A Novel Sulfonylurea Receptor Forms with BIR (Kir6.2) a Smooth Muscle Type ATP-sensitive K+ Channel*

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We have isolated a cDNA encoding a novel isoform of the sulfonylurea receptor from a mouse heart cDNA library. Coexpression of this isoform and BIR (Kir6.2) in a mammalian cell line elicited ATP-sensitive K+ (I\textsubscript{K\textsubscript{ATP}}) channel currents. The channel was effectively activated by both diazoxide and pinacidil, which is the feature of smooth muscle K\textsubscript{ATP} channels. Sequence analysis indicated that this clone is a variant of cardiac type sulfonylurea receptor (SUR2). The 42 amino acid residues located in the carboxyl-terminal end of this novel sulfonylurea receptor is, however, divergent from that of SUR2 but highly homologous to that of the pancreatic one (SUR1). Therefore, this short part of the carboxyl terminus may be important for diazoxide activation of K\textsubscript{ATP} channels. The reverse transcription-polymerase chain reaction analysis showed that mRNA of this clone was ubiquitously expressed in diverse tissues, including heart, brain, liver, and skeletal muscle. These results suggest that this novel isoform of sulfonylurea receptor is a subunit reconstituting the smooth muscle K\textsubscript{ATP} channel.

ATP-sensitive K+ (I\textsubscript{K\textsubscript{ATP}}) channels, which represent a family of K+ channels inhibited by intracellular ATP, have been found in a variety of tissues including heart, pancreatic b-cells, skeletal muscle, smooth muscle, and the central nervous system (1–4). These K\textsubscript{ATP} channels have been associated with diverse cellular functions, such as shortening of action potential duration and cellular loss of K+ ions that occur during metabolic inhibition in heart, insulin secretion from pancreatic b-cells, smooth muscle relaxation, regulation of skeletal muscle excitability, and neurotransmitter release (5, 6). Furthermore, K\textsubscript{ATP} channels in different tissues exhibit considerable variation in response to K+ channel openers. For example, the pancreatic b-cell K\textsubscript{ATP} channel is activated by diazoxide and only weakly by pinacidil. The cardiac K\textsubscript{ATP} channel is activated by pinacidil but not by diazoxide. The smooth muscle K\textsubscript{ATP} channel is activated effectively by both of these compounds (2, 5, 6). Thus, properties of K\textsubscript{ATP} channels vary among tissues, having led to the premise that this K+ channel family may be composed of heterogeneous K+ channel proteins.

Recently, it has been shown that the pancreatic b-cell K\textsubscript{ATP} channel is a complex composed of at least two subunits, a K+ channel subunit (BIR/Kir6.2) and the pancreatic sulfonylurea receptor, SUR1 (7, 8). Coexpression of these two subunits reconstituted inwardly rectifying ATP-sensitive K+ conductance (I\textsubscript{K\textsubscript{ATP}}), which was activated by sulfonylureas and activated by diazoxide. It was also reported that coexpression of BIR and an isoform of SUR isolated from a rat brain cDNA library, designated SUR2, elicited I\textsubscript{K\textsubscript{ATP}}, which was activated by pinacidil and cromakalim but not by diazoxide (9). SUR2 mRNA was expressed at high levels in heart and skeletal muscle as assessed by Northern blot analysis. Thus, the complex of BIR and SUR2 may reconstitute K\textsubscript{ATP} channels described in heart and skeletal muscle. The finding that distinct SURs produce different responses of the reconstituted I\textsubscript{K\textsubscript{ATP}} to K+ channel openers suggests the existence of other isoforms of SURs that could be responsible for the smooth muscle type of response of I\textsubscript{K\textsubscript{ATP}}.

In this study, we have tried to find SURs in a mouse heart cDNA library and obtained a novel isoform. Coexpression of BIR and this novel SUR reconstituted I\textsubscript{K\textsubscript{ATP}} activated by both pinacidil and diazoxide. The reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that mRNA for this SUR was expressed in various tissues, including brain, heart, lung, liver, urinary bladder, and skeletal muscle. These findings suggest that this novel SUR is a subunit that could represent part of the smooth muscle K\textsubscript{ATP} channel.

EXPERIMENTAL PROCEDURES

Cloning a Sulfonylurea Receptor and an Inwardly Rectifying K+ Channel (Kir)—A mouse heart cDNA library (Stratagene, La Jolla, CA) was screened under a mild stringency condition using a 3P-labeled DNA fragment encoding rat SUR1 (nucleotide positions 3486–4589) (10), which was obtained by RT-PCR from rat heart RNA. A rat brain cDNA library (Stratagene) was screened for BIR (Kir6.2) using a 3P-labeled mouse u\textsubscript{K\textsubscript{ATP}}-1 (Kir6.1) cDNA probe. Hybridization and DNA sequencing were performed as described previously (10).

Transfection and Electrophysiology—The coding regions of cloned cDNAs were subcloned into the expression vector (pcDNA3, Invitrogen, San Diego, CA). These subcloned plasmids were transfected into human embryonic kidney (HEK) 293T cells fed with Dulbecco’s modified Eagle’s medium (Nihon, Kyob, Japan) containing 10% fetal calf serum (Life Technologies, Inc.), cells (7 × 10⁶/coverslip) were seeded on glass coverslips (15 mm diam) coated with poly d-lysine (Sigma). After 18–24 h, the cells were washed once with Opti-MEM (Life Technologies, Inc.) and transfected with the plasmids using lipofectAMINE (Life Technologies, Inc.) in Opti-MEM. 6 h after transfection, fetal calf serum was added to the medium to 10%, and the cells were incubated for 18–24 h. After this period, the cells were washed and incubated in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum for further 24–48 h before electrophysiological assay.

Single-channel recordings were made at room temperature in the cell-attached or inside-out configuration of patch clamp technique. The pipette had a tip resistance of 6–7 megaohms when filled with a solution containing (in mM): 140 KCl, 1 CaCl2, 1 MgCl2, and 5 HEPES-KOH (pH 7.4). Channel activity was measured with a patch clamp amplifier.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) D86037, D86038, and D86039.

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1 The abbreviations used are: K\textsubscript{ATP}, ATP-sensitive K+ channel; I\textsubscript{K\textsubscript{ATP}}, K\textsubscript{ATP} conductance; SUR, sulfonylurea receptor; RT, reverse transcription; PCR, polymerase chain reaction; HEK, human embryonic kidney; bp, base pairs; m-, mouse; r-, rat.
Cloning of Smooth Muscle Type Sulfonylurea Receptor

We obtained 49 positive clones after screening approximately 6 × 10⁵ plaques of the mouse heart cDNA library. Two of these clones, named MCS3 and MCS10, were further analyzed by sequencing. The nucleotide sequence of MCS10 revealed a single open reading frame encoding a protein of 1546 amino acid residues (Fig. 1). The amino acid sequence of MCS10 had 67% identity with that of rat SUR1 (7) and 97% identity with that of rat SUR2 (9), indicating that MCS10 is homologous to SUR2. The hydropathy profile of MCS10 was similar to those of SUR1 and SUR2, suggesting that this clone has a similar topology with 13 putative transmembrane regions similar to those of SUR1 and SUR2, suggesting that this clone has a similarmotifsthatareindicatedbyboxedsequences(FIG.1;Ecotransfectedcells.Diazoxide(200mM)orpinacidilinduced single channel current amplitudes were resolved in the single channel containing (in mM): 140 KC1, 5 EGTA, 5 HEPES (pH 7.3), and, unless otherwise indicated, 2 MgCl₂ (free Mg²⁺ concentration, ~1.4 mM). ATP was dissolved in the internal solution with free Mg²⁺ concentration adjusted to 1.4 mM by adding MgCl₂ (referred to as MgATP) or in the internal solution, which contained no MgCl₂, but EDTA (5 mM) instead of EGTA (Mg²⁺-free ATP). In both cases, the concentration of ATP is the total ATP concentration unless otherwise indicated. A continuous record of channel currents was stored for subsequent analysis on video-cassette tapes through a PCM converter system (VR-108, Instrotech Corp., Great Neck, NY). For analysis, data were reproduced, low pass filtered at 1 kHz (~3 decibels) by an 8-pole Bessel filter (Frequency Devices, Harwell, MA) digitized at 3 or 5 kHz by an AD converter (ITC-16, Instrotech Corp.), and analyzed on a computer (Machintosh Quadra 700, Apple Computer Inc., Cupertino, CA) by using Pulse program (HEKA Electronik, Lambrecht, Germany) and Patch Analyst Pro (MT Corporation, Hyogo, Japan). The channel activity was estimated by measuring the mean current amplitude after subtracting a leak current. Statistical data were expressed as means ± S.D.

RT-PCR Assay for SURs—The cDNAs synthesized from total RNAs extracted from various organs with oligo(dT) primers were used as templates for PCR amplification. The sequences of the primers for amplification of the novel SUR were as follows: 5′-ACGCCGCTAACCATAGCT-3′ (forward) and 5′-CATGTCACGCCCTTAAG-3′ (reverse), corresponding to nucleotide positions 4546–4503 and 4847–4864 of MCS10, respectively. The PCR condition was as follows: an initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 1 min, and extension at 72°C for 2 min, with a final extension step at 72°C for 8 min.

RESULTS

The single channel current amplitudes could not be distinguished. Such responses to the K⁺ channel openers were not observed in the cells transfected with either MCS10 or r-BIR alone (not shown). Both diazoxide- and pinacidil-induced channel activity were inhibited by tolbutamide or glibenclamide, specific blockers of KATP channels (Fig. 2, a). On patch excision, maximal channel activity appeared promptly and was almost completely inhibited by 1 mM of intracellular MgATP. Similar data were obtained from five other patches in the cotransfected cells. Synchronous openings of the MCS10/r-BIR channels in the inside-out patches rapidly ran down in the presence of 100 μM intracellular Ca²⁺ (Fig. 2b, b). The channel could be easily reactivated after treating the patch with 1 mM MgATP. MgUDP (10 mM) restored the channel activity after rundown (Fig. 2, b). Single channel recordings of the MCS10/r-BIR channels in a cell-attached patch are shown in Fig. 2b. The channels opened in bursts at all membrane potentials examined. The currents flowing through the channels reversed around 0 mV under the symmetrical K⁺ solutions (Fig. 2b, a). The current-voltage relationship demonstrated weak inward rectification with the single channel conductance of 80.3 pS at ~5 pA at ~60 mV occasionally appeared in bursts in the cotransfected cells. Diazoxide (200 μM), pinacidil (100 μM) added to the bathing solution markedly increased the channel activities. As shown in Fig. 2a, the pinacidil side was indicated by an arrow. Other nucleotide binding sites were shaded, and the Walker A and B consensus sites are boxed. Possible cyclic AMP-dependent protein kinase phosphorylation sites (○) and protein kinase C-dependent phosphorylation sites (▲) are marked above the sequence. Two potential N-glycosylation sites are indicated by arrows.

Amino acid sequence of the novel isoform of mouse SUR. Putative transmembrane regions are overlaid. Putative nucleotide binding sites are shaded, and the Walker A and B consensus sites are boxed. Possible cyclic AMP-dependent protein kinase phosphorylation sites (○) and protein kinase C-dependent phosphorylation sites (▲) are marked above the sequence. Such potential N-glycosylation sites are indicated by arrows.
Cloning of Smooth Muscle Type Sulfonylurea Receptor

% Inhibition = 100(1 + ([ATP]/\(K_a[/ATP]\))^n)  
(Eq. 1)

where [ATP] is the concentration of ATP, \(K_a\) is the apparent dissociation constant, and \(n\) is the Hill coefficient (Fig. 2C, b). The \(K_a\) and \(n\) were estimated as 67.9 \(\mu\)M and 1.85 for \(Mg^{2+}\)-free ATP (Fig. 2C, b, closed circles), and 300 \(\mu\)M and 1.43 for MgATP (Fig. 2C, b, open circles), respectively. When the inhibition evoked by MgATP was replotted by calculating the concentration of ATP not complexed with \(Mg^{2+}\) in this solution (Fig. 2C, b, open diamonds), the apparent \(K_a\) was estimated as 16.9 \(\mu\)M. This value was lower than that in the absence of \(Mg^{2+}\) (67.9 \(\mu\)M). Therefore, these results indicate that both \(Mg^{2+}\)-free ATP and MgATP can inhibit the channel openings.

Intracellular UDP restored the channel openings after rundown of the expressed channel in a concentration-dependent manner (Fig. 2D, a). The concentration-response relationship could be fitted with the following Hill equation:

Relative activation = 100(1 + \((K_a[/UDP]\))^n)  
(Eq. 2)

where [UDP] is the concentration of UDP. The \(K_a\) and \(n\) were estimated as 71.7 \(\mu\)M and 1.74, respectively. Thus, UDP may activate this channel in a positive cooperative manner.

The sequence analysis of another clone obtained, MCS2, showed that this clone was essentially the same as rat SUR2. MCS3, which lacked 5’-untranslated and coding regions, has a sequence identical to MCS10 and possessed an additional 176-bp insertion in the COOH terminus between nucleotide positions 4505 and 4506 of MCS10. The insertion of these 176 bp generated divergent amino acid sequences in the COOH termini between MCS3 and MCS10 (Fig. 3A). Thus, MCS3 had an amino acid sequence identical to MCS10 through Val\(^{1504}\) and then diverged in the COOH-terminal ends. These findings indicated that MCS10 and MCS3 may be formed by alternative splicing of a single mouse gene. A comparison of amino acid sequences in the COOH termini of MCS10, MCS3, and r-SUR1, and r-SUR2 is shown in Fig. 3B. The amino acid sequence of MCS3 was identical to that of r-SUR2 except for one amino acid residue (Val\(^{1508}\) in MCS3 and Met\(^{1507}\) in r-SUR2), suggesting that MCS3 is a mouse homolog of r-SUR2. Based on these results, we designated r-SUR2, MCS3, and MCS10 as r-SUR2A, m-SUR2A, and m-SUR2B, respectively. For the alternative regions composed of 42 amino acid residues located in the ends of their sequences, m-SUR2B showed 74% identity with r-SUR1 and 33% identity with m-SUR2A.

To determine tissue distributions of m-SUR2A and m-SUR2B mRNAs, the RT-PCR assay was performed. The specific primers for amplification of both m-SUR2A and m-SUR2B were designed to produce cDNA fragments of 555 and 379 bp, respectively. As shown in Fig. 4, m-SUR2A mRNA was expressed in cerebellum, eye, atrium, ventricle, urinary bladder, and skeletal muscle. The m-SUR2B mRNA distributed not only in these tissues but in all other tissues examined: forebrain, lung, liver, pancreas, kidney, spleen, stomach, small intestine, colon, uterus, ovary, and fat tissue.

**DISCUSSION**

Recent studies have shown that coexpression of SUR (SUR1 or SUR2A) and BIR constitutes \(I_{KATP}\), but neither of them can express the channel activity on their own (7–9, 11, 12). Likewise, SUR2B only when cotransfected with BIR produced \(I_{KATP}\) channel activity. In this study, BIR was used to provide the gating part of \(I_{KATP}\) channels. However, it has also been shown that Kir clones other than BIR, such as ROMK1 (Kir1.1) and uKATP-1 (Kir6.1) can interact with SUR (12). These Kir clones produce \(K^+\) channel activity for themselves but become sensitive to glibenclamide when the SUR1 clone is cotransfected. Therefore, SUR2B may also be able to couple to several
and/or induce glibenclamide sensitivity. This might be the mechanism responsible for the reported diversity of the gating mechanism important for diazoxide activation of K<sub>ATP</sub> channels. On the other hand, binding sites of pinacidil to SURs may be in the regions different from the COOH-terminal end, because pinacidil activated K<sub>ATP</sub> channels reconstituted from SUR2A or SUR2B but not from SUR1.

In conclusion, our results indicate that SUR2B forms with BIR a K<sub>ATP</sub> channel with a pharmacology representative of smooth muscle, whereas SUR1/BIR and SUR2A/BIR channels form pancreatic and cardiac types, respectively. The successful cloning of this novel SUR should open a novel approach to elucidate the molecular mechanism of smooth muscle regulation and also to develop new vaso relaxants belonging to K<sup>+</sup> channel openers.

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