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Expression of ATP-sensitive Potassium Channel and Sulfonylurea Receptor in Neonate and Adult Rat Tissues

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The ATP-sensitive potassium (K_{ATP}) channel is a member of inward rectifier potassium channel (Kir) that is inhibited by intracellular ATP and functions in close relation to sulfonylurea receptors (SUR). Although the molecular mechanism and physiological function of K_{ATP} channels are well understood, the expression pattern during development or treatment with the channel modulators such as glybenclamide is little known. In this work, we determined mRNA levels of a K_{ATP} channel (Kir6.2) and a sulfonylurea receptor (SUR2) in rat tissues by RNase protection assay. Levels of Kir6.2 and SUR2 mRNA in the rat brain and skeletal muscle were higher in adult (90 ~ 120 days) than in neonate (2 ~ 8 days), whereas those in the heart were not much different between neonate (2 ~ 8 days) and adult (90 ~ 120 days). In addition, none of K_{ATP} channel modulators (opener, pinacidil and nicorandil; blocker, glybenclamide) affected the Kir6.2 mRNA levels in the heart, brain and skeletal muscle. The results indicate that the expression of Kir and SUR genes can vary age-dependently, but the expression of Kir is not dependent on the long-term treatment of channel modulators. The effect of the channel modulators on mRNA level of SUR is remained to be studied further.

Key Words: Kir6.2, SUR2, mRNA, Developmental change, RNase protection assay

INTRODUCTION

The ATP-sensitive potassium (K_{ATP}) channel was first described in cardiac muscle (Noma et al, 1983) and subsequently found in variety of other tissues including pancreatic β-cells, smooth muscle, skeletal muscle and neurons (Ashcroft et al, 1988). K_{ATP} channel is inhibited by intracellular ATP and regulated also by ATP/ADP ratio. So it seems to link metabolic status of cells to the electrical activity of plasma membrane (Ashcroft et al, 1990). Glybenclamide (or glyburide), one of sulfonylurea drugs, has been used in treatment of non insulin-dependent diabetes mellitus and appeared one of potent blockers of K_{ATP} channels (Schmid-Antomarchi et al, 1987; Ashcroft et al, 1992). In addition, several K_{ATP} channel openers such as cromakalim, nicorandil, pinacidil were developed as a potential therapeutic agents for hypertension, asthma, incontinence of urine (Ashcroft, 1988).

K_{ATP} channels are known to play important roles in insulin secretion and various pathophysiological condition such as ischemia and metabolic exhaustion (Ashcroft et al, 1990). In pancreatic β-cell, K_{ATP} channel plays a key role in the insulin secretory response. It was recently reported that mutation in NBF-2 region of the SUR gene causes familial persistent hyperinsulinemic hypoglycemia of infancy (PHHI), an autosomal recessive disorder characterized by unregulated insulin secretion (Thomas et al, 1995). K_{ATP} channel may play an important role in ionic disorders associated with ischemia in the brain during ischemia. It has been proposed that interstitial K^+ concentration increases in the brain during ischemia, indicating that a K^+ efflux pathway has been opened (Hansen et al, 1985). This intracellular K^+ loss may be a direct consequence of the decrease of the intracellular ATP linked to the ischemic situation (Ber-

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nardi et al, 1988). During anoxia, the extent of K^+ increase is different between young and adult animals, that is, extracellular K^+ activity in newborn animal is lower than in adult animal (Jiang et al, 1992) and glibenclamide receptor binding sites are much lower in the newborn animal than in adult animal central nervous system (Miller et al, 1991; Xia et al, 1991). During metabolic blockade, K^+ efflux in heart will lead to vasodilation and reduce action potential duration and will help to preserve cellular ATP level. The fall of ATP concentration during metabolic exhaustion increase the activity of K_{ATP} channels in skeletal muscle, which may reduce excitability (Davies, 1990).

The β-cell K_{ATP} channel has been shown to comprise a complex of (at least) two proteins: a member of the inward rectifier K^+ channel family and a sulfonylurea receptor (Ammala et al, 1996), that is, inward rectifier K^+ channel (Kir6.0) family form the channel pore, whereas sulfonylurea receptor (SUR) is required for activation and regulation (Augular-Bryan et al, 1998). Sulfonylurea binding has been described in the heart (Gopalakrishinan et al, 1991), brain (Xia et al, 1993; Zini et al, 1993), skeletal (Gopalakrishinan et al, 1991) and smooth muscles (Zini et al, 1991). Complementary DNA (cDNA) of sulfonylurea receptor (SUR1) was first cloned in 1995 (Aguilar-Bryan et al, 1995), and is known to be a member of the growing family of ATP- binding cassette (ABC) protein, including multidrug resistance (MDR) related protein, p-glycoprotein, and cystic fibrosis transmembrane conductance regulator (CFTR) (Philipson et al, 1995). Sulfonylurea receptor proteins have 13 membrane-spanning domains and two nucleotide-binding folds (NBF-1, NBF-2) (Aguilar-Bryan et al, 1995). Unlike SUR, other ABC transporter have 12 transmembrane domains. SUR2 was first cloned by screening a rat brain cDNA library with SUR1 cDNA (Inagaki et al, 1995a). SUR2 cDNA sequence has 68% identity with SUR1 (Inagaki et al, 1996). Although the structural features of SUR2 are similar to those of SUR1, SUR1 binds glibenclamide or its analogues with higher affinity than SUR2 does (Inagaki et al, 1996). The biophysical properties of a K_{ATP} channel can be different depending on the type of associated SUR (Inagaki et al, 1995a; Inagaki et al, 1996) as well as the type of Kir6.0 (Seino, 1999; Fujita & Kurachi, 2000).

Previously it has been shown that the extent of sulfonylurea receptor ligand, glyburide binding (Bmax) to K_{ATP} channel is up-regulated about 80% in rat heart and about 24% in rat brain by glyburide and is down-regulated about 20% in rat brain by K_{ATP} channel opener, pinacidil (Gopolakrishnan et al, 1992). But little is known about the mechanisms that regulate the mRNA expression of inward rectifying potassium channels by K_{ATP} channel modulators. In addition, it is still not well known on the age-dependent expression pattern of mRNAs for K_{ATP} channels and SURin the rat. Therefore, in this work, we examined mRNA levels of a subtype of K_{ATP} channels (Kir6.2) and sulfonylurea receptors (SUR2) in neonate, adult and K_{ATP} channel modulator-treated rats. The results suggest that there are age-dependent alterations in K_{ATP} channel expressions, but K_{ATP} channel modulators did not change Kir6.2 mRNA levels in rat heart, brain and skeletal muscle.

METHODS

Animals

Sprague-Dawley rats (neonates, 2~8 days; adults, 200~ 300 g, 90~120 days) for the study of developmental expression of Kir6.2 and SUR2 mRNAs were obtained from Samyuk Laboratory Animal Co. (Osan, Kyonggi, Korea). They were sacrificed by decapitation and heart, brain and skeletal muscle were removed and frozen in liquid nitrogen. For examining the effect of pinacidil, nicorandil and glibenclamide, male Sprague-Dawley rats (250~300 g) were maintained under standard laboratory conditions and had free access to food and water during the course of the experiment. Pinacidil (20 mg/kg/day), nicorandil (20 mg/kg/day) and glibenclamide (3 mg/kg/day) were administered by intraperitoneal injection every 12 hours for 10 days. Since pinacidil administration was associated with hypotension and tachycardia, we also treated a group of rats with hydralazine (20 mg/kg/day, i.p. administered every 12 hours for 10 days). Control animals received equivalent volumes of polyethylene glycol (PEG) or 0.9% saline. Animals were sacrificed 24 hours after the last injection of the drug or vehicle.

Reagents

The following reagents were purchased from Sigma (St. Louis, MO, USA): guanidium thiocyanate, mer-

captoethanol, sodium citrate, sodium acetate, bromophenol blue, xylene cyanol FF, isopropanol, chloroform, hydralazine, glibenclamide, and pinacidil. Nicorandil was kindly provided by C&C Research Laboratory (Hwasung, Gyonggi, Korea).

Bacto-agar and Bacto-trypton were purchased from Difco (Detroit, MI, USA). Phenol and isopropyl-β-D-thiogalactoside (IPTG) were purchased from Boehringer Mannheim (Mannheim, Germany). All the supplies for *in vitro* transcription (MAXIscript) and RNase protection assay (Hybspeed RPA) were from Ambion (Austin, TX, USA). Restriction enzyme (*Eco* RI, *Sal* I, *Hin*d III, *Hin*f I and *Bst* EII), X-gal and Clean-up kit were obtained from Promega (Madison, WI, USA). [^{32}P]-UTP was from Amersham (Little Chalfont, Buckinghamshire, U.K.) or ICN (Costa Mesa, CA, USA). Stock solutions of pinacidil (10 mg/ml, nicorandil (10 mg/ml) and glibenclamide (15 mg/ml) were prepared in polyethylene glycol. Hydralazine (10 mg/ml) was prepared in 0.9% saline.

The cDNA clones of mKir6.2/pCMV6b and rSUR2/pCMV6c were kindly provided by Dr. Susumu Seino, Chiba University in Japan and haSUR/pSK by Dr. Joseph Bryan, Baylor College of Medicine in USA.

Total RNA extraction

Total RNAs were isolated from frozen rat tissues by the method described by Chomczyinski et al (1987). Briefly, the obtained RNA pellet was dissolved in Solution D (4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) and extracted twice with phenol and choloroform-isoamylalchol (49 : 1). The quantity and purity of RNA was checked by measuring the absorbance at 260 and 280 nm. The integrity of RNA was confirmed by visualization of the 28 S and 18 S ribosomal RNA bands in 1% agarose gel stained with ethidium bromide.

Subcloning of Kir6.2 and SUR2 cDNA

The fragment for cRNA probe was selected avoiding transmembrane domains and nucleotide binding folds, which have high homology between related genes.

Kir6.2: The Kir6.2 cDNA containing the entire coding sequence has been cloned previously (Inagaki et al, 1995a). After digestion of the pCMV6b mKir6.2 cDNA and pT7T3 α-19 vector (Life Technology, Grand Island, NY, USA) with *Sal* I and *Eco* RI, the fragment (1486 nucleotides) of Kir6.2 was subcloned into *Sal* I/*Eco* RI restriction site of pT7T3 α-19 vector (Life Technology, Grand Island, NY, USA).

SUR2: The SUR2 cDNA containing the entire coding sequence has been cloned previously (Inagaki et al, 1996). After digesting the pCMV6c rSUR2 cDNA and pT7T3 α-19 vector with *Acc*I and *Hin*dIII, the fragment (440 nucleotides) of SUR2 was subcloned into *Acc* I/ *Hin*dIII restriction site of pT7T3 α-19 vector (Life Technology, Grand Island, NY, USA).

Synthesis of cRNA probes for RNase protection assay

For the production of antisense cRNA probes, Kir6.2/pT7T3 α-19, SUR2/pT7T3 α-19 plasmid was linearized with *Hin*fI, *Bst*EII and *Bam*HI respectively and the antisense RNAs were transcribed by either T7 (SUR2) or T3 (Kir6.2) RNA polymerase from the linearized recombinants. [α-^{32}P]-UTP (Amersham, Little Chalfont, Buckinghamshire, U.K.)-labeled antisense probes were prepared using MAXIscript In Vitro Transcription Kit (Ambion, Austin, TX, USA) following manufactor's protocol. The cRNA probe with expected sizes (Kir6.2, 400 bases; SUR1, 288 bases; SUR2. 447 bases) was purified by electrophoresis in a denaturating gel (7 M urea, 5% polyacrylamide) and eluted in Probe Elution Buffer (Ambion, TX, USA) at 37°C for 2 hours and ethanol precipitated.

The estimated protected mRNA sizes of Kir6.2 and SUR2 were 300 and 440 respectively. Mouse β-actin antisense probe with 188 bases (protected sizes: 125 bases) by in vitro transcription of pTRI-β-actin mouse plasmid DNA (Ambion, Austin, TX, USA) was prepared to normalize the difference in amounts of loaded RNA.

Ribonuclease protection assay (RPA)

The levels of Kir6.2, SUR1 and SUR2 mRNAs were measured by RPA using Hybspeed RPA kit (Ambion, Austin, TX, USA), following the manufacture's protocol. Briefly, hybridization of Kir6.2 and SUR2 probes were performed with 80 μg of total RNA. The ^{32}P-labeled antisense probe (10^5 dpm per tube) were combined with the total RNA, subsequently dissolved in hybridization buffer (Ambion, Austin, TX, USA) and incubated overnight at 68°C. The hybridization mixture was then digested with

RNase T1 (Kir6.2, 200 unit; SUR2, 400 unit) for 1 hour at 37°C. The ribonuclease digestion was terminated by addition of 150 μl of RNase Inactivation/Precipitation Solution (Ambion, Austin, TX, USA) and the protected fragment was precipitated at −20°C for 1 h. The pellet was dissolved in 8 μl of Loading Buffer (Ambion, Austin, TX, USA) and subjected to electrophoresis on a 5% polyacrylamide gel containing 7 M urea at 300 V for 2 h. The gel was dried and exposed to either X-ray film (Agfa Curix RP Plus, Agfa, Mortsel, Belgium) at −70°C for 5 days or Bio-imaging Analysis System (Fuji, Tokyo, Japan) plate for 3 days.

Relative amounts of mRNA levels were analyzed with image analysis computer software (Tina 2.0 program, Fuji, Tokyo, Japan) using β-actin mRNA levels as internal controls. X-ray film image was scanned with GS700 Imaging Densitometor (Bio-Rad, Hercules, CA).

RESULTS

In a preliminary study of tissue distribution of Kir6.2 and SUR2 mRNAs, it appears that Kir6.2 and

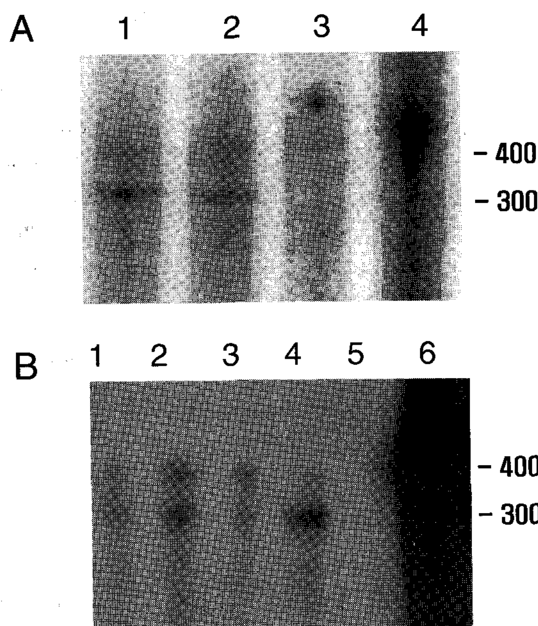

Fig. 1. Representative radiograms of RPA for the developmental expression of Kir6.2 mRNA in the rat heart, brain and skeletal muscle. RPA of total RNA (80 μg/lane) from neonate (2~8 days) and adult (90~120 days) tissues was performed with Kir6.2 antisense probe as described in the Methods. RPA of total RNA (10 μg/lane) from each tissue using β-actin antisense probe was carried out to normalize the difference in amount of loaded RNA. The numbers at the right side indicate size of the probe and the protected bands. (A) Developmental expression in rat heart. Lanes are: 1, neonate (4 days) heart; 2, adult (90 days) heart; 3, yeast RNA with RNase digestion; 4, yeast RNA without RNase digestion. (B) Developmental expression in rat brain and skeletal muscle. Lanes are: 1, neonate (2 days) brain; 2, adult (90 days) brain; 3, neonate (2 days) skeletal muscle; 4, adult (90 days) skeletal muscle; 5, yeast RNA with RNase digestion; 6, yeast RNA without RNase digestion.

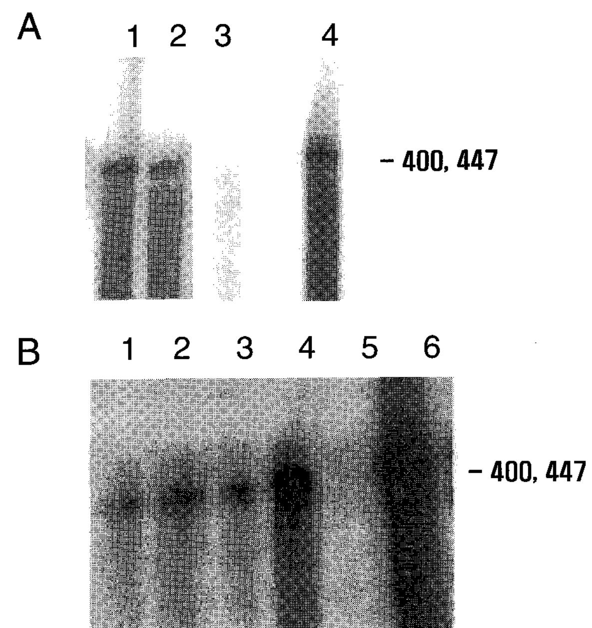

Fig. 2. Representative radiograms of RPA for the developmental expression of SUR2 mRNA in the rat heart, brain and skeletal muscle. RNase protection assay of total RNA (80 μg/lane) from neonate (2~8 days) and adult (90~120 days) tissues was performed with SUR2 antisense probe as described in the Methods. RNase protection assay of total RNA (10 μg/lane) from each tissue using β-actin antisense probe was carried out to normalize the difference in amount of loaded RNA. The numbers at the right side indicates size of the probe and protected bands. (A) Developmental expression in rat heart. Lanes are: 1, neonate (4 days) heart; 2, adult (90 days) heart; 3, yeast RNA with RNase digestion; 4, yeast RNA without RNase digestion. (B) Developmental expression in rat brain and skeletal muscle. Lanes are: 1, neonate (2 days) brain; 2, adult (90 days) brain; 3, neonate (2 days) skeletal muscle; 4, adult (90 days) skeletal muscle; 5, yeast RNA with RNase digestion; 6, yeast RNA without RNase digestion.

SUR2 mRNAs are mainly expressed in rat heart, brain and skeletal muscle (data not shown). We focused on the expression of Kir6.2 and SUR2 in these tissues to find the changes in mRNA levels with age and with treatment of K_{ATP} channel modulators.

Age-dependent expression patterns of Kir6.2 and SUR2 mRNAs

We compared the mRNA levels of Kir6.2 and SUR2 in neonate and adult rat tissues. In adult (90~120 days) brain and skeletal muscle, the mRNA levels of Kir6.2 and SUR2 were higher than in neonate (2~8 days) tissues (n=3, Fig. 1B, 2B). In adult brain, Kir6.2 and SUR2 mRNA level is higher by 261% and 130% respectively than neonate brain. In adult skeletal muscle, Kir6.2 and SUR2 mRNA level is higher by 157% and 133% than neonate respectively. In contrast, the mRNA levels in heart were not significantly different between the neonate and adult rats (Fig. 1A, 2A).

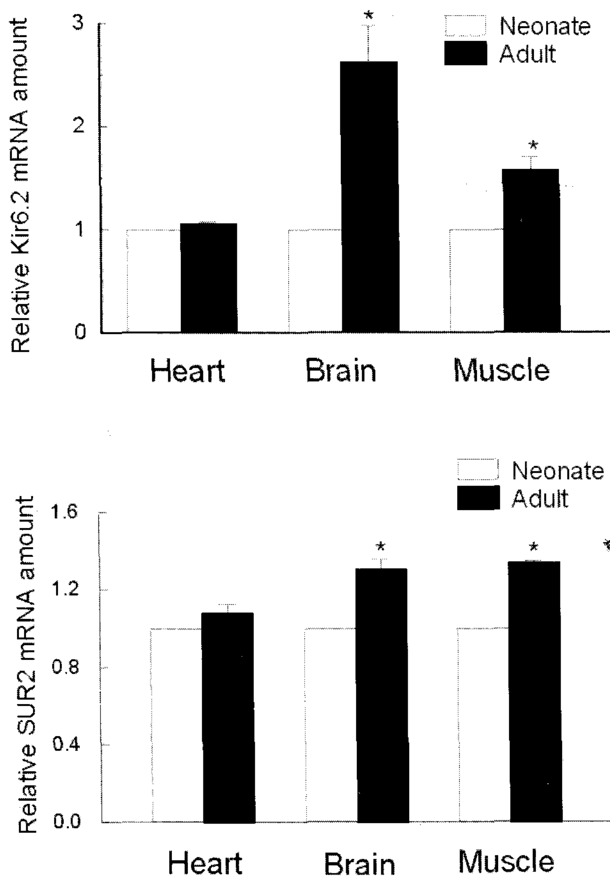

Fig. 3. Quantitative analysis of Kir6.2 and SUR2 mRNAs in the heart, brain, skeletal muscle. The RPA autoradiograms were analyzed by densitometry as described in the Materials and Methods. The amounts of Kir6.2 and SUR2 mRNA were normalized using actin mRNA level as a standard. The concentration of mRNA from each adult rat tissue was expressed relative to the mRNA concentration in neonate rat tissue fixed as 1.0. Error bar represents standrd error (n=3~4). *denotes statistically significant difference from neonate concentration.

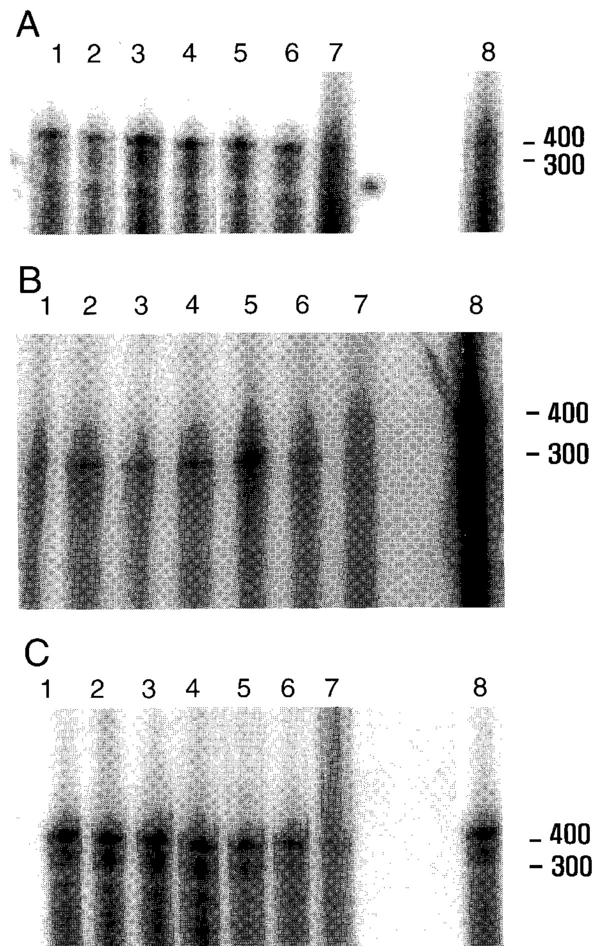

Fig. 4. Kir6.2 mRNA levels of heart (A), brain (B), and skeletal muscle (C) from rats treated with pinacidil, nicorandil and glybenclamide. RPA of total RNAs (80 μg/lane) from rat tissues treated with 3 different drugs was performed with Kir6.2 antisense probe as described in the Materials and Methods. RPA of 10 μg total RNAs using β-actin mRNA band as a standard was carried out to normalize the Kir6.2 mRNA band intensity. The numbers at the right side indicates size of the probe and protected bands. Lanes are 1, saline treated; 2, PEG treated; 3, pinacidil treated; 4, nicorandil treated; 5, hydralazine treated; 6, glibenclamide treated; 7, yeast RNA with RNase digestion; 8, yeast RNA without RNase digestion.

Fig. 3 summarizes the result of relative mRNA levels of Kir6.2 and SUR2 in brain, skeletal muscle and heart.

Effect of pinacidil, nicorandil, and glibenclamide on Kir6.2 mRNA level

We found that heart, brain and skeletal muscle Kir6.2 mRNA expression was not significantly affected by administration of K^+-channel opener, pinacidil and nicorandil, or K^+ channel blocker, glibenclamide for 10 days (Fig. 4).

DISCUSSION

Brain mRNA levels during development

The function of the mammalian brain depends on a continuous supply of O_2 and glucose. It has been previously known that the decrease of intracellular ATP concentration and the increase of extracellular K^+ concentration in the brain occurs during ischemia (Hansen 1985; Jiang 1992; Choi et al, 1997). So a good candidate responsible for K^+ loss could be K_{ATP} channel. Another data supports that K_{ATP} channel is related to extracellular K^+ increase during anoxia and that glibenclamide, a specific blocker of K_{ATP} channels has no effect on K^+ homeostasis during oxygenated states, but almost halves the anoxia-induced increase in extracellular K^+ in the adult rat (Jiang et al, 1992).

It is well established that young animals survive longer periods of ischemia than adults (Kass et al, 1989). It is suggested that the different resistance to anoxia in young and adult animals which is related to differences in the ability to keep near normal potassium gradients across the cells in the brain (Hansen, 1977). In this study, Kir6.2 and SUR2 mRNA levels were increased in adult rat brain than in neonate rat (Fig. 1, 2). Previously, Miller et al (1991) proposed that significant age- dependent development of glibenclamide sites was seen in brain. The consequence of higher expression of functional K_{ATP} channels in adult rat brain may explain its higher sensitivity of adult brain to ischemia. During ischemia ATP was depeleted in brain and K_{ATP} channel is activated, resulting in K^+ efflux through the channel. So intracellular K^+ is lost through K_{ATP} channel but the extent of K^+ loss is different in neonate and adult animal. In other words, the length of time required to attain ionic homeostasis in neonate is shorter than adult. Since multiple subtypes of Kir and SUR are expressed in the brain, in particular hippocampus (Zawar et al, 1999), however, the expression pattern of all known subtypes of both gene family should be evalauated together to understand the coorelation between ischemic sensitivity and the expression pattern of Kir and SUR.

Cardiac mRNA levels during development

In response to ischemia or hypoxia myocardial cells rapidly lose potassium (Weiss et al, 1981). Extracellular potassium accumulation has been implicated as a cause of arrhythmias (Weiss et al, 1981). But the factors that control the rate of cellular K^+ loss during ischemia are not well understood. The two mechanisms that have been hypothesized to explain the increase in extracellular K^+ concentration are increased membrane K^+ conductance via K_{ATP} channels (Kantor et al, 1990) or increase of intracellular concentration of lactate (Baker et al, 1997). In previous studies, glibenclamide has been shown to reduce extracellular K^+ accumulation or net cellular K^+ loss during ischemia (Rub et al, 1996; Venkatesh et al, 1991). Similar to brain, immature heart represent the tolerance to the ischemia than mature heart (Baker et al, 1997; Tani et al, 1997). Another experimental data in relation to ischemic responses, the single channel conductance and channel density of the K_{ATP} channel in neonatal myocytes were significantly smaller than in adult cell (Chen et al, 1992). There is a report that mRNAs for Kir6.2 and SUR2 remained unchanged in rat heart under ischemic conditions (Akao et al, 1997).

However, in contrast to brain ischemia, K^+ channel activation could also cause increase in coronary flow to occur and a decreased afterload which may reduce the sensitivity to ischemia (Grover et al, 1989). So it is of interest to look at the potential mechanisms of cellular K^+ loss during ischemia by measuring Kir6.2 and SURs mRNA levels in neonatal and adult rat hearts. In this work, the mRNA level of Kir6.2 and SUR2 appeared little different between neonate (2~8 days) and adult (90~120 days) hearts. Such results do not seem to fit with the fact of age-dependent ischemic responses of rat heart (Baker et al, 1997; Tani et al, 1997). Therefore, it is possible that other isoforms of Kir and SUR are additionally expressed in adult heart as suggested in skeletal

muscle (Tricarico et al, 1997) or K_{ATP} channel consists of different molar ratio of Kir under different conditions, age and pathological conditions (Akao et al, 1997). Therefore, it is necessary to compare the mRNA levels of all functional K_{ATP} channels such as SUR2A/Kir6.2 and SUR2B/Kir6.1 in the heart (Fujita & Kurachi, 2000).

Skeletal muscle mRNA levels during development

Exercise causes skeletal muscle to lose potassium ions which accumulate in the extracellular space (Medbo et al, 1990). The local rise of external concentration of K^+ ion is possibly mediated by K_{ATP} channels during exercise (Medbo et al, 1990; Lee et al, 1995). The mRNA level of Kir6.2 and SUR2 in neonate (2 ~ 8 days) skeletal muscle is lower than that of adult (90 ~ 120 days). This result is not incompatible with that K_{ATP} channel is related to the extracellular K^+ accumulation in adult rat skeletal muscle. Recently it was proposed that two forms of K_{ATP} channel, juvenile channel type and adult channel type in skeletal muscle. The juvenile channel type has lower single channel conductance in respect to the adult form (Tricarico et al, 1997). Thus, the physiological significance of age-dependent change of Kir6.2 and SUR2 mRNA expression in brain and skeletal muscle remained to be further studied.

Effects of pinacidil, nicorandil, and glibenclamide on Kir6.2 mRNA level

Kir6.2 mRNA levels in heart, brain and skeletal muscle appears not changed by K^+ channel opener, pinacidil or nicorandil or by K^+ channel blocker, glibenclamide. However, in a previous study, treatment of rats with the K_{ATP} channel modulators, glyburide and pinacidil changed the maximum number (Bmax) of specific [^{3}H]glyburide binding sites in cardiac and brain membrane (Gopalakrishnan et al, 1992). Therefore, the regulation of [^{3}H]glyburide binding sites by K^+ channel seems to be unrelated to the level of K_{ATP} channel mRNA expression. However, to draw decisive conclusion, it is necessary to compare mRNA levels of both Kir and SURs under the similar experimental conditions since the gating mechanism of K_{ATP} channels are dependent on both Kir as well as SUR (Seino, 1999).

In conclusion, our results indicate that the expression of Kir and SUR genes can vary age-dependently, but the expression of Kir is not dependent on the treatment of drugs that modulate the activity of K_{ATP} channels. It is remained to be studied further whether the level of SUR is affected by K_{ATP} channel modulators.

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