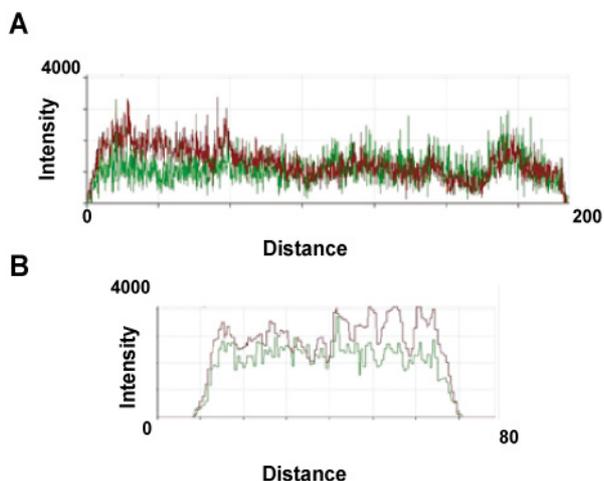


Fig. 2. Analysis of co-localization of sulphonylurea receptors and mitochondria in isolated rat cardiomyocytes. Using the fluorescence image of the cell in Fig. 1D, the fluorescence intensity of glibenclamide-BODIPY (green line) and MitoTracker Red CMXRos (red line) was measured in longitudinal (A) and transverse (B) profiles.

washed with Kraft Brühe (KB) solution for 5 min before the cannula was removed. The atria were discarded, and the ventricular walls and septum were cut into four to six small pieces that were agitated gently in KB solution to dissociate the tissue and to obtain individual cells. The cell

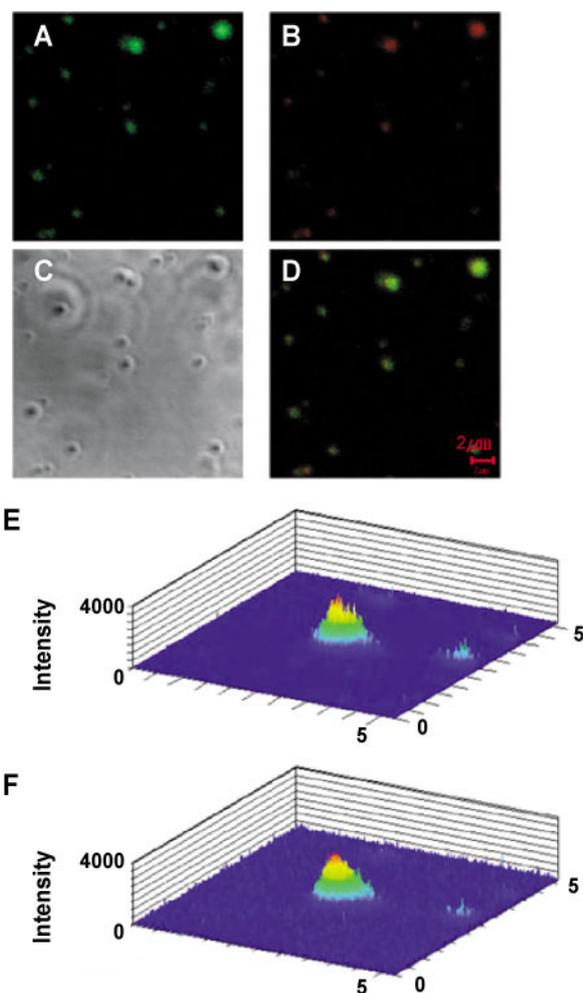


Fig. 3. Fluorescence detection of sulphonylurea receptors in mitochondria extracted from cardiomyocytes. Mitochondria were loaded simultaneously with glibenclamide-BODIPY (A) and MitoTracker Red CMXRos and (B). Images in C is transmitted light with differential interference contrast optics. Overlays of glibenclamide-BODIPY and MitoTracker Red CMXRos fluorescence (D) were created to examine the degree of co-localization. Images in A~D were captured at 400× magnification. Using the fluorescence intensity of the mitochondria preparation that was stained with glibenclamide-BODIPY (E) and MitoTracker Red CMXRos (F), fluorescence intensity was measured in each individual image (in A and B, respectively).

suspension was kept at 4°C until use (within 1 h).

Preparation of mitochondria

Mitochondria were collected and purified from individual cardiomyocytes by discontinuous percoll gradient purification. Individual cardiomyocytes were homogenized in homogenization buffer using a Dounce homogenizer before being centrifuged (3 min, 1,300×g, 4°C) in a swing-out rotor. The supernatant was then centrifuged (10 min, 21,000×g, 4°C) in an angle rotor. The pelleted cells were resuspended in 15% percoll solution, layered in a tube that contained 23 and 40% percoll solution in the same

homogenization buffer, and then centrifuged (5 min, 31,000 ×g, 4°C). Mitochondria were collected (at the lowest interface band), washed, and then diluted in hypertonic solution.

All procedures were carried out on ice or at 4°C, and all implements were cooled in a refrigerator prior to being used. To assess the purity of mitochondrial preparation, samples were stained with the mitochondria-specific red fluorescent probe, MitoTracker Red CMXRos (578/599 nm excitation/emission). As a negative control, samples were stained with the endoplasmic reticulum marker, ER-Tracker Blue-White DPX.

Fluorescence-based immunoassay

MitoTracker Red CMXRos-stained mitochondria were fixed in a solution of 4% paraformaldehyde and 0.05% glutaraldehyde for 4 h. Thereafter, the mitochondria were blocked for 1 h in a washing solution that contained 1% bovine serum albumin (BSA) and 10% normal goat serum. Mitochondria were then incubated overnight with primary antibodies against Kir6.1, Kir6.2, SUR1, and SUR2 (1 : 300 dilution for each). Thereafter, they were washed three times in a solution that contained 1% BSA before being incubated with rabbit anti-goat Alexa Fluor® 488-conjugated antibody (1 : 5000 dilution) for 1 h. After further washing (three times), the samples were visualized using confocal microscopy (see below).

Western blot

To obtain whole-cell extracts, cells were suspended within a lysis buffer for 60 min, homogenized, and then centrifuged (30 min, 13,000×g, 4°C). Protein concentrations in the whole-cell extract (in the supernatant) and enriched mitochondrial fraction were measured using the Bradford method. Equal amounts of protein (20µg) were run on a 7.5~12.0% sodium dodecyl sulfate-polyacrylamide (SDSPAGE) gel. Western blot was carried out with polyclonal goat anti-Kir6.1, anti-Kir6.2, anti-SUR1, and anti-SUR2 primary antibody (1 : 300 dilution for each), and rabbit anti-goat horseradish peroxidase-conjugated secondary antibody (1 : 1000 dilution). Protein-bound antibody was detected using enhanced chemiluminescence. The amino acid sequence that was obtained from GenBank (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=nucleotide>) was used to calculate the molecular weight of each protein.

Confocal microscopy

Purified mitochondria in hypertonic solution were washed and then stained simultaneously with both the red fluorescent probe, MitoTracker Red CMXRos (50 nM), and the green fluorescent probe, glibenclamide-BODIPY FL (488/510 nm excitation/emission; 50 nM), for 20~30 min on ice. Mitochondria were then visualized under a confocal scanning laser microscope (Carl Zeiss 510META) at 400× and 630× with the appropriate laser lines and filter sets. Images were analyzed using LSM510 META software (Carl Zeiss, Jena, Germany).

Drugs and solutions

Tyrode solution contained (in mM): 143.0 NaCl, 5.4 KCl,

1.8 CaCl₂, 0.5 MgCl₂, 5.5 glucose, 5.0 HEPES; pH=7.4, and the solution was oxygenated for 45 min before use. The modified KB solution contained (in mM): 70.0 KOH, 50.0 L-glutamate, 40.0 KCl, 20.0 KH₂PO₄, 20.0 taurine, 3.0 MgCl₂, 10.0 HEPES, 0.5 EGTA, 10.0 glucose; pH=7.4. The whole cell lysis buffer contained (in mM): 50 Tris-HCl, 120 NaCl, 1 EDTA, 1 DTT, 1 PMSF, 0.5% NP-40, 1× protease inhibitor cocktail. The mitochondrial homogenization buffer contained (in mM): 320.0 sucrose, 1.0 K-EDTA, 10.0 Tris-Cl, 0.1% BSA; pH=7.2. The washing solution contained (in mM): 150.0 KCl, 20.0 K-HEPES, 1.0 K-EGTA, 0.1% BSA; pH=7.2. The hypertonic solution contained (in mM): 750 KCl, 100 K-HEPES, 1 K-EGTA; pH=7.2. Fluorescent probes were purchased from Molecular Probes (Eugene, OR, USA). Rabbit anti-goat Alexa Fluor 488 was supplied at a concentration of 2 mg/ml. Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were obtained from Sigma (St. Louis, MO, USA).

RESULTS

Fluorescence detection of K_{ATP} channels in cardiomyocytes

Prior to the experiments, we checked the viability of individual cardiomyocytes by staining with Trypan Blue, and found that more than 60~70% of the isolated cardiomyocytes were healthy. After simultaneously staining the isolated cardiomyocytes with the sulfonylurea receptor-binding green fluorescent probe glibenclamide-BODIPY FL (Fig. 1A), and the mitochondria-specific red fluorescent probe, MitoTracker Red CMXRos (Fig. 1B), the cells were visualized under a confocal microscope. Both the MitoTracker Red and glibenclamide-BODIPY produced intense fluorescence in the isolated cardiomyocytes (Figs. 1D and 2A and B).

Fluorescence detection of K_{ATP} channels in mitochondria

The purity of the mitochondrial preparation was assessed using ER-Tracker Blue-White DPX, which specifically stains endoplasmic reticulum. As expected, the intensity of the ER-Tracker Blue-White DPX staining was reduced significantly in the purified mitochondrial preparation (data not shown). As in the isolated cardiomyocytes (see above), MitoTracker Red CMXRos (Fig. 3A and E) and glibenclamide-BODIPY FL (Fig. 3B and F) produced intense fluorescence in the mitochondrial preparation. In addition, two fluorescent probes were co-localized in the mitochondria (Fig. 3D).

Fluorescence-based immunoassay of K_{ATP} channels in mitochondria

Incubation of purified mitochondria from isolated cardiomyocytes with antibodies against Kir6.1, Kir6.2, SUR1, and SUR2 and the fluorescent secondary antibody, Alexa Fluor 488, revealed intense immunoreactivity for Kir6.1, Kir6.2, and SUR2. These data suggested that mito K_{ATP} channel subunits included Kir6.1, Kir6.2, and SUR2 (Fig. 4).

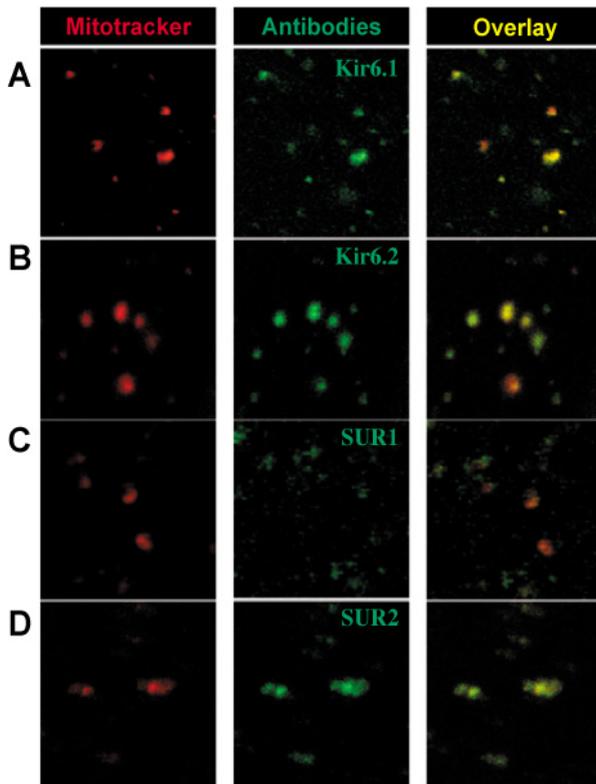


Fig. 4. Fluorescence-based immunoassay of mitoK_{ATP} channel subunit composition. Mitochondria labeled with Alexa Fluor 488 secondary antibody were brightly fluorescent in the samples that were incubated with anti-Kir6.1 (A), anti-Kir6.2 (B), and anti-SUR2 (D) antibodies. By contrast, no fluorescent signal was detected in samples that were incubated with anti-SUR1 antibody (C). Right, middle and left panels correspond to mitochondria that were visualized with the filter for MitoTracker Red CMXRos (578/599 nm excitation/emission), Alexa Fluor 488 (488/519 nm excitation/emission), and an overlay of the individual images. Scale bars=2 μ m.

Western blot analysis of mitochondrial K_{ATP} channel subunit composition

To confirm the subunit composition of the mitoK_{ATP} channels that we observed with fluorescence imaging, Western blot analysis was undertaken (Fig. 5). We detected bands that corresponded to the molecular mass of Kir6.1 (~46 kDa), Kir6.2 (~46 and 44 Da), and SUR2 (~140 kDa) protein in both whole-cell extracts from cardiomyocytes and the mitochondrial preparation. In addition, we detected a band at ~30 kDa that corresponded to Kir6.2 protein. SUR1 protein was not detected.

DISCUSSION

The K_{ATP} channel in the inner mitochondrial membrane was first identified in 1991 (Inoue et al, 1991), but its functional significance has remained unknown, even though it clearly allows K⁺ cycling across the cell membrane. There is much evidence that both sarcK_{ATP} and mitoK_{ATP} channels play an important role in cardioprotection, particular in regards to ischemia/reperfusion-induced injury. Gross (2002)

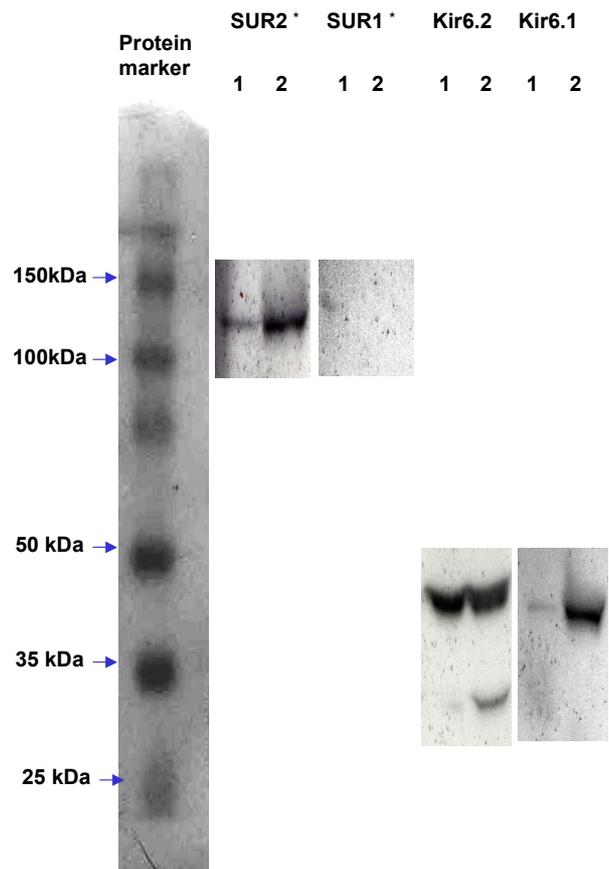


Fig. 5. Immunoblot analysis of Kir6.1, Kir6.2, SUR1, and SUR2 protein expression in rat cardiomyocytes and mitochondria. An equal amount of protein was run in a 7.5% (*) and 12% sodium dodecyl sulfate-polyacrylamide (SDSPAGE) gel. Extracts of cardiomyocytes and enriched mitochondrial preparations are shown in lanes 1 and 2, respectively. Western blot was carried out with antibodies to Kir6.1, Kir6.2, SUR1, and SUR2. The specific ~46-kDa immunoreactive band corresponded to Kir6.1 (A). For Kir6.2, three bands (~44, ~46, and ~30 kDa) were detected (B). A specific ~140-kDa immunoreactive band that corresponded to SUR2 (D) was detected. No band was detected with the anti-SUR1 antibody (C).

provided evidence on a role of sarcK_{ATP} and mitoK_{ATP} channels in IPC, and demonstrated that mitoK_{ATP} channels act as both triggers and end effectors. One of the most striking features of mitoK_{ATP} channels that distinguishes these channels from sarcK_{ATP} channels is a remarkable sensitivity to the K_{ATP} channel opener, 7-chloro-3-methyl-1,2,4-benzothiadiazine-1,1-dioxide (diazoxide). Garlid et al (1997) showed that pre-administration of diazoxide at a concentration that was mitochondrion-specific conferred strong protection against infarction, and this protection was abolished by the presence of either glibenclamide or 5-HD.

SarcK_{ATP} channels are hetero-octameric complexes that comprise four pore-forming subunits (Kirs) and four sulfonylurea receptor subunits (SURs) (Inagaki et al, 1997; Aguilar-Bryan et al, 1998). These complexes belong to the ATP-binding cassette. To date, Kir6.1, Kir6.2, SUR1, SUR2A, and SUR2B have been cloned (Tokuyama et al, 1996; Aguilar-Bryan et al, 1998), and it has been reported

that the mRNA of each of the aforementioned K_{ATP} channel subunits is expressed in heart muscle.

By contrast, relatively few studies have been carried out to investigate the identity of mito K_{ATP} subunits. By using immunogold electron microscopy, Suzuki et al (1997) suggested that Kir6.1 protein is expressed in mitochondria in brain. Seharaseyon et al (2000) used viral gene transfer to probe mito K_{ATP} channels, but found no evidence that Kir6.1 and Kir6.2 are part of the channel structure. Other approaches, such as using pharmacological compounds with higher affinity for mito K_{ATP} channels than for sarc K_{ATP} channels, are limited by nonspecific side-effects. To overcome this disadvantage, we used fluorescence microscopy and Western blot immunodetection to analyze the composition of K_{ATP} channels in intact mitochondria from isolated cardiomyocytes.

In the present study, we detected in purified preparations of mitochondria a single specific Western blot band at ~46 kDa that corresponded to Kir6.1 protein. Surprisingly, the anti-Kir6.2 antibody revealed three bands (at ~44, ~46 and ~30 kDa). Our observation in the Western blot analysis that both Kir6.1 and Kir6.2 proteins are expressed in cardiomyocytes is contrary to that of Kuniyasu et al (2003), who detected only Kir6.2 protein in rat cardiac mitochondria using a C-terminus-binding anti-Kir6.1 antibody and an N-terminus-binding anti-Kir6.2 antibody. Our fluorescence microscopy analysis of Kir6.1 and Kir6.2 protein expression in mitochondria in the present study corroborated the results of our Western blot analysis; fluorescent images of Kir6.1 and Kir6.2 overlapped, indicating that both of these proteins are expressed in rat cardiac mitochondria. In addition, direct visualization of individual mitochondria revealed that the mitochondria-specific fluorescent staining and that of glibenclamide, a sulfonylurea receptor-binding probe, were co-localized, suggesting that SUR protein subunits are expressed in rat cardiac mitochondria. The results of our Western blot analysis concurred with our fluorescence microscopy observations; we found evidence that SUR2, but not SUR1, is expressed in the mitochondria of cardiomyocytes. Therefore, it would appear that the SUR protein that is expressed in cardiomyocyte mitochondria is a SUR2-related (~140 kDa) protein. Lacza et al (2003), using Western blot analysis, found bands at ~50 and ~25 kDa that corresponded to SUR1 and SUR2, respectively, in rat brain mitochondria. Bajgar et al (2001) implied that mito K_{ATP} channel subunits include a 55-kDa Kir and a 63-kDa SUR subunit, which is contrary to the findings of the present study. This apparent discrepancy might be due to the non-specificity of the antibodies that were used in each study. For instance, besides detecting Kir6.1 and SUR2 subunits, the antibody that we used to probe Western blots for Kir6.2 protein revealed three bands at ~46, ~44, and ~30 kDa. To assess the specificity of the antibody, we carried out fluorescent immunohistochemistry, and this provided better evidence that Kir6.1, Kir6.2, and SUR2 are among the mito K_{ATP} channel subunits that are expressed in cardiac mitochondria.

To the best of our knowledge, this is the first study in which the identity of mito K_{ATP} channel subunits has been investigated in rat cardiomyocytes using immunofluorescence microscopy and Western blot analysis. Nevertheless, to confirm our conclusion that mito K_{ATP} channel subunits in cardiomyocytes include Kir6.1, Kir6.2, and SUR2 proteins, further studies are required, such as sequencing

of the subunit proteins and pharmacological and electrophysiological characterization of mito K_{ATP} channels in isolated mitochondria.

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