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## ATP-sensitive K<sup>+</sup>-channel Subunits on the Mitochondria and Endoplasmic Reticulum of Rat Cardiomyocytes

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**SUMMARY** ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channel subunits on the subcellular structures of rat cardiomyocytes were studied with antibodies against Kir6.1 and Kir6.2. According to the results of Western blot analysis, Kir6.1 was strongly expressed in mitochondrial and microsome fractions, and faintly expressed in cell membrane fraction, whereas Kir6.2 was mainly expressed in the microsome fraction and weakly in cell membrane and mitochondrial fractions. Immunohistochemistry showed that Kir6.1 and Kir6.2 were expressed in the endocardium, atrial and ventricular myocardium, and in vascular smooth muscles. Immunoelectron microscopy revealed that Kir6.1 immunoreactivity was mainly localized in the mitochondria, whereas Kir6.2 immunoreactivity was mainly localized in the endoplasmic reticulum and a few in the mitochondria. Both Kir6.1 and Kir6.2 are candidates of mitochondrial K<sub>ATP</sub> channel subunits. The data obtained in this study will be useful for analyzing the composition of K<sub>ATP</sub> channels of cardiomyocytes and help to understanding the cardioprotective role of K<sub>ATP</sub> channels during heart ischemia.

(*J Histochem Cytochem* 53:1491–1500, 2005)

**KEY WORDS**

ATP-sensitive K<sup>+</sup> channels  
mitochondria  
endoplasmic reticulum  
rat  
cardiomyocytes  
immunoelectron microscopy

ATP-SENSITIVE K<sup>+</sup> (K<sub>ATP</sub>) CHANNELS were originally discovered in the heart (Noma 1983), and have since been found in many tissues, including pancreatic  $\beta$ -cells, skeletal muscle, kidney, and brain (Inagaki et al. 1995). K<sub>ATP</sub> channels close at high intracellular ATP concentrations and open during ischemia (lower intracellular ATP concentrations) (Yokoshiki et al. 1998). They are hetero-octameric and consist of four pore-forming subunit molecules, Kir6.x (Kir6.1 or Kir6.2), and four regulatory subunit molecules, sulfonylurea receptors (SUR): SUR1, SUR2A, or SUR2B (Clement et al. 1997).

Brief exposure of myocardium to ischemia and reperfusion, named ischemia preconditioning, can dramatically reduce lethal injury to the heart followed by prolonged severe ischemia, but the mechanism of such

a cardioprotection remains controversial—on the one hand being considered to be attributable to the function of sarcolemmal K<sub>ATP</sub> channels (Suzuki et al. 2002), and on the other to be attributable to mitochondrial K<sub>ATP</sub> channels (Das and Sarkar 2003). Kir6.1 mRNA and protein are specifically upregulated during myocardial infarction (Akao et al. 1997). Mice lacking the Kir6.1 gene experience spontaneous ST elevation and atrioventricular block followed by sudden death (Miki et al. 2002). Kir6.1 may be an important candidate to explain the mechanism of cardioprotection, but its precise localization remains to be determined.

Two conflicting views still exist. By the patch-clamp technique, K<sub>ATP</sub> channels were revealed to be localized in the inner membrane of mitochondria with fused giant mitoplasts (Inoue et al. 1991). Later, Kir6.1 was revealed to be localized in the mitochondria in skeletal muscles (Suzuki et al. 1997), and in neurons and glial cells of rat brain (Zhou et al. 1999), whereas the results of the gene transfer technique indicated that neither Kir6.1 nor Kir6.2 was localized in the mitochondria (Seharaseyon et al. 2000). Recently, Kir6.1 and

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Received for publication May 18, 2005; accepted June 1, 2005 [DOI: 10.1369/jhc.5A6736.2005].

Kir6.2 were expressed in mitochondria in the isolated mitochondrial fraction of mouse cardiomyocytes (Lacza et al. 2003) and isolated rat ventricular myocytes (Singh et al. 2003). In contrast, there were deniable results that showed no expression of  $K_{ATP}$  subunits Kir6.1, Kir6.2, SUR2A, and SUR2B in mitochondrial fractions of rat cardiomyocytes by Western blot analysis (Kuniyasu et al. 2003). The specificity of antibodies used and the purity of mitochondrial fractions obtained seemed convincing, but the reason for the discrepancy among the results still remains unknown. Thus it is still necessary to determine the exact cellular and subcellular localization of Kir6.1 or Kir6.2 in cardiomyocytes to identify the molecular identity of  $K_{ATP}$  channels.

In the present study, we used a new rabbit anti-rat Kir6.1 antibody and a commercial goat anti-human Kir6.2 antibody to analyze the localization of Kir6.1 and Kir6.2 in the mitochondria or endoplasmic reticulum (ER) of rat cardiomyocytes by immunoblot analysis and immunoelectron microscopy. We paid special attention to the characterization of antibody used. Fractions of mitochondria, cell membrane, and microsome of rat heart were carefully purified and distinguished with anti-cytochrome c, anti- $Na^+/K^+$  ATPase  $\beta$ 1 subunit, and anti-calreticulin antisera. Immunohistochemistry and immunoelectron microscopy were used to detect the localization of  $K_{ATP}$  subunit proteins on rat heart sections.

## Materials and Methods

### Generation of Anti-Kir6.1 Antibody

Rabbit anti-Kir6.1 antiserum was raised against a synthetic 14 amino acid peptide,  $NH_2$ -(C)QFMTPEGNQCPSSES-OH, which corresponds to amino acid residues 411–424 of rat Kir6.1 (Gene No. D42145). The polyclonal peptide antiserum production was processed according to Van Bueren et al. (1993), with some modification. In brief, the synthetic peptide representative to rat Kir6.1 was coupled to the carrier protein keyhole limpet hemocyanin via the cysteine residue added to the N-terminal. Two Japanese white rabbits (Japan SLC; Hamamatsu, Japan) weighing 2.5–3.0 kg were injected with  $\sim 200$   $\mu$ g of peptide-keyhole limpet hemocyanin conjugate emulsified with an equal volume of Freund's complete adjuvant (Rockland Immunochemicals; Gilbertsville, PA) at multiple intradermal sites, followed by three boosters at 2-wk intervals and later by injection with the same dosage of the peptide conjugate emulsified in Freund's incomplete adjuvant (Rockland Immunochemicals) three times. The antiserum was harvested 1 wk after the final injection. The antiserum was purified by immunoaffinity column chromatography before using for Western blot analysis and immunohistochemistry.

### Animals

Male Wistar rats (4–8 wk) were used. The protocols for animal experimentation described were approved by the Ani-

mal Research Committee, Akita University. All subsequent animal experiments were conducted in accordance with the Guidelines for Animal Experimentation of the University.

The animals used for immunohistochemistry and immunoelectron microscopy were anesthetized by diethyl ether inhalation and then perfused through left ventricle with 4% paraformaldehyde in 0.1 M PBS, pH 7.4. The hearts were quickly excised and placed in the same fixative for 6 hr at 4C, after which they were transferred to 30% sucrose in PBS. Cryosections 7–8  $\mu$ m thick were cut, thaw-mounted on MAS-coated glass slides (Matsunami Glass Ind. Ltd.; Kishiwada-city, Japan), and stored at  $-80$ C until used.

### Subcellular Fractionation of Rat Heart

All operations were carried out at 0–4C. Adult Wistar rats were anesthetized by inhalation of diethyl ether. Their hearts were immediately excised and quickly washed with 0.9% NaCl solution. The hearts were then cut into small pieces and homogenized with 0.25 mM sucrose/50 mM Tris-HCl buffer, pH 7.4, containing proteinase inhibitor cocktail tablets (Roche Diagnostics GmbH; Mannheim, Germany). Subcellular fractions were extracted as described elsewhere (Itoh et al. 2002). Briefly, after centrifugation at  $600 \times g$  for 10 min, the precipitate was discarded, and the  $600 \times g$  supernatant was centrifuged at  $7000 \times g$  for an additional 10 min. The  $7000 \times g$  precipitate (P1) was redissolved in the buffer and centrifuged at  $5000 \times g$  for 10 min, and the  $5000 \times g$  precipitate was used as the mitochondrial fraction. The  $7000 g$  supernatant (S1) was centrifuged at  $54,000 \times g$  for 60 min, and the supernatant (S2) was centrifuged at  $105,000 \times g$  for an additional 60 min. The  $54,000 \times g$  precipitate was used as the cell membrane fraction, the  $105,000 \times g$  precipitate as the microsome fraction, and the supernatant as the cytoplasm fraction.

### Western Blot Analysis

SDS/PAGE was carried out according to the procedure of Laemmli (Laemmli 1970), using 10% polyacrylamide. Proteins of cellular fractions were electrophoresed (10  $\mu$ g per lane). After electrophoresis, proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad; Hercules, CA) with a semi-dry transfer unit (Hoefer TE70 series; Amersham Pharmacia Biotechnology, Little Chalfont, Buckinghamshire, England). After blocking with 5% (w/v) skim milk in PBS and 0.1% Tween-20, the polyvinylidene difluoride membranes were incubated with rabbit anti-rat Kir6.1, goat anti-human Kir6.2 (Sc-11228; Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit anti-cytochrome c (Sc-7159; Santa Cruz Biotechnology Inc.), rabbit anti-calreticulin (NB600-101; Novus Biologicals, Littleton, CO), or rabbit anti  $Na^+/K^+$  ATPase  $\beta$ 1 (Sc-16053; Santa Cruz Biotechnology Inc.), diluted in 1:500 to 1:1000, respectively, for 60 min. After rinsing with PBS, they were then exposed to horseradish peroxidase-conjugated donkey anti-rabbit IgG (NA9340; Amersham Pharmacia Biotechnology), or donkey anti-goat IgG (AP180P; Chemicon International, Temecula, CA), diluted in 1:3000 to 1:5000 for 30 min. The antigen-antibody complexes were visualized with chemiluminescence detection reagents (Amersham Pharmacia Biotechnology), according to the manufacturer's instructions.

### Immunohistochemistry

The heart sections were maintained in PBS containing 0.3% Tween-20 for 45 min or pretreated with 0.4% pepsin in 0.2N HCl for 3 or 5 min at 37°C. After preincubation with 5% normal serum (goat or rabbit) for 60 min, the sections were incubated with rabbit anti-rat Kir6.1 at a dilution of 1:200, goat anti-human Kir6.1 (Sc-11224, Santa Cruz Biotechnology) at a dilution of 1:500, or goat anti-human Kir6.2 at a dilution of 1:200 for 12 hr at room temperature. Before incubation, sections were treated with a 0.3% solution of H<sub>2</sub>O<sub>2</sub> in methanol for 10 min to reduce endogenous peroxidase reactions. After thoroughly rinsing with PBS, the sections were exposed to biotinylated goat anti-rabbit IgG or biotinylated rabbit anti-goat IgG (BA-1000 and BA-5000; Vector Laboratories Inc., Burlingame, CA), diluted in 1:200 for 30 min, and then with ABC complex (Vector Laboratories Inc.) according to the manufacturer's instructions. Reaction sites were visualized by the DAB (3,3'-diaminobenzidine tetrahydrochloride) reaction, and counterstaining was performed with methyl green.

### Immunofluorescence Double Staining

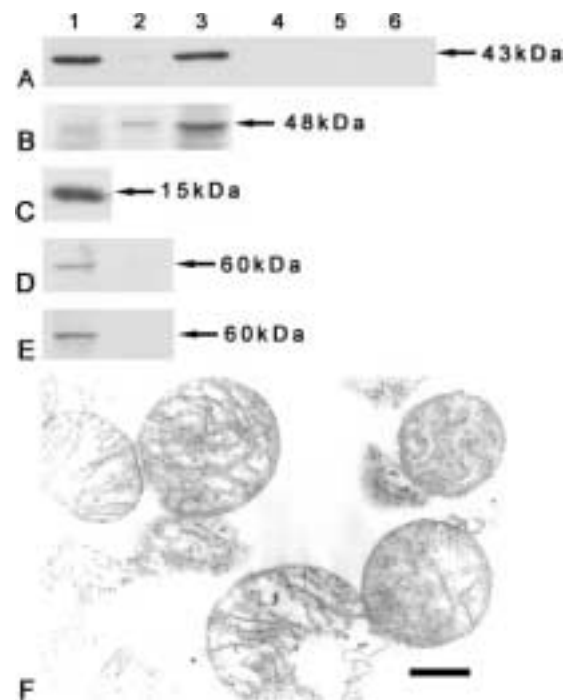
The heart sections were maintained in PBS containing 0.3% Tween-20 for 45 min. After preincubation with 5% normal donkey serum in PBS for 1 hr, the sections for double labeling with Kir6.1 and cytochrome c were incubated with goat anti-human Kir6.1 antibody (1:200), and rabbit anti-cytochrome c antibody (1:200) diluted together in PBS, and those for double labeling with Kir6.2 and calreticulin were incubated with goat anti-human Kir6.2 antibody (1:200) and rabbit anti-calreticulin antibody (1:100) diluted together in PBS for 12 hr at room temperature. After rinsing with PBS, the sections were then reacted with Alexa 488-conjugated donkey anti-goat IgG (A11055) and with Alexa 594-conjugated donkey anti-rabbit IgG (A21207; Molecular Probes Inc., Eugene, OR: green for Kir6.1 and Kir6.2, red for cytochrome c and calreticulin) diluted together at 1:1000 in PBS for 30 min. The sections were then cover slipped with PermaFluor aqueous mounting medium (Thermo; Pittsburgh, PA) after counterstaining with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI). Fluorescence immunolabeling signals were detected by a laser-scanning microscope (LSM510; Carl Zeiss, Oberkochen, Germany).

### Immunoelectron Microscopy

**Preembedding Method.** Tissue sections showing immunopositive reactions to Kir6.1, and those to Kir6.2 were postfixed in 1% osmium tetroxide (O<sub>3</sub>O<sub>4</sub>) for 30 min, dehydrated in a graded ethanol series, and embedded in Quetol 812 (Nissin EM Co.; Tokyo, Japan). Thin sections were cut and examined with an electron microscope without uranyl acetate and lead citrate staining.

**Postembedding Method.** After perfusion fixation tissue blocks were washed with PBS, dehydrated in an ethanol series, and embedded in Lowicryl K4M at -20°C in an ultraviolet polymerization chamber (Nissin EM) according to the manufacturer's instructions. Thin sections were placed on nickel grids and immunostained by incubating in 1% BSA/PBS for 60 min and then incubated for 12 hr at room temperature

with rabbit anti-rat Kir6.1 or goat anti-human Kir6.2 antisera diluted to 1:200 with PBS containing 1% BSA. Normal goat or rabbit serum was used as a negative control. After washing several times with PBS containing 0.1% BSA, the sections exposed to rabbit antisera were incubated with goat anti-rabbit IgG-labeled 5-nm colloidal-gold (G7277; Sigma-Aldrich, Tokyo, Japan) at a dilution of 1:40 with 1% BSA/PBS; those exposed to goat antisera were incubated with rabbit anti-goat IgG-labeled 5-nm colloidal-gold (G5528, Sigma-Aldrich), at a dilution of 1:40 in 1% BSA/PBS for 6 hr at room temperature. After rinsing several times with PBS containing 0.1% BSA, the sections were fixed in 2% glutaraldehyde/PBS for 10 min, rinsed with distilled water, and stained with 2% uranium acetate, and then examined with an electron microscope.



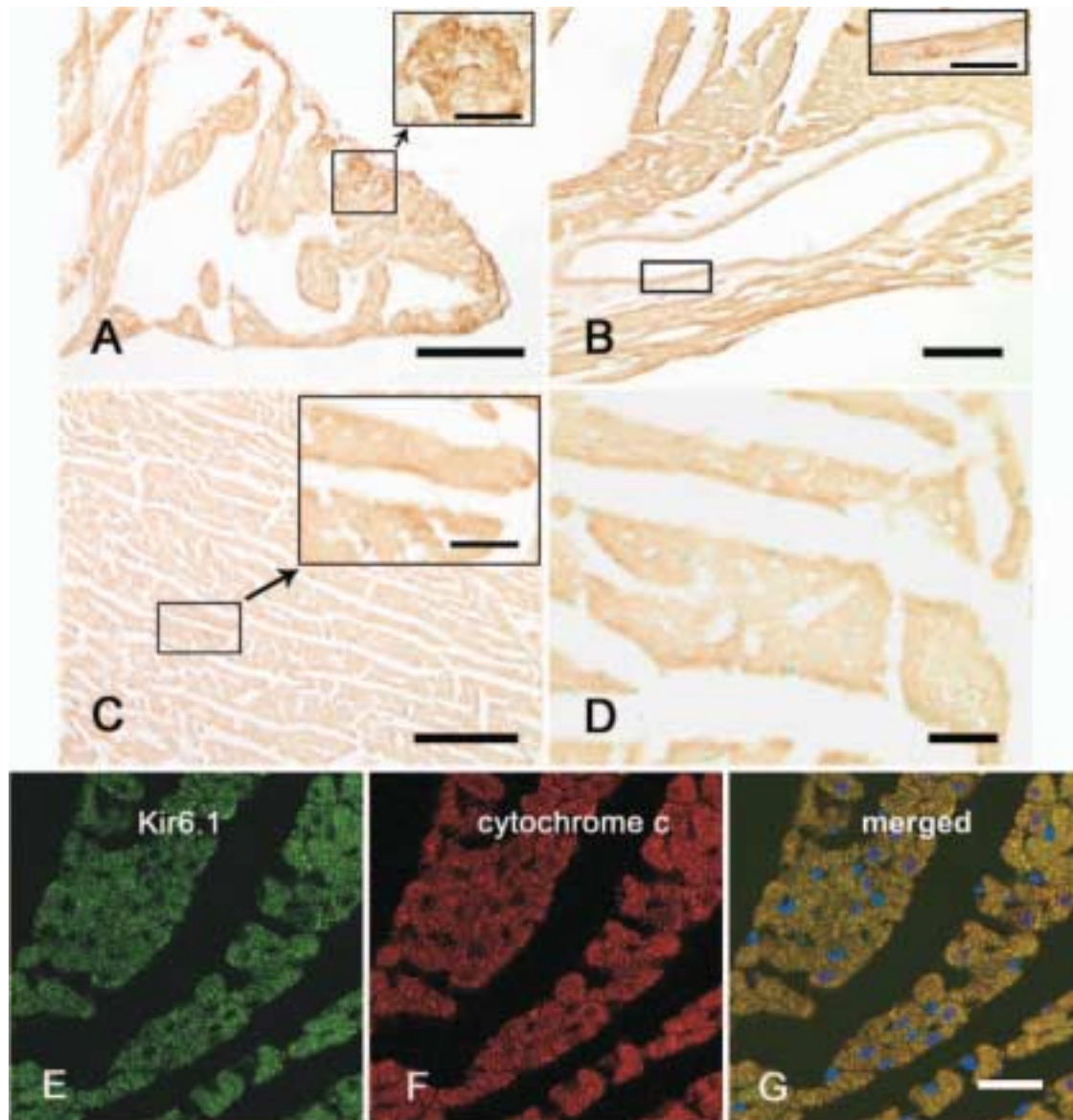
**Figure 1** Western blot analysis of Kir6.1 and Kir6.2 in the rat heart and purification of isolated cellular fractions. (A) In the three left lanes, the rabbit anti-rat Kir6.1 antibody recognizes a prominent ~43 kDa band in the mitochondrial (Lane 1) and microsome (Lane 3) fractions, but very weak in the cell membrane fraction (Lane 2). In the three right lanes, after preincubation with immunizing peptide antigen the detection bands have disappeared (Lanes 4–6). (Lanes 1,4) Mitochondrial fraction; (Lanes 2,5) plasma membrane fraction; and (Lanes 3,6) microsome fraction. (B) A corresponding band (~48 kDa) is detected by the goat anti-human Kir6.2 antibody in the microsome fraction (Lane 3) of the rat heart, weakly in the cell membrane (Lane 2) and the mitochondrial (Lane 1) fractions. (C) A corresponding band (15 kDa) is strongly detected in the mitochondrial fraction by the rabbit anti-cytochrome c antibody. (D) The anti-calreticulin antibody recognizes a corresponding band (60 kDa) only in the microsome fraction (Lane 1), but not in the mitochondrial fraction (Lane 2). (E) The anti-Na<sup>+</sup>/K<sup>+</sup> ATPase  $\beta$ 1 subunit antibody recognizes a corresponding band (60 kDa) in the plasma membrane fraction (Lane 1), but not in the mitochondrial fraction (Lane 2). (F) Most mitochondria were intact shown in the electron micrograph, proving the purity of the mitochondrial fraction. Bar = 500 nm.

### Electron Microscopy of the Mitochondrial Fraction

To confirm the purity of the mitochondrial fraction used in the Western blot, the  $5000 \times g$  precipitate obtained by subcellular fractionation was fixed with 2% glutaraldehyde for 2 hr, followed by 1%  $\text{OsO}_4$  for 2 hr, and then dehydrated with acetone and embedded in Quetol 812. Thin sections were cut and directly examined with an electron microscope after uranyl acetate staining.

### Quantitative Evaluation

Unit areas of mitochondria and areas outside the mitochondria were measured with an image-analyzing computer and software (version 1.62, NIH Image; Bethesda, MD) in each of 20 electron micrographs (original magnification  $\times 15,000$  or  $\times 20,000$ ) randomly taken in the postembedded sections stained with anti-Kir6.1 antibody or anti-Kir6.2 antibody. The numbers of labeled colloidal gold particles per unit area



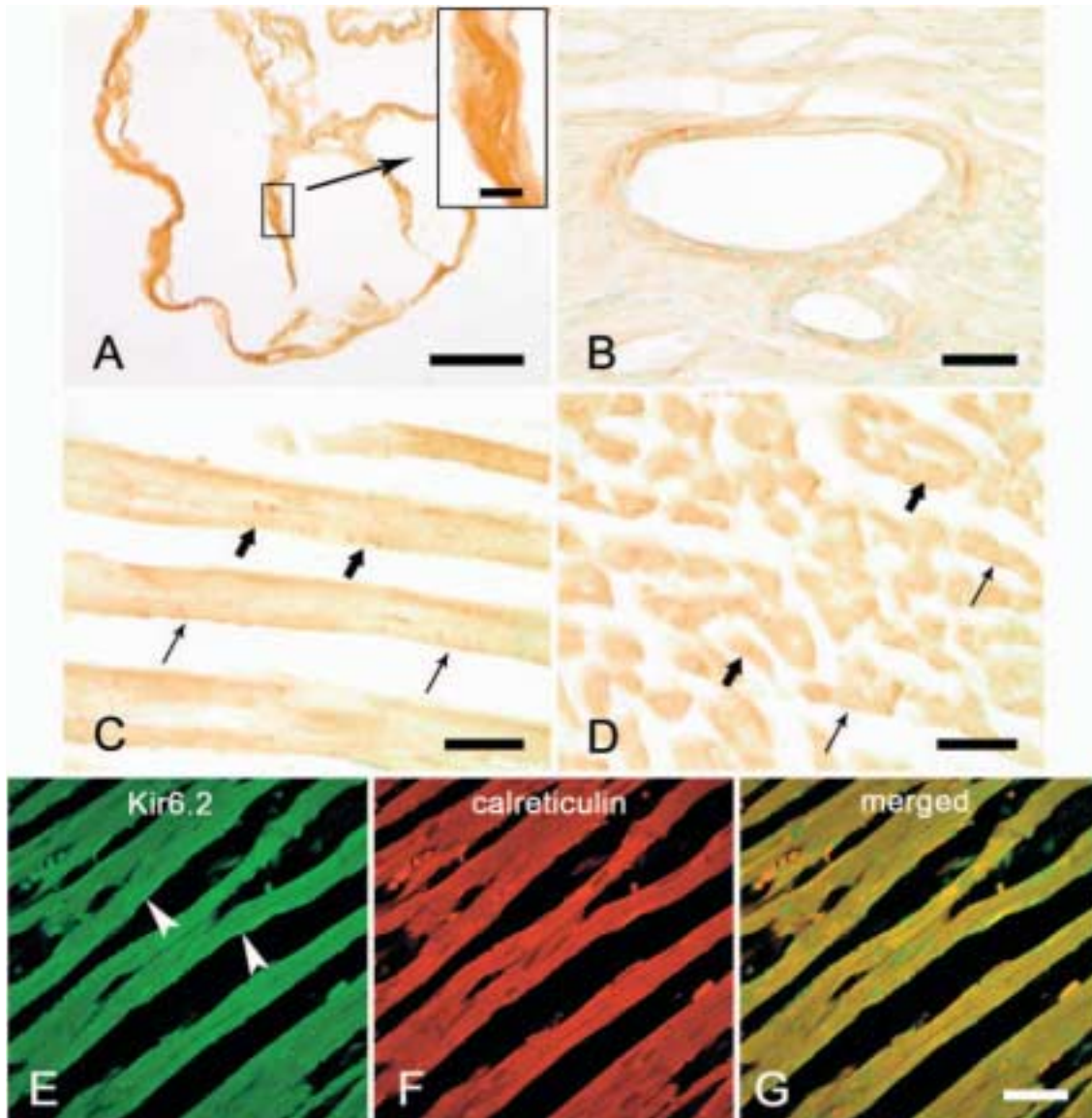
**Figure 2** Immunohistochemistry showing expression of Kir6.1 protein in rat cardiomyocytes. Immunoreactivity with anti-rat Kir6.1 is seen in the cardiomyocytes of the atrium (A), ventricle (B,C), and smooth muscle of coronary blood vessel (B). Punctate reaction products are seen in the cytoplasm (insets in A,C). A goat anti-human Kir6.1 antibody yielded the same results (D). Immunofluorescence double staining shows the expression of Kir6.1 and cytochrome c in rat cardiomyocytes (E–G). The green fluorescence (Alexa 488) represents the Kir6.1 in the cytoplasm of rat cardiomyocytes (E); the red fluorescence (Alexa 594) represents the cytochrome c in the mitochondria (F). The merged image (G) shows that the Kir6.1 was colocalized with cytochrome c in the mitochondria with yellow. Bars: A–C = 0.1 mm; D = 20  $\mu\text{m}$ ; E–G = 20  $\mu\text{m}$ . Inset bars: A,B = 10  $\mu\text{m}$ ; C = 20  $\mu\text{m}$ .

of mitochondria and area outside the mitochondria, including the cytoplasm, myofilaments, and ER, were calculated. All data were input into an access database by Excel 2000 and analyzed with SPSS software (version 10.0J, SPSS Inc.; Chicago, IL). The data were reported as means  $\pm$  SE. Differences in the mean particle density between the two groups were analyzed by the unpaired Student's *t*-test. All statistical tests and *p* values were two-tailed, and the results were considered significant when the *p* value was less than 0.05.

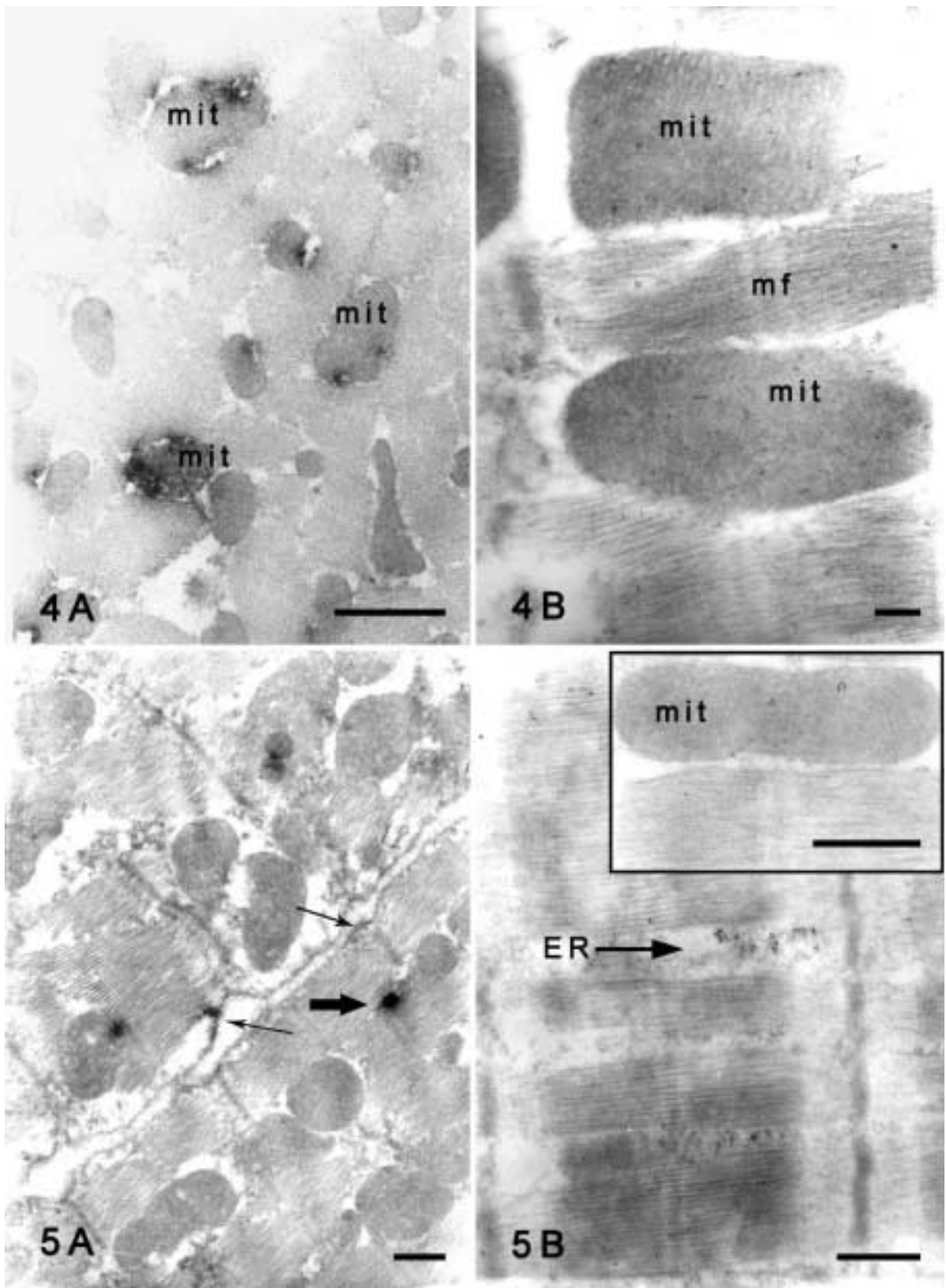
## Results

### Western Blot Analysis

Polyclonal antiserum generated in a rabbit against rat Kir6.1 was affinity-purified to investigate the distribution of Kir6.1 protein in rat cardiomyocytes. The anti-Kir6.1 antibody recognized a prominent  $\sim$ 43 kDa protein band in the mitochondrial fractions (Figure 1A, Lane 1), microsome fraction (Figure 1A, Lane 3), and



**Figure 3** Immunohistochemistry showing expression of Kir6.2 protein in rat cardiomyocytes. Immunoreactivity with anti-Kir6.2 antibody is seen in cardiomyocytes in the atrium (A). No prominent punctate reaction products were detected in the cytoplasm (inset). Immunoreactivity was also observed in the wall of coronary blood vessels in the rat heart (B). In the longitudinal (C) and transversal (D) section of rat ventricle, weak immunoreactivity to Kir6.2 is seen in the cell membrane (thin arrows); some granular immunoreaction products are shown in the cytoplasm (bold arrows). Immunofluorescence double staining shows the expression of Kir6.2 as green (Alexa 488, E) in the cytoplasm and plasma membrane (arrowheads) and calreticulin as red (Alexa 594, F). The merged image (G) indicates that Kir6.2 is partly colocalized with calreticulin in the endoplasmic reticulum. Bars: A = 0.1 mm; B = 50  $\mu$ m; C, D = 20  $\mu$ m; E–G = 20  $\mu$ m. Inset bar = 10  $\mu$ m.



a very weak signal was detected in the cell membrane fraction (Figure 1A, Lane 2). The detection signals were completely removed (Figure 1A, Lanes 4–6) by preabsorption with the immunizing peptide antigen. The anti-human Kir6.2 antibody, which crossreacts with rat Kir6.2, recognized a prominent band in the microsome fraction (Figure 1B, Lane 3), and that was weak in the mitochondrial fraction (Figure 1B, Lane 1), and the cell membrane fraction (Figure 1B, Lane 2).

#### Confirmation of the Cellular Fractions

Anti-cytochrome c antibody was used to confirm the purity of the mitochondrial fraction. The mitochondrial fraction showed intense immunoreactivity to anti-cytochrome c antibody in the Western blot analysis (Figure 1C). The microsome fractions was confirmed by anti-calreticulin antibody (an ER-specific antibody), which reacted to microsome fraction (Figure 1D, Lane 1), but not to mitochondrial fraction (Figure 1D, Lane 2). The purity of the cell membrane fraction was confirmed by anti-Na<sup>+</sup>/K<sup>+</sup> ATPase  $\beta$ 1 subunit antibody (a membrane-specific antibody), which reacted to plasma membrane fraction (Figure 1E, Lane 1), but not to the mitochondrial fraction (Figure 1E, Lane 2). The purity isolated mitochondrial fraction was also confirmed by electron microscopy. In the electron photograph, most of the visual field was covered with complete or some broken mitochondria (Figure 1F).

#### Immunohistochemistry of Kir6.1 and Kir6.2

In the atria, intense immunoreactivity with anti-Kir6.1 antibodies was observed in the atrial cardiomyocytes (Figure 2A). In the ventricles, moderate to intense immunoreactivity with Kir6.1 antibodies was detected in both the transverse sections and longitudinal sections of cardiomyocytes (Figure 2B). Kir6.1 protein was also detected in the papillary muscles, endocardium, interatrial septum, and interventricular septum. The immunoreactivity with anti-Kir6.1 antibodies in cardiomyocytes was prominent because of punctate-immunoreactive products in the cytoplasm (Figure 2C). Weak to moderate immunoreactivity with Kir6.1 was also observed in the smooth muscle and endothelium of blood vessels (Figure 2B). The anti-human Kir6.1 anti-

body, which crossreacts with rat Kir6.1, also exhibited the similar immunoreactivity in rat cardiomyocytes as observed with rabbit anti-rat Kir6.1 antibody (Figure 2D).

Rat cardiomyocytes in the atria (Figure 3A), ventricles (Figure 3B), papillary muscles, interatrial septum, interventricular septum, and vascular smooth muscle (Figure 3B) showed immunoreactivity with Kir6.2, but the characteristics of Kir6.2 expression were some different from those of Kir6.1 expression. In longitudinal sections, the cell membrane of the cardiomyocytes showed weak immunoreactivity with Kir6.2 (Figure 3C), and some granular or punctate immunoreaction products were also observed in the cytoplasm both in longitudinal and transversal sections (Figures 3C and 3D).

#### Immunofluorescence Double Staining

To determine whether the Kir6.1 is localized in the mitochondria of the rat cardiomyocytes, immunofluorescence double staining was performed. Immunoreactivity of Kir6.1 was detected as green fluorescence (Alexa 488, Figure 2E), and immunoreactivity of cytochrome c as red fluorescence (Alexa 594, Figure 2F). When both images were merged, the yellow fluorescence was detected in the cytoplasm (Figure 2G), indicating that Kir6.1 is overlapped on the mitochondria.

Immunofluorescence double staining was also performed to determine whether the Kir6.2 is localized in the ER. The expression of Kir6.2 was detected as green fluorescence in the cytoplasm and also detectable on the plasma membrane (Alexa 488, Figure 3E), and the expression of calreticulin, a multifunctional, highly conserved Ca<sup>2+</sup>-binding protein localized to the ER, detected as red fluorescence (Alexa 594, Figure 3F). When both images were merged, the yellow fluorescence was detected in the cytoplasm (Figure 3G), indicating that Kir6.2 is overlapped on the ER.

#### Immunoelectron Microscopy for Kir6.1 and Kir6.2

Electron microscopy after the preembedding method revealed the subcellular localization of Kir6.1 in the cardiomyocytes. The punctate immunoreaction products of Kir6.1 observed by light microscopy were de-

#### Figures 4 and 5

**Figure 4** Immunoelectron micrographs showing Kir6.1 protein in rat cardiomyocytes. (A) Preembedding method. The immunoreactivity with Kir6.1 is shown in the mitochondria (mit) of the transverse section. (B) Postembedding method. Most gold particles representing Kir6.1 have been deposited in the mitochondria (mit), but some are present in the cytoplasm and myofilaments (mf) in a back-ground level. Bars: A = 1  $\mu$ m; B = 200 nm.

**Figure 5** Immunoelectron micrographs showing Kir6.2 protein in rat cardiomyocytes. (A) Preembedding method. Immunoreactivity with Kir6.2 was shown in the endoplasmic reticulum (ER, bold arrow) and cell membrane (thin arrows) of cardiomyocytes. (B) Postembedding method. Dense deposition of colloidal gold particles representing Kir6.2 is seen in the ER. Inset micrograph shows that a few gold particles have been deposited in the mitochondria (mit). Bars = 500 nm.

tected in the mitochondria of the cardiomyocytes (Figure 4A). No specific labeling was observed on the cell membrane or ER of the rat cardiomyocytes. With the postembedding method, immunogold particles representing Kir6.1 were clearly observed in the mitochondria (Figure 4B), and fewer particles were seen in the myofilaments, nucleus, or cytoplasm of the cardiomyocytes. No specific labeling was observed on the cell membrane.

By contrast, Kir6.2 immunoreactivity was observed in the ER and the cell membrane of the rat cardiomyocytes with preembedding method (Figure 5A). Immunogold particles representing Kir6.2 were concentrated in the ER (Figure 5B) with a postembedding method. A few particles were also observed in the mitochondria (Figure 5B, inset).

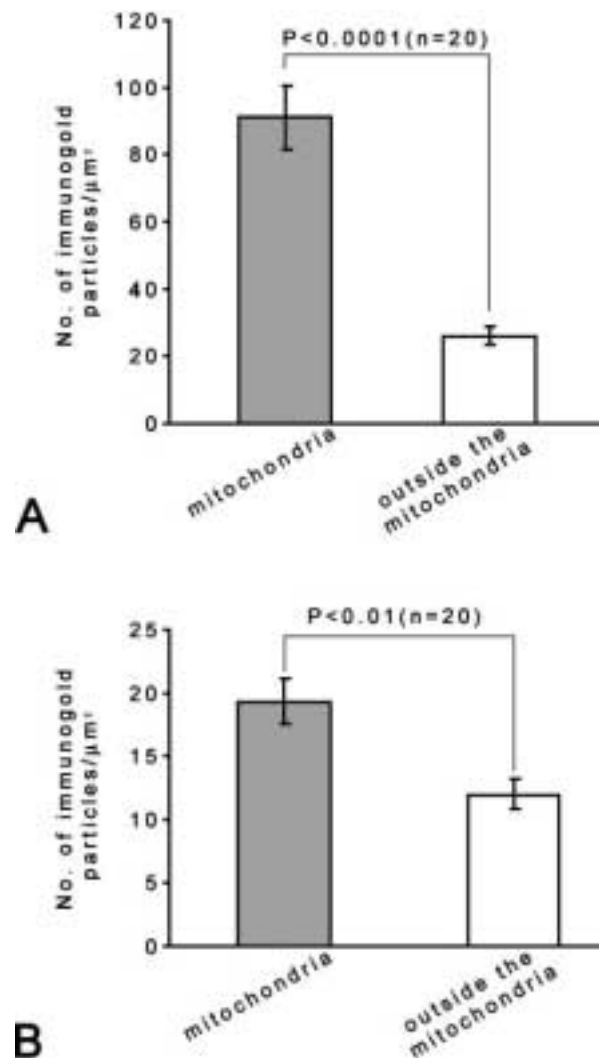
### Statistical Analysis

The density of colloidal gold particle labeling was calculated in the mitochondria and outside the mitochondria. With anti-Kir6.1 antibody, particle density in the mitochondria—that is, the number of labeled gold particles per unit area of mitochondria ( $91.03 \pm 9.57/\mu\text{m}^2$ )—was significantly higher than that of outside the mitochondria ( $25.88 \pm 2.81/\mu\text{m}^2$ ,  $p < 0.0001$ , Figure 6A). With anti-Kir6.2 antibody, particle density in the mitochondria ( $19.33 \pm 1.80/\mu\text{m}^2$ ) was also significantly higher than outside the mitochondria ( $12.0 \pm 1.25/\mu\text{m}^2$ ,  $p < 0.01$ , Figure 6B).

### Discussion

Biochemical and immunohistochemical methods revealed the localization of Kir6.1 and Kir6.2 in the subcellular fractions and structures of rat cardiomyocytes. The cellular fractions of mitochondria, cell membrane and microsome were confirmed by their marker antibodies, such as cytochrome c, a marker of mitochondrion;  $\text{Na}^+/\text{K}^+$  ATPase  $\beta 1$ , a marker of cell membrane; and calreticulin, a marker of ER. Each cellular fraction only reacted to its own marker antibody without to the other antibodies. The mitochondrial fraction was also confirmed by electron microscopy. With these cellular fractions, Western blot analysis was processed.

A polyclonal antibody was generated in a rabbit against the C-terminal domain of rat Kir6.1. The antibody was affinity-purified to investigate the distribution of Kir6.1 protein in the rat heart. The specificity of the antibody was assessed by Western blot analysis. This antibody recognized a prominent band ( $\sim 43$  kDa) in mitochondrial and microsome fractions, and a faint band in cell membrane fraction. The immunopositive bands were eliminated after preabsorption with immunizing peptide antigen. Kir6.2 was prominently detected in the microsome fraction, weakly in the cell



**Figure 6** Statistical analysis of Kir6.1 and Kir6.2 density in rat cardiomyocytes detected by the postembedding method. (A) The density of colloidal gold particles representing Kir6.1 in the mitochondria ( $91.03 \pm 9.57$  per  $\mu\text{m}^2$ ) was significantly higher than outside the mitochondria ( $25.88 \pm 2.81$  per  $\mu\text{m}^2$ ,  $p < 0.0001$ ). (B) The density of colloidal gold particles representing Kir6.2 in the mitochondria ( $19.33 \pm 1.80$  per  $\mu\text{m}^2$ ) was significantly higher than outside the mitochondria ( $12.0 \pm 1.25$  per  $\mu\text{m}^2$ ,  $p < 0.01$ ).

membrane and mitochondrial fractions. Immunohistochemistry showed that Kir6.1 and Kir6.2 were widely expressed in the cardiomyocytes in the atria and the ventricles as well as smooth muscles of coronary artery. Commercial goat anti-human Kir6.1 antibody showed similar distribution of Kir6.1 in the cardiomyocytes as our new antiserum. Double immunofluorescence staining showed that the fluorescence image of Kir6.1 was overlapped with cytochrome c, but not detected on the cell membrane, and the fluorescence image of Kir6.2 was overlapped with calreticulin and on the cell membrane. Preembedding immunoelectron



microscopy showed that the punctate Kir6.1 immunoreaction products observed by light microscopy were localized in the mitochondria, and Kir6.2 immunoreaction products were localized in the ER and cell membrane. With the postembedding method, the density of colloidal gold particles representing Kir6.1 that deposited on the mitochondria was significantly higher than outside the mitochondria. The colloidal gold particles representing Kir6.2 were mainly concentrated in the ER and also in the mitochondria. These data strongly indicate that the Kir6.1 was mainly localized in the mitochondria and Kir6.2 was mainly localized in the ER and also in the mitochondria.

The expression of Kir6.1 protein in rat cardiomyocytes and coronary arteries was consistent with the wide distribution of Kir6.1 mRNA in rat heart (Inagaki et al. 1995; Pountney et al. 2001; van Bever et al. 2004) and in the smooth muscle of blood vessels (Yamada et al. 1997; Suzuki et al. 2001; Miki et al. 2002; Li et al. 2003; Miura et al. 2003). The subcellular localization of Kir6.1 in the mitochondria was also consistent with the results in isolated mitochondrial fraction analysis (Lacza et al. 2003) and in isolated ventricular myocytes analysis by colocalized with mitochondrial marker MitoFluor red (Singh et al. 2003). The localization of Kir6.2 in ER and cell membrane in rat cardiomyocytes was similar to the results obtained in our previous immunoelectron microscopy study in neurons and glial cells of rat brain (Zhou et al. 2002). The Kir6.2 immunoreactivity on the ER may represent the site of production before the formation in functional octamers with sulphonylurea receptors on the plasma membrane (Clement et al. 1997; Inagaki et al. 1997; Inagaki and Seino 1998; Zerangue et al. 1999).

Recently, a gene transfer method did not show evidence that mitochondria contain Kir6.1 or Kir6.2 (Sekaraseyon et al. 2000). Furthermore, an immunoblot analysis denied that mitochondria contain Kir6.1 or Kir6.2 (Kuniyasu et al. 2003). Thus it was advocated that both Kir6.1 and Kir6.2 were not localized in the mitochondria. On the other hand, Kir6.1 and Kir6.2 were contained in mitochondria by immunoblot analysis and immunoelectron microscopy with mitochondrial fractions (Lacza et al. 2003) and with rat-isolated ventricular myocytes by immunocytochemistry (Singh et al. 2003). The methods that Kuniyasu et al. (2003) used to prepare their antibodies and show the specificities by immunoblot analysis with fusion proteins by gene vector transfer are almost the same as those used by Singh et al. (2003), but they reached different conclusions. The reason for such sharp discrepancies on the subcellular localizations of Kir6.1 and Kir6.2 is still unknown, but interestingly, Singh et al. (2003) and Lacza et al. (2003) pointed out by computer analysis that purified mitochondrial targeting sequence of amino acids colocalizes with Kir6.1 and Kir6.2 proteins.

In the present study, we showed that mitochondria contain Kir6.1 and Kir6.2 by both Western blot analysis and immunohistochemistry. We emphasize that Kir6.1 was mainly localized in the mitochondria and Kir6.2 mainly localized in the ER and also in the mitochondria and plasma membrane. Both Kir6.1 and Kir6.2 may be candidates for mitoK<sub>ATP</sub> channel subunits. Further study is required to determine whether Kir6.1 or Kir6.2 assembles with sulphonylurea receptors on these subcellular structures and forms functional channels, because coexpression with SUR2A or SUR2B on mitochondria still remains to be revealed.

### Acknowledgments

This work was supported by a part of Research Grants of Akita University to H.A. and by a part of the Kitasato Graduate School of Medical Sciences (2002–2003) to K.K.

The authors wish to thank Prof. H. Kondo (Tohoku University, School of Medicine) for his kind suggestion for this manuscript. The authors also thank the staff of Bioscience Research-Education Center of Akita University School of Medicine for their useful help and Mr. Nibe for his technical assistance in this study.

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