Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 147–156
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Selective expression of Kir6.1 protein in different vascular and non-vascular tissues

Xianfeng Sun[1], Kun Cao[1], Guangdong Yang, Yuan Huang, Salma Toma Hanna, Rui Wang[*]

Department of Physiology, College of Medicine, University of Saskatchewan, Saskatoon, Sask., Canada S7N 5E5

Received 14 May 2003; accepted 5 August 2003

Abstract

K_{ATP} channels are composed of pore-forming subunits Kir6.x and auxiliary subunits SURx. These channels play important roles in modulating the contractility of vascular smooth muscle cells (SMCs) by altering membrane potentials. The molecular basis of K_{ATP} channels in vascular SMCs is unclear and the expression of different K_{ATP} channel subunits at protein level in various tissues still undetermined. In this study, using an anti-Kir6.1 antibody, we detected the expression of Kir6.1 proteins in rat vascular tissues including mesenteric artery, pulmonary artery, aorta, and tail artery. Kir6.1 proteins were also identified in heart and other non-vascular tissues including spleen and brain, but they were undetectable in liver and kidney. Immunocytochemical study revealed the expression of Kir6.1 proteins in cultured rat thoracic aortic SMCs. Using the whole-cell patch-clamp technique, it was found that the intracellularly applied anti-Kir6.1 antibody significantly inhibited K_{ATP} channel currents in HEK-293 cells that were stably transfected with Kir6.1 cDNA. A better understanding of differential expression of Kir6.1 proteins in various vascular and non-vascular tissues may help discern different molecular basis and functions of K_{ATP} channel complexes in these tissues.
© 2003 Elsevier Inc. All rights reserved.

Keywords: Anti-Kir6.1 antibody; HEK-293; COS-1; K_{ATP} channels; Heart; Vascular smooth muscles

1. Introduction

K_{ATP} channels are composed of four pore-forming subunits (Kir6.x) encircled by four sulfonylurea receptor (SURx) subunits [1–4]. Molecular and compositional properties of K_{ATP} channels in vascular SMCs, however, have been unclear. Biophysical and pharmacological properties of native K_{ATP} channels in vascular SMCs resemble those reconstituted from heterologous co-expression of cloned Kir6.1 and SUR2B subunits [5]. The transcript of Kir6.1 subunit has been identified in vascular SMCs [6]. Whether this pore-forming K_{ATP} subunit gene is translated into protein in vascular SMCs and whether the expression of this K_{ATP} subunit protein is ubiquitous across a wide spectrum of vascular SMCs remain to be determined. Answers to these questions would greatly help device-specific therapeutic strategies targeting on specific K_{ATP} channel subunits under different pathophysiological conditions. The functional roles of K_{ATP} channels in a tissue-specific fashion in cardiovascular system will also be better understood.

Subunit-specific anti-K_{ATP} channel antibodies are important tools for examining the molecular composition and structure–function relationship of native K_{ATP} channels in vascular SMCs. To date, in limited studies anti-Kir6.1 antibodies were produced using either synthetic peptides based on short sequences of Kir6.1 gene [7,8] or Kir6.1–GST fusion protein as antigen [9]. None of these antibodies has been used to study the expression of Kir6.1 proteins in cardiovascular system. Availability of more specific antibody for Kir6.1 subunit would greatly facilitate in depth analysis of expression abundance of K_{ATP} channel proteins and the structure–function relationship of K_{ATP} channels in vascular SMCs. In the present study, we generated an anti-Kir6.1 antibody using GST-tagged

[*] Corresponding author. Tel.: +1-306-966-6592; fax: +1-306-966-6532.
E-mail address: wangrui@duke.usask.ca (R. Wang).
[1] They made equal contributions to this work.

Abbreviations: K_{ATP}, ATP-sensitive potassium channels; Kir, inwardly rectifying potassium channel subunit; SUR, sulfonylurea receptor; SMCs, smooth muscle cells; GST, glutathione S-transferase; OPD, *o*-phenylenediamine; IPTG, isopropyl β-D-thiogalactopyranoside; GSSG, glutathione disulfide; GSH, glutathione.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2003.08.041

Kir6.1C fusion protein. With this antibody we systematically examined the expression patterns of Kir6.1 proteins in different vascular and non-vascular tissues. Our finding showed that Kir6.1 subunit proteins were differentially expressed in vascular tissues and non-vascular tissues.

2. Materials and methods

2.1. Generation of Kir6.1C fusion protein

rvKir6.1 cDNA (Genbank Accession No.: AB043637) (rv stands for rat vascular tissue) was isolated from mesenteric artery tissues of Sprague–Dawley rats in our previous study [6]. A 494 bp Kir6.1 cDNA fragment was amplified with a GeneAmp. PCR system 2400 (Perkin-Elmer) from the full-length cDNA using a forward primer 5′-CAGGATCCTGTACGATATCTCAGCCACT-3′ (containing a *Bam*H I site) and a reverse primer 5′-TAGCGGCCGCAGTGAGTGGCTGTCTAGGGT-3′ (containing a *Not* I site). The fragment was cleaved with corresponding endonucleases and subcloned into a pGEX-5X-1 prokaryotic expression vector (Pharmacia Biotech) to form pGEX-Kir6.1C recombinant construct. The construct was further modified with *Sma* I and *Not* I endonucleases and self-ligated after filling in 5′ overhanging end of *Not* I with DNA polymerase I (New England Biolab), resulting in a new construct that encodes a GST tag and an in-framed 79 aa Kir6.1C fragment (L277-A355) (pGEX-Kir6.1C). The recombinant construct was verified by DNA sequencing analysis prior to bacterial expression.

The pGEX-Kir6.1C construct was transformed into the competent *Escherichia coli* BL21 (DE3) cells (Pharmacia Biotech) for bacterial expression, which was induced by 0.5 mM IPTG for 3 hr. Cells were then harvested by centrifugation. The pellets were re-suspended in phosphate-buffered saline (PBS) containing 1 mg/mL of lysozyme and 1% Triton X-100 (Sigma). Bacteria were broken down by sonication at 4°. Inclusion bodies were obtained after centrifugation at 10,000 *g* for 10 min [10].

The inclusion body-containing pellets were disrupted and solubilized in 8 M urea containing 0.1 M sodium phosphate (pH 7.8) and 0.3 M sodium chloride with overnight stirring. After centrifugation at 10,000 *g* for 10 min, collected supernatant was transferred to a dialysis tubing (Spectrum Lab, Inc.) to be dialyzed against gradient concentrations of 7, 6, 5, 4, 3, 2, 1, and 0 M urea each for 6 hr. GSSG (0.1 mM) and GSH (0.9 mM) were added in when 1 M urea solution was used for dialysis to help recover the original protein structure [11,12].

Dialyzed supernatant was mixed with Sepharose Glutathione 4B and stirred 1 hr at room temperature for protein purification. The pellets were collected after centrifugation at 1000 *g* for 10 min and washed three times with 10 volumes of 1× PBS. Glutathione Elution Buffer was added and kept at room temperature for 10 min. After centrifugation at 1000 *g* for 5 min, supernatant was collected and the procedure was repeated three times. Purified protein was stored at −20° for later use.

2.2. Antibody production and purification

Male New Zealand white rabbits of eleven weeks old were used for antibody generation. Animal use protocol was approved by The University of Saskatchewan Animal Use and Care Committee. Briefly, 200 μg of Kir6.1C bacterial fusion protein was emulsified in complete Freund's adjuvant for the primary immunization and injected into the rabbit subcutaneously. In subsequent immunizations, antigens emulsified in incomplete Freund's adjuvant were injected every 3 weeks for three repeats. Blood was withdrawn on the day immediately prior to first injection, 1 week later, and 10 days after the last injection for later analysis.

Three milliliters of protein A Sepharose CL-4B column (column Ø: 0.5 cm, 15 cm) (Pharmacia Biotech) was prepared for antibody purification according to manufacturer's instruction. The column was washed using 3× bed volume of 50 mM Tris–Cl (pH 7.0). Antisera (3 mL) was added into the column and washed using Tris–Cl containing 1% Triton X-100 at a speed of 0.5 mL/min until unspecific proteins were washed out. Purified antibody was eluted with 0.1 M glycine buffer (pH 2.6). Fractions were collected and stored at −80° for later use.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Anti-Kir6.1 antibody titer was determined by ELISA using 96U MaxiSorp Nunc-Immuno plates (Nalge Nunc) that were coated overnight with a coating solution (0.05 M sodium carbonate buffer containing 20 μg/mL of purified antigenic protein, pH 9.6) [13]. The plate was washed three times with PBST (0.05 M PBS, pH 7.2; 0.05% Tween 20), blocked with PBST containing 1% BSA for 2 hr at 37°, and washed three more times with PBST. The properly diluted primary antibody (100 μL) was added to the plate and incubated at 37° for 1 hr, and the plate was washed three times with PBST. Secondary antibody (1:5000 diluted goat anti-rabbit IgG–HRP conjugate) (Bio-Rad) was added and incubated at 37° for 45 min followed by three times of washing with PBST. OPD solution was used to develop reaction and the absorbance value measured at 492 nm [14].

2.4. Cell culture and transfection with K_{ATP} subunit cDNA

HEK-293 cells (passage 15) and COS-1 cells (passage 17) (American Type Culture Collection) were cultured in 35 mm Petri dishes at 37° in a humidified incubator with 95% air and 5% CO_2 in RPMI-1640 medium and Dulbecco's modified Eagle's medium (DMEM), respectively. The media also contained 4 mM L-glutamine, 10% fetal bovine

serum (FBS), and 1% penicillin/streptomycin. Cultured cells reached 70–80% confluence prior to transfection experiments. HEK-293 cells and COS-1 cells were stably transfected with pcDNA3.1-rvKir6.1 cDNA construct, following the protocols described before [6].

2.5. Electrophysiological recording of whole-cell K_{ATP} channel currents

The Petri dish with transfected HEK-293 cells was mounted on the stage of an inverted phase-contrast microscope. Pipettes were pulled from microhematocrit capillary tubes (Fisher) with tip resistance of 2–4 MΩ when filled with the pipette solution, which permitted dialysis of antibody into cytosol [15,16]. K_{ATP} currents were recorded using an Axopatch-200B patch clamp amplifier. The output signals were filtered at 1 kHz with an 8-pole filter, and stored on the hard disk of a computer for off-line analysis. Data acquisition and analysis were performed using pClamp software (version 7, Axon instruments). At the beginning of each experiment, junction potential between pipette solution and bath solution was adjusted to zero [17]. Leakage subtraction was not performed and the cells with obvious leakage were not used in this study. The pipette solution contained (mM): 132 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, and 5 Hepes. Na_2ATP (0.3 mM) was included in the pipette solution immediately before the experiments. The bath solution contained (mM): 40 KCl, 100 NaCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, and 5 Hepes. The pipettes were dipped in an antibody-free intracellular solution and then back filled with the pipette solution containing anti-Kir6.1 antibody at dilution of 1:500. All experiments were performed at room temperature (20–22°).

2.6. Protein isolation from rat tissues and Western blotting

Various tissues (300 mg each) from Sprague–Dawley rats were homogenized using a Polytron homogenizer with 0.5 mL of Tris–EDTA solution (EDTA 0.5 M; Tris–Cl 1 M, pH 7.4; and sucrose 0.3 M), antipain hydrochloride (1 μg/mL), benzamidine hydrochloride hydrate (1 mmol/mL), leupeptin hemisulfate (1 μg/mL), pepstatin A (1 μmol/mL), 1,10-phenanthroline monohydrate (1 mmol/mL), phenylmethylsulfonyl fluoride (0.1 mmol/mL), and iodoacetamide (1 μmol/mL). Supernatants containing crude cellular proteins were collected by centrifugation at 15,000 *g* for 15 min and used for Western blotting studies. Briefly, samples were denatured in a denaturing buffer (Tris–HCl 10 mM, 1% 2-mercaptoethenol, 1% SDS, 10% glycerol, and 0.005% Methylene blue) at 95° for 5 min before electrophoresis on a 10% SDS–polyacrylamide gel. Protein samples separated by electrophoresis were transferred onto nitrocellulose membrane. The membrane was blocked with 3% milk powder solution at room temperature for 2 hr and rinsed three times with PBS before incubated with anti-Kir6.1 antibody (dilution 1:4000). After the incubation, the membrane was washed three times with blocking solution for 5 min and incubated with the secondary antibody (goat anti-rabbit IgG antibody) for 45 min at room temperature on a shaker. Finally, the membrane was washed consecutively with blocking solution and PBS for three times followed by enhanced chemiluminescence staining (Perkin-Elmer Life Sci.) to detect positive bands [18,19].

2.7. Immunocytochemical detection of Kir6.1 proteins in A10 cells

The distribution of Kir6.1 proteins in A10 cells was investigated. A10 cells were cultured on cover-glass, and fixed with acetone for 20 min and washed with PBST three times prior to immunocytochemical analysis. A10 cells were incubated with anti-Kir6.1 antibody (1:200 in PBST containing 1% BSA) for 1 hr at room temperature, and then washed with PBST three times, followed by 40 min incubation at 37° with anti-rabbit IgG-FITC (1:500 in PBST containing 1% BSA). Glycerol was added to the cover-glass after washing with PBST. The green fluorescence emitted from A10 cells were observed with a fluorescent confocal microscopy (Zeiss Lsm 510meta) using argon laser excitation 488 nm and emission filter 505–530 nm.

2.8. Chemicals and statistical analysis

T4 DNA Ligase was purchased from Promega, and hygromycin was from Invitrogen. All other chemicals and culture media were purchased from Sigma. Data were expressed as means ± SEM and analyzed using Student's *t*-test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Characterization of anti-Kir6.1 antibody

pGEX-Kir6.1C construct containing partial Kir6.1 gene sequence was expressed in *E. coli* BL21 (DE3) cells with IPTG induction. No expression of the fusion protein in *E. coli* was detected without IPTG induction (Fig. 1A, lane 1). High expression level of Kir6.1C–GST fusion protein, 38 kDa in molecular mass including a 29 kDa GST tag, was detected after IPTG induction (Fig. 1A, lane 2). Because the fusion protein was in inclusion bodies when expressed in *E. coli* cells, necessary steps were taken to solubilize and re-nature the protein (see Section 2). The solubilized protein was then purified with Sepharose Glutathione 4B column. The purification procedure greatly eliminated non-Kir6.1 background (Fig. 1A, lanes 3–4). Subsequently, this purified Kir6.1C–GST fusion protein was used for rabbit immunization.

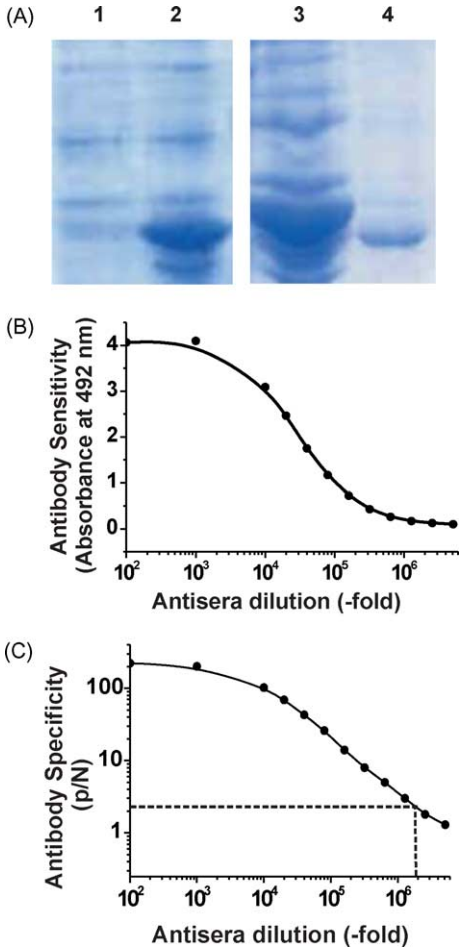

Fig. 1. Generation and purification of Kir6.1C antigenic protein and anti-Kir6.1 antibody. (A) SDS–PAGE (10%) analysis of Kir6.1C expression in *E. coli* BL21 (DE3). Lane 1, bacterial cells transformed with construct containing Kir6.1C cDNA before IPTG induction. Lane 2, bacterial cells transformed with construct containing Kir6.1C cDNA after IPTG induction. Lane 3, bacterial cells transformed with construct containing Kir6.1C with induction before purification. Lane 4, expressed Kir6.1C fusion protein after purification. (B) Determination of the sensitivity of anti-Kir6.1 antisera. Kir6.1C fusion protein (20 μg/mL) as antigen was used to coat the assay plate. Absorbance was measured at 492 nm. (C) Determination of the specificity of anti-Kir6.1 antisera. OPD absorbance values were measured and compared when Kir6.1C fusion protein and BSA were used to coat ELISA assay plates and *P* and *N* values were respectively determined. The dashed line indicates the threshold level of $P/N \geq 2.1$.

Anti-Kir6.1 antisera collected from rabbits 2 weeks after the last booster inoculation were subjected to an ELISA assay. The sensitivity of anti-Kir6.1 antisera was documented in Fig. 1B as arbitrary absorbance units determined from the chemiluscence of OPD. OPD is activated by HRP, which occurs when specific interaction between anti-Kir6.1 antibody and Kir6.1 antigen allows the binding of HRP-conjugated secondary antibody. The titer of antibody was evaluated based on the value of *P*/*N* in which *P* stands for antibody sensitivity as determined in Fig. 1B, and *N* for OPD absorbance when using BSA instead as a control. Figure 1C indicated that the titer of anti-Kir6.1 antibody was as high as 1:1,280,000 with *P*/*N* value ≥2.1.

Since GST-tagged Kir6.1C fusion protein was used as antigen to raise antisera, the antisera may also detect GST proteins, in addition to Kir6.1 proteins, in the examined samples. Therefore, the antisera were firstly pre-absorbed with GST protein before being used in the following Western blot experiments. A single 41 kDa band of Kir6.1 protein was detected in HEK-293 and COS-1 cell lines, which were permanently transfected with rvKir6.1 coding cDNA (lanes 2 and 4 in Fig. 2A and B). Interestingly, a band with the same molecular mass was also detected in non-transfected native HEK-293 and COS-1 cells (lanes 1 and 3 in Fig. 2A and B). Quantitative analysis revealed that the expression levels of Kir6.1 proteins in the transfected HEK-293 and COS-1 cells were 5.3- and 1.9-folds higher than that in non-transfected HEK-293 and COS-1 cells, respectively, when normalized with β-actin expression level ($P < 0.01$) (Fig. 2B). Furthermore, the antibody specifically recognized a single 38 kDa bacterial Kir6.1C–GST fusion protein (Fig. 2A, lane 5) and did not cross-react with a Kir6.2C–GST fusion protein (Fig. 2A, lane 6). After the antibody was pre-absorbed with Kir6.1 bacterial fusion protein prior to Western blot experiment, positive bands in HEK-293 and COS-1 cells disappeared

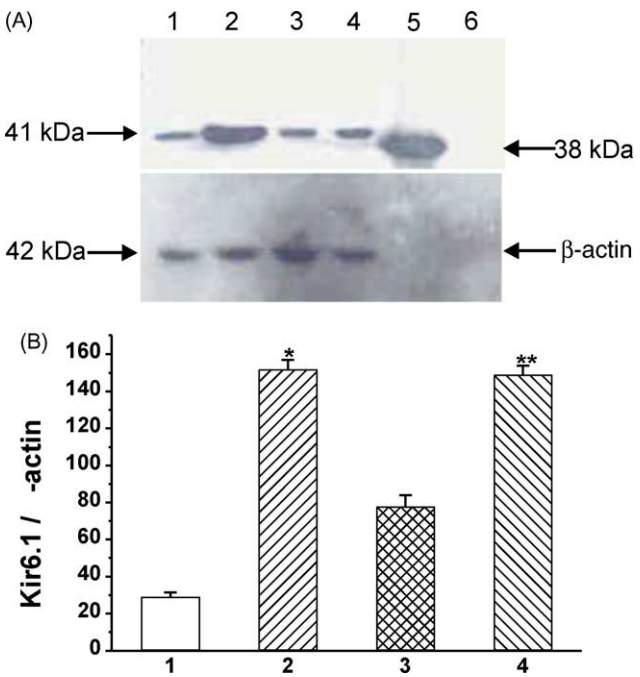

Fig. 2. Detection of Kir6.1 protein in HEK-293 and COS-1 cells. (A) Representative Western blots with anti-Kir6.1 antibodies. Lane 1, non-transfected HEK-293 cells; lane 2, HEK-293 cells transfected with rvKir6.1 cDNA; lane 3, non-transfected COS-1 cells; lane 4, COS-1 cells transfected with rvKir6.1 cDNA; lane 5, Kir6.1C–GST fusion protein; lane 6, Kir6.2C–GST fusion protein. (B) Normalized expression levels of Kir6.1 proteins. Column numbers bear the same meaning as the corresponding lane numbers in (A). N = 3 for each group. $^{*}P < 0.01$ for transfected vs. non-transfected HEK-293 cells. $^{**}P < 0.01$ for transfected vs. non-transfected COS-1 cells.

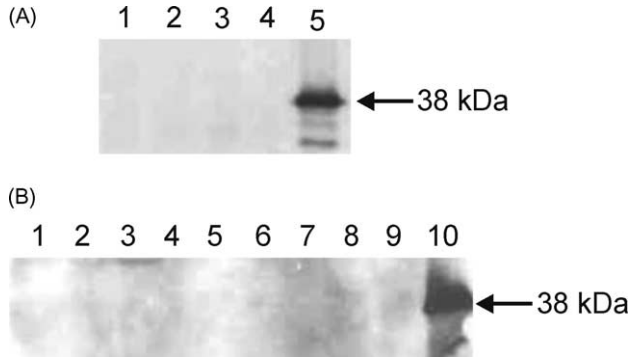

Fig. 3. Immunological reactions of different preparations to anti-GST antibody. (A) Representative Western blots with anti-GST antibodies on cultured cells. Lane 1, non-transfected HEK-293 cells; lane 2, HEK-293 cells transfected with rvKir6.1 cDNA; lane 3, non-transfected COS-1 cells; lane 4, COS-1 cells transfected with rvKir6.1 cDNA; lane 5, Kir6.1C–GST fusion protein (38 kDa). (B) Representative Western blots with anti-GST antibody on different tissues. Lane 1, mesenteric artery; lane 2, tail artery; lane 3, pulmonary artery; lane 4, aorta; lane 5, heart; lane 6, liver; lane 7, spleen; lane 8, kidney; lane 9, brain; and lane 10, Kir6.1C–GST fusion protein (38 kDa).

(data not shown). When anti-GST antibody was used as primary antibody instead of anti-Kir6.1 antibody, only Kir6.1–GST fusion protein was detected (Fig. 3A). No reaction was observed between anti-GST antibody with any tested cells or tissues (Fig. 3A and B).

3.2. Detection of Kir6.1 proteins in various rat tissues

Cellular proteins (including plasma membrane fraction) were prepared from various rat tissues. Kir6.1 proteins were detected as a single 41 kDa band in all four vascular smooth muscle tissues, i.e. mesenteric artery, tail artery, pulmonary artery, and aorta using the anti-Kir6.1 antibody (Fig. 4A, lanes 1–4). Their expression levels were normalized with β-actin level in the same tissue preparations. The expression level of Kir6.1 proteins in tail artery was the lowest in these four vascular smooth muscle tissues ($P < 0.05$, $N = 3$ for each group) (Fig. 4B, lanes 1–4). Although there were no statistical differences among mesenteric artery, pulmonary artery, and aorta tissues ($P > 0.05$, $N = 3$ for each group), the expression level of Kir6.1 in pulmonary artery tissues seems greater. A positive band was detected in cardiac tissues, but the molecular weight of this band was lower (35 kDa) than that in vascular smooth muscle tissues (Fig. 4A, lane 5). We did not further quantitate this Kir6.1-like protein in heart using the same β-actin as the house-keeping control as for other tissues because of the acknowledged difficulty to detect β-actin in cardiac tissues using Western blot analysis. A single band of 41 kDa was detected repeatedly in spleen tissue preparation (Fig. 4A, lane 7). In contrast, no visible band corresponding to Kir6.1 proteins was observed in liver and kidney tissues (Fig. 4A, lanes 6 and 8). In brain tissue, a specific 51 kDa band was detected with the antibody (Fig. 4A, lane 9). There were

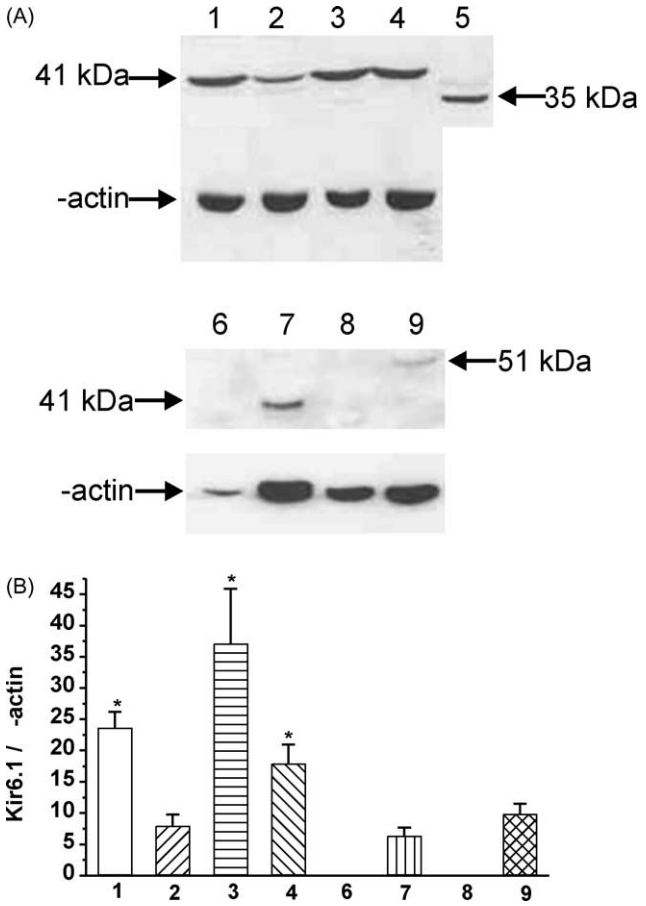

Fig. 4. Expression of Kir6.1 proteins in various rat tissues. (A) Representative Western blots with anti-Kir6.1 antibody. Lane 1, mesenteric artery; lane 2, tail artery; lane 3, pulmonary artery; lane 4, aorta; lane 5, heart; lane 6, liver; lane 7, spleen; lane 8, kidney; and lane 9, brain. (B) Normalized expression levels of Kir6.1 proteins. Column numbers bear the same meaning as the corresponding lane numbers in (A). $N = 3$ for each group. $^{*}P < 0.05$ vs. tail artery.

no significant differences in terms of Kir6.1 expression levels between tail artery and spleen or brain tissues ($P > 0.05$, $N = 3$ for each group). In another set of experiments, 58 μg anti-Kir6.1 antibody was mixed with 1.16 mg purified GST-tagged Kir6.1C bacterial fusion protein (ratio, 1:20) and incubated at 37° for 30 min followed by overnight incubation at 4°. This preabsorbed anti-Kir6.1 antibody was used in Western blotting against tissue preparations. Figure 5 shows that the specific Kir6.1 bands in vascular tissues, heart, liver, and brain were no longer detectable after pre-absorption of anti-Kir6.1 antibody.

3.3. Electrophysiological studies using anti-Kir6.1 antibody

K_{ATP} currents in HEK-293 cells stably transfected with Kir6.1 cDNA were recorded. Two minutes after the formation of the whole-cell configuration, the cells were repetitively hyperpolarized to −150 mV from a holding potential of −20 mV with 800 ms pulses. In the presence of anti-Kir6.1 antibody (1:500) in the pipette solution,

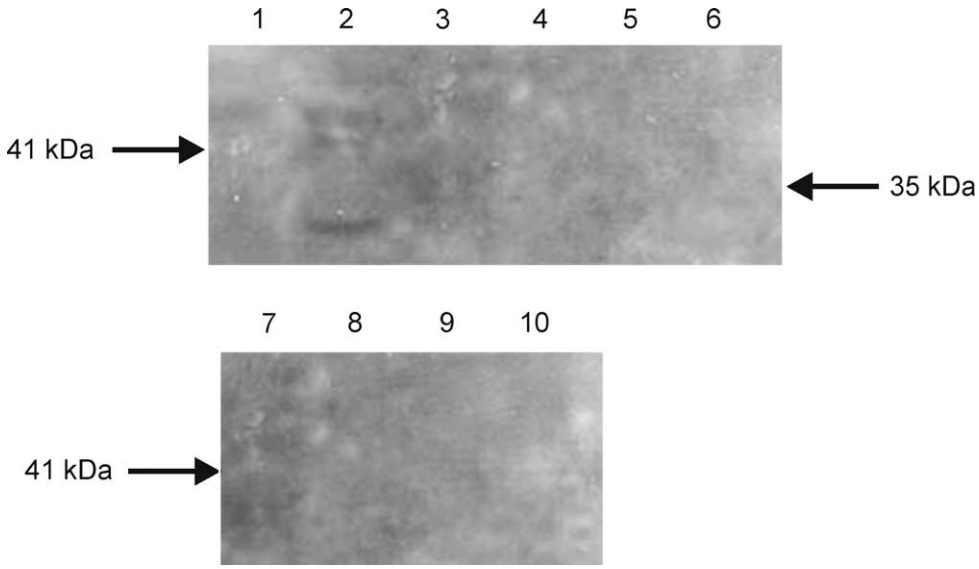

Fig. 5. Pre-absorption abolished the specific Kir6.1 band in Western blot assay. Lane 1, mesenteric artery; lane 2, tail artery; lane 3, pulmonary artery; lane 4, aorta; lane 5, Heart; lane 6, Kir6.1C–GST fusion protein; lane 7, liver; lane 8, spleen; lane 9, kidney; and lane 10, brain. All specific bands disappeared when anti-Kir6.1 antibody has been pre-absorbed with purified Kir6.1 antigen.

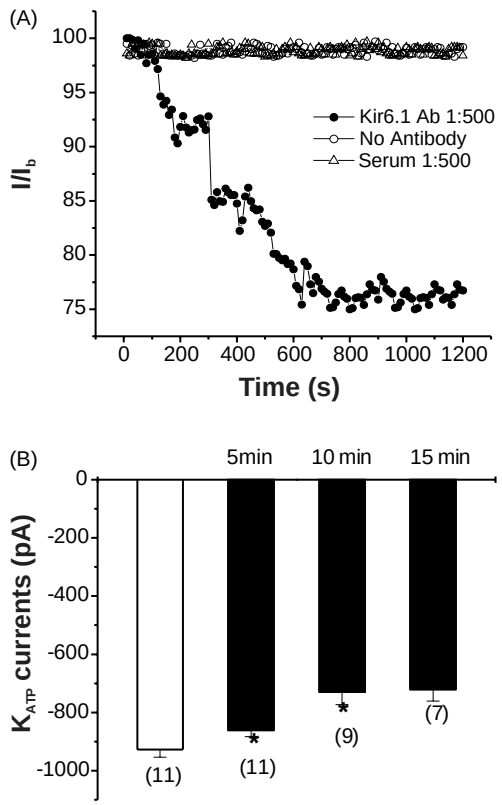

Fig. 6. Effects of anti-Kir6.1 antibody (Kir6.1 Ab) on K_{ATP} currents in stably transfected HEK-293 cells. Test potential, −150 mV; holding potential, −20 mV. (A) The time-dependent effect of Kir6.1 Ab on whole-cell K_{ATP} currents. Current amplitude (I) at −150 mV at each time point was compared with those obtained at the beginning of the recording (I_b). (B) Summary of the effect of Kir6.1 Ab on K_{ATP} currents recorded at −150 mV for 15 min. $^*P < 0.05$ vs. the first 1 min of recording (open column). Number of cells for each experiment is denoted respectively.

K_{ATP} currents were inhibited within 1 min after the establishment of the whole-cell configuration by 21.3 ± 4.6% (from −927.7 ± 25.9 pA to −729.7 ± 42.9 pA). The inhibition of K_{ATP} currents became stable 10–15 min thereafter. K_{ATP} currents were stable for 15 min (Fig. 6) in the absence of antibody, or in the presence of 1:500 control sera that was taken from the same rabbit before immunization with Kir6.1 antigenic protein, in the pipette solution.

3.4. Localization of Kir6.1 proteins in cloned smooth muscle cells using the anti-Kir6.1 antibody

The localization of Kir6.1 subunit was investigated in A10 cells (rat thoracic aortic smooth muscle cell line). Immunofluorescence staining from confocal microscopy imaging of the cultured cells revealed the presence of Kir6.1 proteins in A10 cells. Intensified green fluorescence was observed on plasma membranes and in the cytosol (Fig. 7A), but not in nuclear region. In order to exclude the contamination from nonspecific background fluorescence, anti-Kir6.1 antibody was replaced with sera that were taken from the same rabbit before immunized with Kir6.1 antigenic protein. Under this condition, no fluorescence was observed in A10 cells (Fig. 7C).

4. Discussion

Electrophysiological and pharmacological features of different K_{ATP} channel subunits have been characterized in great details in heterologous expression systems. Co-expressed rvKir6.1 and rvSUR2B yielded functional K_{ATP}

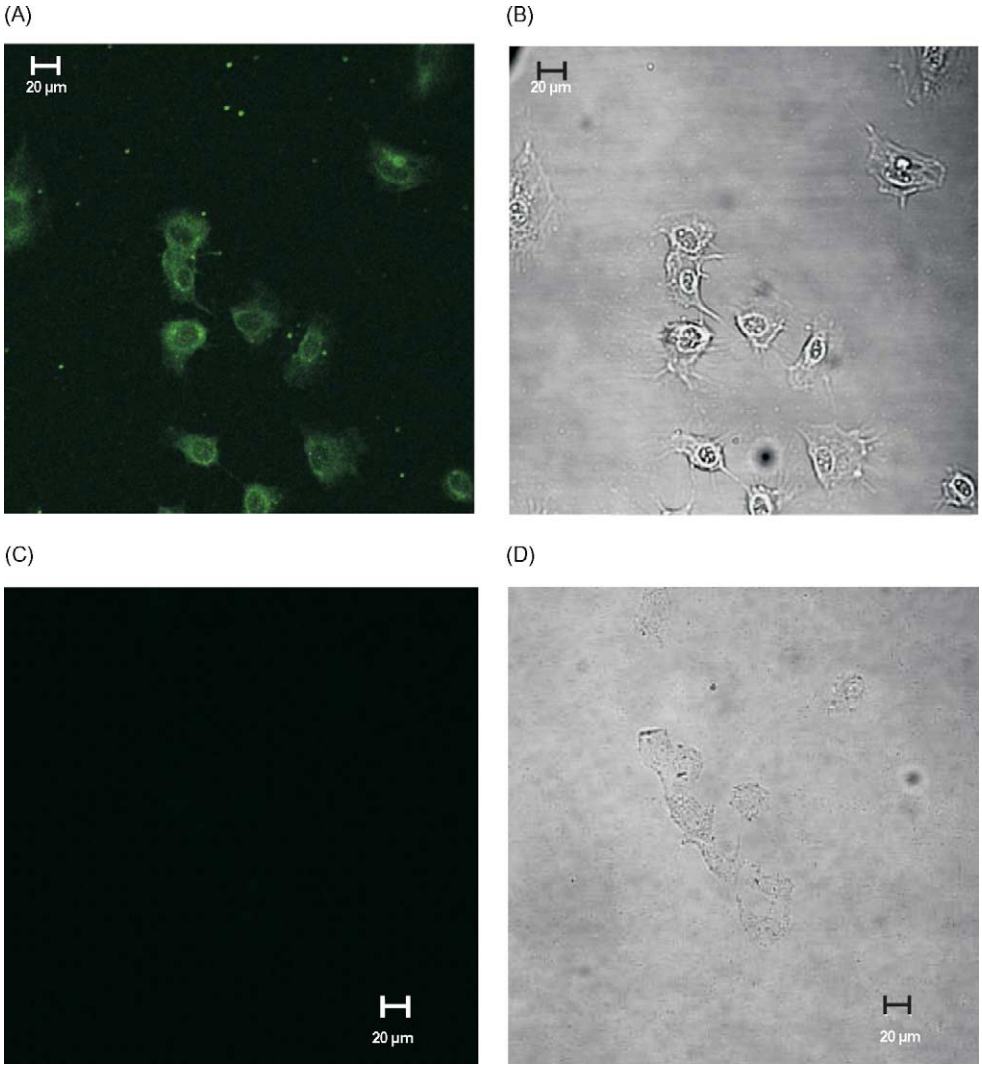

Fig. 7. Localization of Kir6.1 protein in A10 (thoracic aortic smooth muscle) cells using the anti-Kir6.1 antibody. Images in (A) and (B) and those in (C) and (D) were taken from the same field, respectively. The cells stained with anti-Kir6.1 antibody exhibited strong fluorescence (A). In the absence of anti-Kir6.1 antibody no fluorescence was detectable (C). The transmitted light images of the cells were shown in (B) and (D). Scale bars, 20 μm.

channels that were inhibited by glibenclamide, and opened by pinacidil [6]. Kir6.1/SUR2B channels are relatively insensitive to intracellular ATP concentration changes and activated by intracellular nucleotide diphosphates in the presence of Mg^{2+} [5]. Kir6.1/SUR2B channels are also characterized with weak inward rectification, flickering activity within burst, and intermediate unitary conductance (around 33 pS under symmetrical K^+ in the presence of pinacidil) [5]. Interestingly, Kir6.1/SUR2B channels do not spontaneously open on patch excision in the absence of intracellular ATP. These channels in inside-out patches are induced by uridine diphosphate and guanosine diphosphate [5]. Molecular compositions of K_{ATP} channel complex in various tissues, however, remain to be determined. The present study was aimed at examining distribution of Kir6.1 proteins in different rat vascular and non-vascular tissues. Generation of specific antibody against Kir6.1 protein was the critical prerequisite for the present study. In our study, the specificity of anti-Kir6.1 antibody was verified with the absorption experiment and additionally with anti-GST antibody detection experiment in which positive bands were detectable only in bacteria expressing Kir6.1–GST fusion proteins, not in other cellular and tissue preparations.

K_{ATP} channels in plasma membrane are composed by Kir6.x and SURx in different combinations in different types of cells. Up to now, there were only a limited number of literatures describing the expression patterns of K_{ATP} channel subunits in vascular smooth muscle cells at mRNA level. K_{ATP} channel subunit expressions at protein level were largely unknown in vascular smooth muscles or other non-vascular tissue [20]. For instance, Cui *et al.* demonstrated the presence of Kir6.1 transcript with RT-PCR in rat pulmonary artery [20]. Suzuki *et al.* detected Kir6.1 mRNA in mouse aorta using Northern blot technique [21]. Cao *et al.* identified transcripts of Kir6.1 in rat aorta,

tail artery, pulmonary artery, and mesenteric artery tissues [6]. Using the anti-Kir6.1 antibody prepared in the present study, Kir6.1 proteins were identified in four rat vascular smooth muscle tissues including mesenteric artery, tail artery, pulmonary artery, and aorta. This is the first description of Kir6.1 protein expressions in various vascular smooth muscles. These results would help reveal the molecular composition of native K_{ATP} channels in vascular SMCs and the contributions of different K_{ATP} channel subunits to the functions of native K_{ATP} channels in different vascular SMCs. Our results will also pave the way for future studies by targeting the specific subunit of K_{ATP} channels in vascular SMCs.

In contrast to its ubiquitous expression in rat vascular tissues, Kir6.1 protein was not detectable in rat liver and kidney tissues. Suzuki *et al.* reported a pool of Kir6.1 proteins with a variety of molecular sizes in rat liver tissues. The molecular weights of detected bands were 51 and 49 kDa in total homogenate, 51 kDa in mitochondrial fraction, and 47 kDa in plasma membrane vesicles [22]. A recent paper from the same group [23], on the other hand, demonstrated the absence of Kir6.1 mRNA in mice liver using Northern blot technique. These seemingly conflict results on the expression of Kir6.1 in hepatocytes need to be reconciled.

A single band of 35 kDa, smaller than that in vascular tissues (41 kDa), was detected by the anti-Kir6.1 antibody in rat heart tissue homogenate. It is generally believed that cardiac K_{ATP} channels are composed of Kir6.2 and SUR2A subunits [21]. There was no report on the expression of Kir6.1 proteins in heart except the study by Akao *et al*., in which Kir6.1 protein was detected in rat heart tissue with a molecular weight of 49 kDa [24]. On the other hand, Kir6.1 mRNA in heart from both rat and human had been detected by many laboratories [25–27]. The detected cardiac Kir6.1 proteins in our study may result from a truncated Kir6.1 protein, a mitochondrial fraction of the protein, or a distinctive isoform of Kir6.1 gene. These possibilities warrant further investigations. A single band of 51 kDa of Kir6.1 was detected in rat brain tissues in our study. This result is in line with a previous report on the expression of Kir6.1 protein with molecular weight of about 50 kDa in rat astrocytes [9]. Studies by other groups have showed the existence of several potential cAMP-dependent, protein kinase C-dependent, casein kinase-II-dependent phosphorylation sites, and several N-linked glycosylation sites in Kir6.1 protein structure. Largely owing to these post-translational modifications, matured Kir6.1 proteins have been detected with a wide spectrum in term of molecular weight in different tissues or even in different subcellular fractions of cells within the same tissue [9,22]. Therefore, Kir6.1 protein in brain tissues detected in our study may have been modified by glycosylation or phosphorylation. In addition, Kir6.1 protein was not detected in rat kidney tissues in our study. Sgard *et al.* observed a faint band of Kir6.1 mRNA using Northern blot technique and this mRNA expression was significantly up-regulated after renal hypoxic injury [27]. It is possible that Kir6.1 translation is limited in kidney, but it may be up-regulated by hypoxic or ischemic stresses. Finally, Kir6.1 protein identified in spleen tissue has not been reported before. The significance of this finding needs to be elucidated in the future.

Although a much stronger 41 kDa band was detected in HEK-293 or COS-1 cells transfected with rvKir6.1 cDNA, endogenous Kir6.1 proteins were detected in non-transfected HEK-293 or COS-1 cells (Fig. 2). Using RT-PCR technique, we had also detected transcripts of Kir6.1 subunit in non-transfected HEK-293 and COS-1 cells (unpublished observation). Suzuki *et al.* [22] did not detect Kir6.1 proteins, using anti-Kir6.1 antibody, in COS-1 cells transfected with pCMV vector alone. The amount of protein (15 μg) used in their experiments was smaller than what we used (45 μg), which may underline their negative results. Thomzig *et al.* also reported the absence in non-transfected COS-7 cells of endogenous Kir6.1 proteins using an anti- Kir6.1 antibody [9]. While we examined the expression of Kir6.1 proteins in pool of cells, Thomzig *et al.* [9] studied the expression in individual cells. Together with potential difference in the affinities between antibodies produced in different laboratories, a higher sensitivity of the experimental approach may explain the positive identification of Kir6.1 in our study. It is worth to point out that low endogenous expression level of Kir6.1 proteins in native HEK-293 or COS-1 cells may not suffice to yield functional K_{ATP} channel currents in these cells. Our previous eletrophysiological study on non-transfected HEK-293 or COS-1 cells did not found detectable K_{ATP} channel currents [28].

The contribution of individual K_{ATP} channel subunits to native K_{ATP} channels in vascular smooth muscle cells has been largely unknown. Lack of K_{ATP} subunit-specific antibody is one of the reasons for this gap of knowledge. The present study demonstrated that K_{ATP} channels in HEK-293 cells transfected with Kir6.1 cDNA were significantly inhibited by anti-Kir6.1 antibody. The inhibitory effect of anti-Kir6.1 antibody was specific since the recorded K_{ATP} currents did not change their amplitude over the same time frame in the absence of the antibody in the pipette solution or with the inclusion of control serum. Within 10 min of application, the inhibition of K_{ATP} currents by the antibody became stable. The existence of other endogenous K_{ATP} channel subunits in native HEK-293 cells, other than Kir6.1, may explain the incomplete inhibition of K_{ATP} currents induced by anti-Kir6.1 antibody. Furthermore, our antibody is targeted against a 79 aa fragment of Kir6.1 protein at its C terminus. This specific epitope of Kir6.1 may participate in, but not fully responsible for, the formation of K^+ permeation pore. Thus, designing and applying different antibodies against specific epitopic antigenities would help unmask the contribution of different epitopes of K_{ATP} channel subunits to the function of

K_{ATP} channels by probing different regions for pore formation, ATP binding, and specific modulation domains. By comparing changes in conductance of native K_{ATP} currents after the application of anti-Kir6.1 antibody, it will also become possible to identify the participation of Kir6.1 proteins in the formation of native K_{ATP} channel complex in different types of cells.

In summary, Kir6.1 proteins were identified in all vascular tissues examined with higher abundance level in pulmonary artery, aorta, and mesenteric artery than in tail artery. The expression of this K_{ATP} channel subunit protein was also detected in heart, spleen, and brain, but not liver and kidney. In cultured A10 cells, Kir6.1 proteins were located in plasma membrane. Different expression patterns of Kir6.1 proteins in various vascular and non-vascular tissues indicate the relative importance of Kir6.1 proteins in participating in the formation of K_{ATP} channel complex and in the regulation of cellular functions in different tissues.

Acknowledgments

The authors thank Dr. Weimin Zhao, Mrs. Koleen Safiniuk, and Mrs. Ginger Beal for technical assistance. The assistance and guidance from Dr. Y. Wei and Mr. X. Wu regarding the use of confocal microscope are greatly appreciated. This study was supported by an operating grant from Canadian Institutes of Health Research (CIHR). X. Sun and K. Cao received post-doctoral fellowship awards from Health Services and Utilization Research Council of Saskatchewan, Canada. Y. Huang received a post-doctoral fellowship award from College of Medicine, University of Saskatchewan. S.T. Hanna is supported by a CIHR\ Canadian Hypertension Society Rx & D studentship. R. Wang has been supported by an Investigator award from CIHR/Regional Partnership Program.

References

[1] Babenko AP, Aguilar-Bryan L, Bryan J. A view of SUR/KIR6.X, K_{ATP} channel. Annu Rev Physiol 1998;60:667–87.

[2] Clement JPT, Kunjilwar K, Gonzalez G, Schwanstecher M, Panten U, Aguilar Bryan L, Bryan J. Association and stoichiometry of K_{ATP} channel subunits. Neuron 1997;18:827–38.

[3] Inagaki N, Gonoi T, Seino S. Subunit stoichiometry of the pancreatic beta-cell ATP sensitive K^+ channel. FEBS Lett 1997;409:232–6.

[4] Krapivinsky G, Gordon EA, Wickman K, Velimirovic B, Krapivinsky L, Clapham DE. The G-protein-gated atrial K^+ channel I_{KACh} is a heteromultimer of two inwardly rectifying K^+-channel proteins. Nature 1995;37:4135–41.

[5] Yamada M, Isomoto S, Matsumoto S, Kondo C, Shindo T, Horio Y, Kurachi Y. Sulfonylurea receptor 2B and Kir6.1 form a sulfonylurea-sensitive but ATP-insensitive K^+ channel. J Physiol 1997;49:9715–20.

[6] Cao K, Tang G, Hu D, Wang R. Molecular basis of ATP-sensitive K^+ channels in rat vascular smooth muscles. Biochem Biophys Res Commun 2002;296:463–9.

[7] Cui Y, Giblin JP, Clapp LH, Tinker A. A mechanism for ATP-sensitive potassium channel diversity: functional coassembly of two pore-forming subunits. Proc Natl Acad Sci USA 2000;98:729–34.

[8] Pountney DJ, Sun ZQ, Porter LM, Nitabach MN, Nakamura TY, Holmes D, Rosner E, Kaneko M, Manaris T, Holmes TC, Coetzee WA. Is the molecular composition of K_{ATP} channels more complex than originally thought? J Mol Cell Cardiol 2001;33:1541–6.

[9] Thomzig A, Wenzel M, Karschin C, Eaton MJ, Skatchkov SN, Karschin A, Veh RW. Kir6.1 is the principal pore-forming subunit of astrocyte but not neuronal plasma membrane K-ATP channels. Mol Cell Neurosci 2001;18:671–90.

[10] Ceciliani F, Caramori T, Ronchi S, Tedeschi G, Mortarino M, Galizzi A. Cloning, overexpression, and purification of *Escherichia coli* quinolinate synthetase. Protein Exp Purif 2000;18:64–70.

[11] Hurt JD, Tu C, Laipis P. Isolation and expression of murine carbonic anhydrase IV. Protein Exp Purif 1998;12:7–16.

[12] Gupta P, Waheed SM, Bhatnagar R. Expression and purification of the recombinant protective antigen of *Bacillus anthracis*. Protein Exp Purif 1999;16:369–76.

[13] Maurice S, Hadge D, Dekel M, Friedman A, Gertler A, Shoseyov O. A-protein from achromogenic atypical aeromonas salmonicida: molecular cloning, expression, purification, and characterization. Protein Exp Purif 1999;16:396–404.

[14] Haqqani AS, Cowling RT, Maroun JA, Birnboim HC. Characterization of a polyclonal antibody to human thymidylate synthase suitable for the study of colorectal cancer specimens. J Histochem Cytochem 1999;47:1563–73.

[15] Archer SL, Souil E, Sinh-Xuan AT, Schremmer B, Mercier JC, Elyaagoubi A, Nguyen L, Reeve HL, Hampl V. Molecular identification of the role of voltage-gated K^+ channels, Kv1.5 and Kv1.2, in hypoxic pulmonary vasoconstriction and control of resting membrane in rat pulmonary artery myocytes. J Clin Invest 1998;101:2319–30.

[16] Conforti L, Bodi I, Nisbet JW, Millhorn DE. O_2-sensitive K^+ channels: role of the Kv1.2 α-subunit in mediating the hypoxic response. J Physiol 2000;524:783–93.

[17] Hanna ST, Wang R. Effect of calyculin A on the outward K^+ channel current in rat tail artery smooth muscle cells. J Cardiovasc Pharmacol 2002;40:660–8.

[18] Scott VES, Muniz ZM, Sewing S, Lichtinghagen R, Parcej DN, Pongs O, Dolly JO. Antibodies specific for distinct Kv subunits unveil a heterooligomeric basis for subtypes of α-dendrotoxin-sensitive K^+ channels in bovine brain. Biochemistry 1994;33:1617–23.

[19] Stonehouse AH, Pringle JH, Norman RI, Stanfield PR, Conley EC, Brammar WJ. Characterisation of Kir2.0 proteins in the rat cerebellum and hippocampus by polyclonal antibodies. Histochem Cell Biol 1999;11:2457–65.

[20] Cui Y, Tran S, Tinker A, Clapp LH. The molecular composition of K_{ATP} channels in human pulmonary artery smooth muscle cells and their modulation by growth. Am J Respir Cell Mol Biol 2002;26: 135–43.

[21] Suzuki M, Li RA, Miki T, Uemure H, Sakamoto N, Ohmoto-Sekine Y, Tamagawa M, Ogura T, Seino S, Marban E, Nakaya H. Functional roles of cardiac and vascular ATP sensitive potassium channels clarified by Kir6.2-knockout mice. Circ Rec 2001;88:570–7.

[22] Suzuki M, Kotake K, Fujikura K, Inagaki N, Suzuki T, Gonoi T, Seino S, Takata K. Kir6.1: a possible subunit of ATP-sensitive K^+ channels in mitochondria. Biochem Biophys Res Commun 1997; 241:693–7.

[23] Miki T, Suzuki M, Shibasaki T, Uemura H, Sato T, Yamaguchi K, Koseki H, Iwanaga T, Nakaya H, Seino S. Mouse model of Prinzmetal angina by disruption of the inward rectifier Kir6.1. Nat Med 2002; 8:466–72.

[24] Akao M, Otani H, Horie M, Takoto M, Kuniyasu A, Nakayama H, Kouchi I, Sasayama S, Murakami T. Myocardial ischemia induces differential regulation of K_{ATP} channel gene expression in rat hearts. J Clin Invest 1997;100:3053–9.

[25] Inagaki N, Tsuura Y, Namba N, Masuda K, Gonoi T, Horie M, Seino Y, Mizuta M, Seino S. Cloning and functional characterization of a novel ATP-sensitive potassium channel ubiquitously expressed in rat tissues, including pancreatic islets, pituitary, skeletal muscle, and heart. J Biol Chem 1995;270:5691–4.

[26] Unaltuna-Erginel N, Yang W, Blanar M. Genomic organization and expression of KCNJ8/Kir6.1, a gene encoding a subunit of an ATP-sensitive potassium channel. Gene 1998;211:71–8.

[27] Sgard F, Faure C, la Rochelle CD, Graham D, O'Connor SE, Janiak P, Besnard F. Regulation of ATP-sensitive potassium channel mRNA expression in rat kidney following ischemic injury. Biochem Biophys Res Commun 2000;269:618–22.

[28] Jiang B, Sun XF, Cao K, Wang R. Transcriptional expression of KV channel genes and the characteristics of the endogenous KV channels in native human embryonic kidney (HEK-293) cells. Mol Cell Biochem 2002;23:869–79.