

# Molecular Mechanisms Underlying Ketamine-mediated Inhibition of Sarcolemmal Adenosine Triphosphate-sensitive Potassium Channels

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**Background:** Ketamine inhibits adenosine triphosphate-sensitive potassium ( $K_{ATP}$ ) channels, which results in the blocking of ischemic preconditioning in the heart and inhibition of vasorelaxation induced by  $K_{ATP}$  channel openers. In the current study, the authors investigated the molecular mechanisms of ketamine's actions on sarcolemmal  $K_{ATP}$  channels that are re-associated by expressed subunits, inwardly rectifying potassium channels (Kir6.1 or Kir6.2) and sulfonylurea receptors (SUR1, SUR2A, or SUR2B).

**Methods:** The authors used inside-out patch clamp configurations to investigate the effects of ketamine on the activities of reassociated Kir6.0/SUR channels containing wild-type, mutant, or chimeric SURs expressed in COS-7 cells.

**Results:** Ketamine racemate inhibited the activities of the reassociated  $K_{ATP}$  channels in a SUR subtype-dependent manner: SUR2A/Kir6.2 ( $IC_{50} = 83 \mu M$ ), SUR2B/Kir6.1 ( $IC_{50} = 77 \mu M$ ), SUR2B/Kir6.2 ( $IC_{50} = 89 \mu M$ ), and SUR1/Kir6.2 ( $IC_{50} = 1487 \mu M$ ). S-(+)-ketamine was significantly less potent than ketamine racemate in blocking all types of reassociated  $K_{ATP}$  channels. The ketamine racemate and S-(+)-ketamine both inhibited channel currents of the truncated isoform of Kir6.2 (Kir6.2 $\Delta$ C36) with very low affinity. Application of  $100 \mu M$  magnesium adenosine diphosphate significantly enhanced the inhibitory potency of ketamine racemate. The last transmembrane domain of SUR2 was essential for the full inhibitory effect of ketamine racemate.

**Conclusions:** These results suggest that ketamine-induced inhibition of sarcolemmal  $K_{ATP}$  channels is mediated by the SUR subunit. These inhibitory effects of ketamine exhibit specificity for cardiovascular  $K_{ATP}$  channels, at least some degree of stereoselectivity, and interaction with intracellular magnesium adenosine diphosphate.

ADENOSINE triphosphate-sensitive potassium ( $K_{ATP}$ ) channels are inhibited by intracellular adenosine triphosphate (ATP) and activated by magnesium adenosine diphosphate (MgADP) and thus provide a link between the cellular metabolic state and excitability.<sup>1,2</sup>  $K_{ATP}$  channels are composed of an ATP-binding cassette protein, sulfonylurea receptor (SUR), and an inwardly rectifying  $K^+$  channel (Kir) subunit, Kir6.0; SUR acts as a regulatory subunit whereas Kir subunits form the ATP-

sensitive channel pore.<sup>3</sup> These channels, as metabolic sensors, are associated with such cellular functions as insulin secretion, cardiac preconditioning, vasodilatation, and neuroprotection.<sup>4-7</sup>

In cardiac myocytes, intravenous general anesthetics, such as ketamine racemate, propofol, and thiamylal, directly inhibit native sarcolemmal  $K_{ATP}$  channels.<sup>8-10</sup> Although these observations suggest that intravenous anesthetics may impair the endogenous organ protective mechanisms mediated by  $K_{ATP}$  channels, the possibility that  $K_{ATP}$  channel inhibition by intravenous anesthetics might have adverse consequences in clinical practice remains controversial. Indeed, propofol and thiamylal are known to possess cardioprotective and neuroprotective properties, respectively, with these being mediated by other well-established mechanisms that do not involve  $K_{ATP}$  channels.<sup>11,12</sup> In recent *in vitro* studies, however, ketamine racemate, but not the stereoisomer S-(+)-ketamine, was found to block early and late preconditioning in rabbit hearts and inhibit vasorelaxation induced by a  $K_{ATP}$  channel opener.<sup>13-16</sup> It is therefore possible that the mechanisms underlying ketamine-induced inhibition of  $K_{ATP}$  channel activity may differ from those of propofol and thiamylal. A previous mutagenesis study demonstrated that the major effects of both propofol and thiamylal on  $K_{ATP}$  channel activity are mediated *via* the Kir6.2 subunit.<sup>17</sup> However, organ specificity and the molecular site of action of ketamine have not been investigated in detail. In addition, although it is now well established that intracellular MgADP can modulate the sensitivity of  $K_{ATP}$  channel activators and inhibitors,<sup>18,19</sup> there is no evidence that intracellular MgADP modulates intravenous anesthetics inhibitory effects on sarcolemmal  $K_{ATP}$  channels.

In the current study, we used patch clamp techniques to examine the electrophysiological effects and molecular mechanisms of racemic ketamine and S-(+)-ketamine on different types of reassociated  $K_{ATP}$  channels containing wild-type, mutant, or chimeric SURs expressed in COS-7 cells (African green monkey kidney cells). We also investigated the effects of intracellular MgADP on the inhibitory actions of ketamine racemate.

## Materials and Methods

### Molecular Biology

cDNAs (The human Kir6.2, rat Kir6.1, rat SUR1, rat SUR2A, and rat SUR2B) and expression vector pCMV6C

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were kindly provided by Susumu Seino, MD., Ph.D. (Professor and Chairman, Department of Cellular and Molecular Medicine, Chiba University, Chiba, Japan). Coexpressing SUR1 and Kir6.2 (SUR1/Kir6.2) forms the pancreatic  $\beta$  cell  $K_{ATP}$  channel, SUR2A and Kir6.2 (SUR2A/Kir6.2) forms the cardiac  $K_{ATP}$  channel, SUR2B and Kir6.2 (SUR2B/Kir6.2) forms the nonvascular smooth muscle  $K_{ATP}$  channel, and SUR2B and Kir6.1 (SUR2B/Kir6.1) forms the vascular smooth muscle  $K_{ATP}$  channel.<sup>3,20,21</sup> A truncated form of human Kir6.2 lacking the last 36 amino acids at the C terminus was obtained by polymerase chain reaction amplification as previously described.<sup>17</sup> Chimeric cDNA constructs were produced by splicing, using the overlap extension polymerase chain reaction technique.<sup>22</sup> The exact amino acid composition of the SUR1-SUR2A chimeric constructs was: chimera SUR1-2A = (1-1035, SUR1)-(1013-1261, SUR2A)-(1297-1581, SUR1); SUR2A-1 = (1-1013, SUR2A)-(1035-1277, SUR1)-(1241-1545, SUR2A). All DNA products were sequenced using BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA), and an ABI PRISM 377 DNA sequencer (Applied Biosystems) was used to confirm the sequence.

#### Cell Culture and Transfection

$K_{ATP}$  channel-deficient COS-7 cells were plated at a density of  $3 \times 10^5$  per dish (35 mm diameter) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. A full-length Kir cDNA and a full-length SUR cDNA were subcloned into the mammalian expression vector pCMV6c. For electrophysiological recordings, mutated pCMV6c Kir alone (1  $\mu$ g) or either wild-type or mutated pCMV6c Kir (1  $\mu$ g) plus pCMV6c SUR (1  $\mu$ g) were transfected into COS-7 cells with green fluorescent protein cDNA (pEGFP-N1; Clontech Laboratories, Palo Alto, CA) as a reporter gene by using lipofectamine and Opti-MEM 1 reagents (Life Technologies Inc., Rockville, MD) according to the manufacturer's instructions. After transfection, cells were cultured for 48 to 72 h before being subjected to electrophysiological recordings.

#### Electrophysiological Measurements

Membrane currents were recorded in the inside-out configurations using a patch-clamp amplifier as described previously.<sup>9,10,17</sup> Transfected cells were identified by their green fluorescence under a microscope. The intracellular solution contained 140 mM KCl, 2 mM EGTA, 2 mM MgCl<sub>2</sub>, and 10 mM HEPES (pH = 7.3). The pipette solution contained 140 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 10 mM HEPES (pH = 7.4). Recordings were made at  $36^\circ \pm 0.5^\circ\text{C}$ . Patch pipettes were pulled with an electrode puller (PP-830; Narishige, Tokyo, Japan). The resistance of pipettes filled with internal solution and immersed in the Tyrode's solution was 5–7 M $\Omega$ .

The sampling frequency of the single-channel data were 5 KHz with a low-pass filter (1 KHz).

#### Electrophysiological Data Analysis

Channel currents were recorded with a patch clamp amplifier (CEZ 2200; Nihon Kohden, Tokyo, Japan) and stored in a personal computer (Aptiva; IBM, Armonk, NY) with an analog-to-digital converter (DigiData 1200; Axon Instruments, Foster City, CA). pClamp version 7 software (Axon Instruments) was used for data acquisition and analysis. The open probability ( $P_o$ ) was determined from current amplitude histograms and was calculated as follows:

$$P_o = \frac{\sum_{j=1}^N t_j \cdot j}{T_d \cdot N} \quad (1)$$

where  $t_j$  is the time spent at current levels corresponding to  $j = 0, 1, 2, N$  channels in the open state,  $T_d$  is the duration of the recording, and  $N$  is the number of the channels active in the patch. Recordings of 2–3 min were analyzed to determine  $P_o$ . The channel activity was expressed as  $NP_o$ . The  $NP_o$  in the presence of drugs was normalized to the baseline  $NP_o$  value obtained before drug administration and presented as the relative channel activity. When the concentration-dependent effects of drugs were studied, the superfusion was stopped for approximately 1 min at each concentration, and these drugs were injected into the cell bath using a glass syringe to five final concentrations in a cumulative manner (total volume injected was approximately 10–20  $\mu$ l). Therefore, the superfusion was stopped for approximately 5 min; preliminary studies showed that the stopping of superfusion for approximately 5 min had no significant effects on electrophysiological measurements. The average percent recovery of  $K_{ATP}$  channel activities after washout of Ketamine racemate or S-(+)-ketamine was  $94 \pm 6\%$  of the  $NP_o$  measured before drug treatment.

The drug concentration needed to induce half-maximal inhibition of the channels ( $IC_{50}$ ) and the Hill coefficient were calculated as follows:

$$y = \frac{1}{1 + ([D]/K_i)^H} \quad (2)$$

where  $y$  is the relative  $NP_o$ ,  $[D]$  is the concentration of drug,  $K_i$  is  $IC_{50}$ , and  $H$  is the Hill coefficient.

#### Drugs

The following drugs were used: ketamine racemate, S-(+)-ketamine, glibenclamide, and pinacidil (Sigma-

Aldrich Japan, Tokyo, Japan). Glibenclamide and pinacidil were dissolved in dimethylsulfoxide (the final concentration of solvent was 0.01%); preliminary studies showed that 0.02% of dimethylsulfoxide, a twofold higher concentration than we used in the current study, had no significant effects on all types of reassociated  $K_{ATP}$  channel currents.

### Statistics

All data were presented as means  $\pm$  SD. Differences between data sets were evaluated either by repeated-measures one-way analysis of variance followed by the Scheffé *F* test or by Student *t* test.  $P < 0.05$  was considered significant.

## Results

Sarcolemmal  $K_{ATP}$  channels SUR2A/Kir6.2 (cardiac type), SUR2B/Kir6.1 (vascular smooth muscle type), SUR2B/Kir6.2 (nonvascular smooth muscle type), and SUR1/Kir6.2 (pancreatic  $\beta$ -cell type) were heterologously expressed in COS-7 cells. Our previous experiments have shown that the single-channel characteristics of all types of reassociated  $K_{ATP}$  channels were similar to those of native  $K_{ATP}$  channels.<sup>17</sup>

### *Effects of Ketamine Racemate and S-(+)-ketamine on Sarcolemmal $K_{ATP}$ Channels in the Absence of Intracellular ADP*

To assess the effects of ketamine racemate and S-(+)-ketamine on reassociated  $K_{ATP}$  channels in the absence of intracellular ADP, we measured single-channel currents on inside-out patches in the presence of these drugs. Application of 100  $\mu$ M ketamine racemate to the intracellular membrane surface inhibited the SUR2A/Kir6.2, SUR2B/Kir6.1, and SUR2B/Kir6.2 channel currents, with relative channel activities decreasing to  $0.45 \pm 0.11$ ,  $0.41 \pm 0.07$ , and  $0.47 \pm 0.18$ , respectively (fig. 1A). However, 100  $\mu$ M ketamine racemate did not significantly inhibit the SUR1/Kir6.2 channel currents. On the contrary, S-(+)-ketamine at 100  $\mu$ M did not significantly inhibit all of the reassociated  $K_{ATP}$  channel currents (fig. 1B). The inhibitory effects of ketamine racemate and S-(+)-ketamine on  $K_{ATP}$  channel activities were readily reversible (fig. 1A).

The concentration-dependent effects of ketamine racemate and S-(+)-ketamine on the activities of various types of reassociated  $K_{ATP}$  channels, in the absence of intracellular ADP, are shown in figure 2. The  $IC_{50}$  values and Hill coefficients of ketamine and S-(+)-ketamine for the SUR2A/Kir6.2, SUR2B/Kir6.1, SUR2B/Kir6.2, and SUR1/Kir6.2 channels are summarized in table 1. The  $IC_{50}$  value of ketamine racemate for SUR2A/Kir6.2 ( $83 \pm 8 \mu$ M) (table 1) was very similar to that obtained by Ko *et al.*<sup>8</sup> for the native cardiac  $K_{ATP}$  channel in the absence of

intracellular ADP (63  $\mu$ M). Ketamine racemate inhibited the activity of all types of reassociated  $K_{ATP}$  channels with higher potency than S-(+)-ketamine. In addition, ketamine inhibited the SUR2A/Kir6.2, SUR2B/Kir6.1, and SUR2B/Kir6.2 channels with higher potency than the SUR1/Kir6.2 channel.

### *Effects of Ketamine Racemate and S-(+)-ketamine on Kir6.2 $\Delta$ C36 Channel Activity*

A C-terminal truncated pore-forming subunit of Kir6.2 (Kir6.2 $\Delta$ C36), lacking the last 36 amino acids, is capable of forming a functional channel in the absence of SUR.<sup>23</sup> This has proved to be a useful tool for discriminating the site of action of various agents on  $K_{ATP}$  channels.

Ketamine racemate and S-(+)-ketamine inhibited the Kir6.2 $\Delta$ C36 channel current with very low affinity, with 1 mM concentrations of both anesthetics producing less than 30% inhibition (fig. 3, A and B). This result indicates that the SUR subunit, rather than Kir6.2, is primarily responsible for the effects of ketamine racemate and S-(+)-ketamine on wild-type  $K_{ATP}$  channels, a view that is supported by the differential actions of ketamine on  $K_{ATP}$  channels containing different types of SUR subunit.

### *Effects of Ketamine Racemate in Presence of Intracellular ADP*

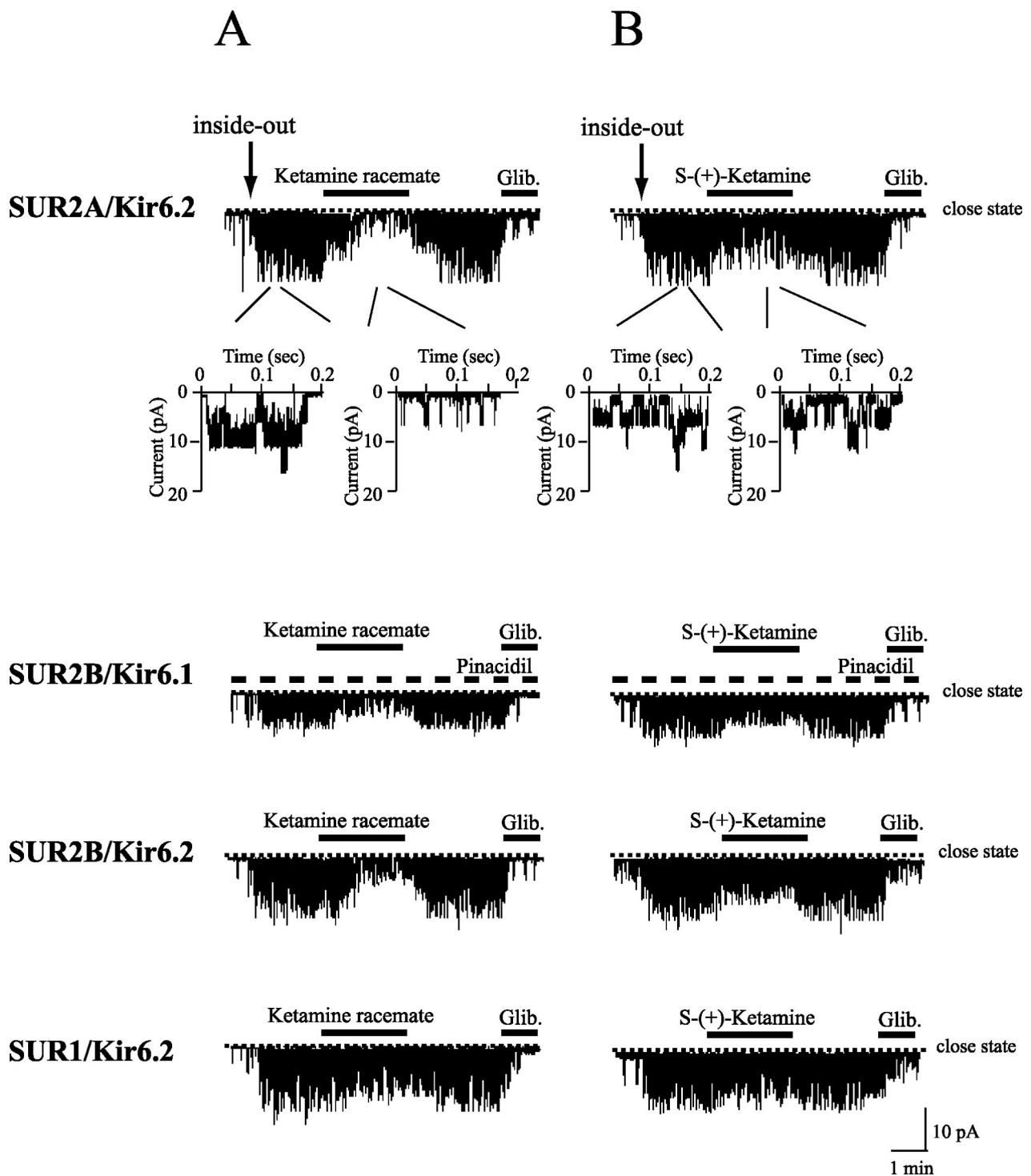
The effects of several sulfonylureas and  $K_{ATP}$  channel openers are modified by interaction of MgADP with SURs.<sup>18,19</sup> Indeed, it has been previously reported that MgADP simultaneously activates the  $K_{ATP}$  channels strongly *via* SUR and inhibits them weakly *via* the ATP binding site on Kir6.2.<sup>23,24</sup> Therefore, we next examined whether the inhibitory effects of ketamine on reassociated  $K_{ATP}$  channel currents are affected in the presence of intracellular MgADP.

Figure 4 shows the effects of 100  $\mu$ M ketamine racemate on SUR1/Kir6.2, SUR2A/Kir6.2, and SUR2B/Kir6.2 channel activities in the absence or presence of 100  $\mu$ M MgADP. The  $IC_{50}$  for these channels are given in table 2. MgADP was significantly enhanced the inhibitory potency of ketamine racemate on all types of reassociated  $K_{ATP}$  channels.

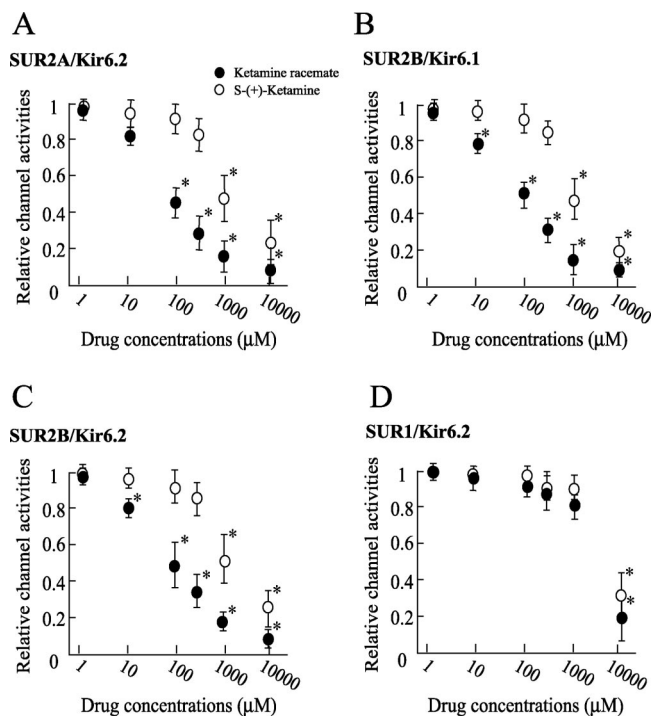
### *Effects of Ketamine Racemate on SUR Chimeras*

The differential effects of ketamine on SUR1 and SUR2 enabled us to use a chimeric approach in identifying regions of SUR2 critical for inhibition by ketamine racemate. SUR is a member of the ATP-binding cassette transporter family and is predicted to possess two intracellular nucleotide binding domains and three transmembrane domains (TMD0, TMD1, and TMD2) that contain five, six, and six transmembrane helices (TMs), respectively.<sup>25</sup> It has been reported recently that TMD2 of SUR1 and SUR2 is crucial for the action of several SUR1-selective sulfonylureas,  $K_{ATP}$  channel blocker, and SUR2-selective  $K_{ATP}$  channel openers, respectively.<sup>22,26</sup>





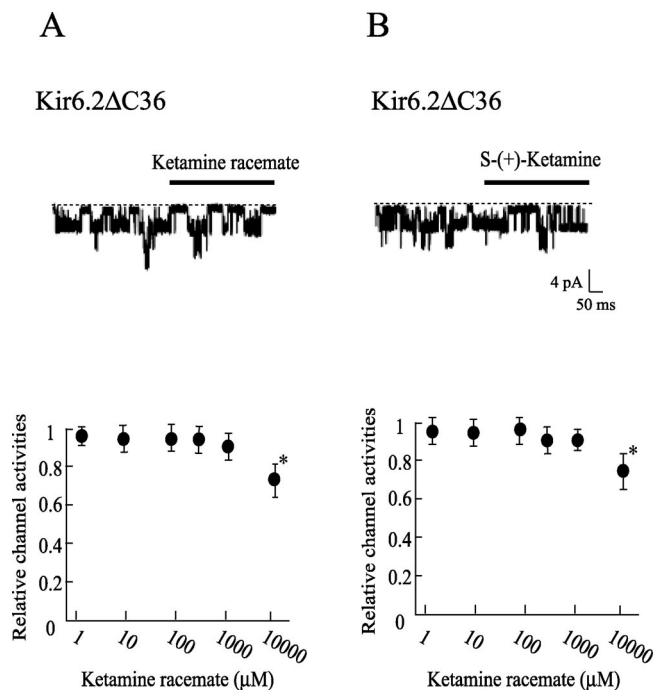
**Fig. 1.** Effects of ketamine racemate and S-(+)-ketamine on the currents of different reassociated adenosine triphosphate-sensitive potassium channels in the excised inside-out configuration. Membrane potentials were clamped at  $-60$  mV. Shown are representative examples of sulfonylurea receptor (SUR) 2A/inwardly rectifying potassium channel (Kir) 6.2, SUR2B/Kir6.1, SUR2B/Kir6.2, and SUR1/Kir6.2 currents obtained before and after the application of ketamine racemate ( $100 \mu\text{M}$ ) (A) or S-(+)-ketamine ( $100 \mu\text{M}$ ) (B). Because SUR2B/Kir6.1 channels are not activated in the inside-out patch clamp configurations, pinacidil ( $100 \mu\text{M}$ ) was used to activate them. This figure shows that washout of ketamine racemate and S-(+)-ketamine restores channel activities. In all cases, the channel activity was inhibited by glibenclamide (Glib;  $10 \mu\text{M}$ ). The periods of ketamine racemate or S-(+)-ketamine administration are marked with *horizontal solid bars*. The periods of pinacidil administration are marked with *horizontal dashed bars*.



**Fig. 2.** Concentration-dependent effects of ketamine racemate (●) and S-(+)-ketamine (○) on the activities of reconstituted sulfonylurea receptor (SUR) 2A/inwardly rectifying potassium channel (Kir) 6.2 (A), SUR2B/Kir6.1 (B), SUR2B/Kir6.2 (C), and SUR1/Kir6.2 (D) channels. Each vertical bar constitutes measurements from 9–18 patches (mean  $\pm$  SD). \*  $P < 0.05$  versus baseline (before drug).

Thus, we hypothesized that TMD2 of SUR might be involved in the SUR2-selective inhibition of ketamine. To test this hypothesis, we constructed chimeric SURs, in which portions of TMD2 were swapped between SUR1 and SUR2A and coexpressed with Kir6.2 in COS-7 cells (fig. 5A).

Ketamine racemate sensitivity could be introduced into SUR1 by transferring TMs 13–17 from SUR2A to SUR1 (chimera SUR1–2) (fig. 5B). The reverse chimera, in which TMs 13–16 were swapped from SUR1 to SUR2A (chimera SUR2–1), was also sensitive to inhibition by ketamine racemate but to a lesser extent than wild-type SUR2A. These results suggest that the region within TM



**Fig. 3.** Effects of ketamine racemate and S-(+)-ketamine on the channel activities of the truncated isoform of inwardly rectifying potassium channel 6.2 (Kir6.2 $\Delta$ C36), which can form functional adenosine triphosphate-sensitive potassium channels in the absence of sulfonylurea receptor molecules, in the excised inside-out configuration. Membrane potentials were clamped at  $-60$  mV. (A) Representative examples of Kir6.2 $\Delta$ C36 currents obtained before and after the application of ketamine racemate (100  $\mu\text{M}$ ) and S-(+)-ketamine (100  $\mu\text{M}$ ). The periods of drug treatment are marked with horizontal bars. (B) Dose-dependent effects of ketamine racemate and S-(+)-ketamine on the activities of Kir6.2 $\Delta$ C36 channels. Each vertical bar constitutes measurements from 10–15 patches (mean  $\pm$  SD). \*  $P < 0.05$  versus baseline (before drug).

13–17 is essential for the full inhibitory effect of ketamine racemate.

## Discussion

In the current study, we transiently expressed the different subtypes of reassociated sarcolemmal  $K_{ATP}$  channels in COS-7 cells and demonstrated that ketamine

**Table 1.** Effects of ketamine and S-(+)-ketamine on different types of re-associated  $K_{ATP}$  channels

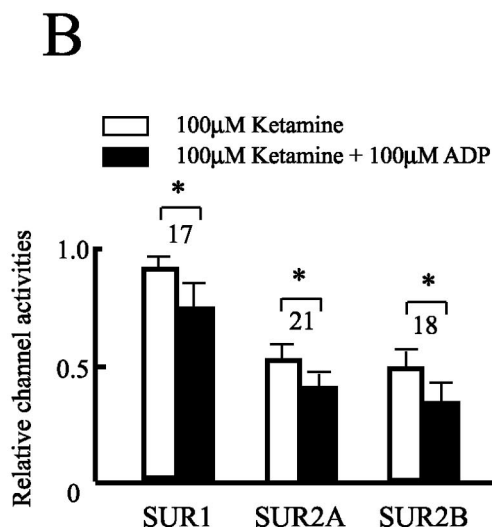
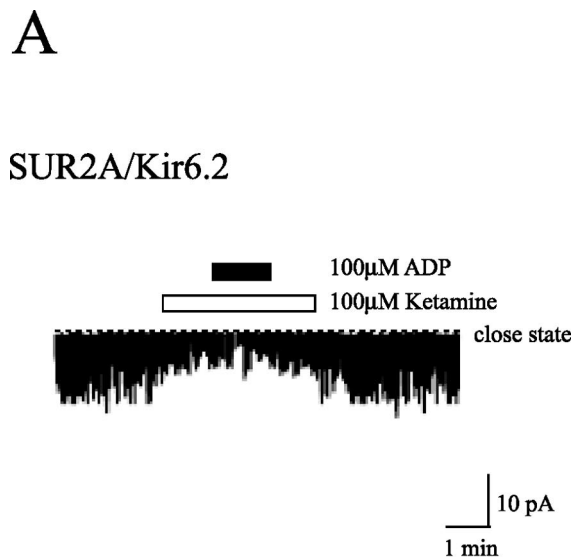
	Ketamine racemate			S-(+)-Ketamine		
	IC <sub>50</sub> ( $\mu\text{M}$ )	n	h	IC <sub>50</sub> ( $\mu\text{M}$ )	n	h
SUR2A/Kir6.2	83 $\pm$ 8*	15	0.92	889 $\pm$ 92*	16	0.89
SUR2B/Kir6.1	77 $\pm$ 9*	12	1.03	703 $\pm$ 76*	13	0.94
SUR2B/Kir6.2	89 $\pm$ 10*	9	0.98	925 $\pm$ 101*	15	0.91
SUR1/Kir6.2	1487 $\pm$ 345	18	1.12	1826 $\pm$ 393	18	1.08

SUR2A/Kir6.2 = re-associated sulfonylurea receptor 2A and inwardly rectifying potassium channel 6.2 channels; SUR2B/Kir6.1 = re-associated sulfonylurea receptor 2B and inwardly rectifying potassium channel 6.1 channels; SUR2B/Kir6.2 = re-associated sulfonylurea receptor 2B and inwardly rectifying potassium channel 6.2 channels; SUR1/Kir6.2 = re-associated sulfonylurea receptor 1 and inwardly rectifying potassium channel 6.2 channels.

IC<sub>50</sub> values are presented as mean  $\pm$  SD.

h indicates the Hill coefficient value.

\*  $P < 0.05$  against SUR1/Kir6.2 (for IC<sub>50</sub> value).



**Fig. 4.** Effects of anesthetics and magnesium adenosine diphosphate (MgADP) on reassociated sulfonyleurea receptor (SUR) 1/inwardly rectifying potassium channel (Kir) 6.2, SUR2A/Kir6.2, and SUR2B/Kir6.2 channels in the excised inside-out configuration. Membrane potentials were clamped at  $-60$  mV. (A) Representative examples of SUR2A/Kir6.2 currents in the absence or presence of anesthetics and MgADP are shown. Ketamine racemate ( $100 \mu\text{M}$ ), propofol ( $100 \mu\text{M}$ ), thiamylal ( $300 \mu\text{M}$ ), and MgADP ( $100 \mu\text{M}$ ) are added to the intracellular solution as indicated by horizontal bars. (B) The relative channel activities in the presence of anesthetics without MgADP (open columns) and with MgADP (closed columns). The relative channel activities in the presence of anesthetics are expressed as a fraction of that observed in the absence of the drugs. The number of patches is given above each bar. The columns and bars indicate the mean and SD, respectively. \* $P < 0.05$  versus without MgADP value.

racemate and S-(+)-ketamine inhibited these channels in a concentration-dependent manner. Our results also indicated that ketamine racemate specifically inhibits cardiovascular type  $K_{\text{ATP}}$  channels. Notably, ketamine's potency in blocking  $K_{\text{ATP}}$  channels containing SUR2 was approximately 18-fold higher than its ability to block  $K_{\text{ATP}}$  channels containing SUR1. Furthermore, ketamine

**Table 2.**  $\text{IC}_{50}$ s of inhibition of re-associated  $K_{\text{ATP}}$  channels by ketamine racemate in the absence and presence of  $100 \mu\text{M}$  magnesium adenosine diphosphate

	Ketamine racemate ( $\mu\text{M}$ )	
	MgADP (-)	$100 \mu\text{M}$ MgADP (+)
SUR1/Kir6.2	$1487 \pm 345$ (18)	$789 \pm 43$ (n = 6)*
SUR2A/Kir6.2	$83 \pm 8$ (15)	$58 \pm 8$ (6)*
SUR2B/Kir6.2	$89 \pm 10$ (9)	$49 \pm 5$ (6)*

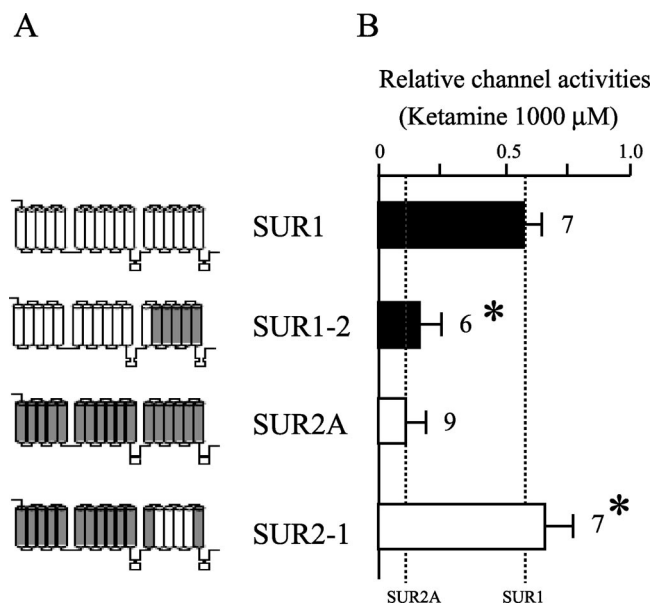
MgADP = magnesium adenosine diphosphate; SUR1/Kir6.2 = re-associated sulfonyleurea receptor 1 and inwardly rectifying potassium channel 6.2 channels; SUR2A/Kir6.2 = re-associated sulfonyleurea receptor 2A and inwardly rectifying potassium channel 6.2 channels; SUR2B/Kir6.2 = re-associated sulfonyleurea receptor 2B and inwardly rectifying potassium channel 6.2 channels.

$\text{IC}_{50}$  values are presented as means  $\pm$  SD (n).

\*  $P < 0.05$  against  $\text{IC}_{50}$  value in the absence of MgADP.

racemate was significantly more potent than S-(+)-ketamine in blocking all types of reassociated  $K_{\text{ATP}}$  channels. Because ketamine racemate is a mixture of S-(+) and R(-)-ketamine stereoisomers, this observation would suggest that block of  $K_{\text{ATP}}$  channels by ketamine enantiomers may be at least partly stereoselective.

$K_{\text{ATP}}$  channels are formed from pore-forming Kir6.0 and regulatory SUR subunits, arranged with 4:4 stoichio-



**Fig. 5.** Effects of ketamine racemate on sulfonyleurea receptor (SUR) chimeras. (A) Schematic representation of wild-type and chimeric SURs. Open regions indicate SUR1 and solid regions indicate SUR2A. (B) Effects of ketamine racemate ( $1 \text{ mM}$ ) on single-channel currents of reconstituted inwardly rectifying potassium channel (Kir) 6.2 with SUR1, SUR2A, chimera SUR1-2, and chimera SUR2-1. The dotted lines indicate the mean inhibition of SUR1/Kir6.2 and SUR2A/Kir6.2 currents. The numbers in brackets indicate the number of patches. For statistical analysis, the chimeras based on SUR1 (□) were compared with SUR1, and the chimeras based on SUR2A (■) were compared with SUR2A. \* $P < 0.05$ .

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metries.<sup>3</sup> The SUR subunit is encoded by two different genes, SUR1 and SUR2; SUR1 serves as the regulatory subunits of the pancreatic  $\beta$ -cell  $K_{ATP}$  channel, and splice variants of SUR2 act as the cardiac (SUR2A) and smooth muscle (SUR2B) SURs.<sup>3</sup>  $K_{ATP}$  channel activators and inhibitors show variable tissue specificity, the different types of  $K_{ATP}$  channel exhibit differential ATP sensitivity and pharmacologic properties, which are endowed by their different molecular composition of Kir6 and SUR subunits.<sup>20</sup> In the current study, the effects of ketamine racemate and S-(+)-ketamine were influenced by the type of SUR subunits, indicating that these anesthetics may interact with the SUR2 rather than the SUR1 subunits of the channels (table 1, figs. 1 and 2). In addition, the results obtained in the current study showing that both ketamine racemate and S-(+)-ketamine at concentrations up to 1 mM had no significant effects on the current generated by expressing Kir6.2 $\Delta$ C36 in the absence of SUR (fig. 3) suggest that the inhibitory effects of these drugs on  $K_{ATP}$  channel activities are not mediated through Kir6.0 subunits. That is, it is unlikely that binding to SUR1 or Kir6.0 subunits contributes significantly to the inhibitory effect of these anesthetics. Furthermore, the inhibitory effects of ketamine racemate on SUR2/Kir6.0 channels were significantly larger than those of S-(+)-ketamine (table 1, figs. 1 and 2). It is, therefore, suggested that specific binding to SUR2A and SUR2B subunits may be the major mechanism underlying the tissue-specific and stereoselective inhibitory effects of ketamine.

The specificity of ketamine for  $K_{ATP}$  channels containing SUR2 isoforms enabled us to use a chimeric approach to identify regions of SURs important for activity of the drug. Chimeric sulfonylurea receptors were constructed in which isolated domains were swapped between SUR1 and SUR2 to identify regions of SUR2 that are required for the high affinity action of ketamine. In this way, we showed that high-affinity inhibition by ketamine racemate could be introduced into SUR1 by transferring parts of TMD2 (TMs 13–17) from SUR2A to SUR1 (fig. 5) (chimera SUR1-2). Furthermore, the reverse chimera, in which parts of TMD2 (TMs 13–16) were swapped from SUR1 to SUR2A (fig. 5) (chimera SUR2-1), abolished high-affinity ketamine racemate inhibition in SUR2. These results suggest that this region within TMD2 of SUR2A is essential for high-affinity inhibition by ketamine racemate.

It has been reported that clinical plasma concentrations for ketamine racemate are 20–50  $\mu\text{M}$ <sup>27</sup> or 3–60  $\mu\text{M}$ ,<sup>8</sup> and the percentage of ketamine bound to plasma protein are 12%<sup>27</sup> or 45–50%,<sup>8</sup> suggesting that plasma concentrations of free ketamine racemate are 2.4–6  $\mu\text{M}$  or 1.4–30  $\mu\text{M}$ , respectively, during surgical anesthesia. The threshold concentrations at which ketamine racemate inhibits reassociated  $K_{ATP}$  channels containing

SUR2 are close to this range (>10–100  $\mu\text{M}$ ) (fig. 2, A–C), but this is not the case for SUR1-containing channels (>10 mM) (fig. 2D). It is possible, therefore, that inhibition of sarcolemmal  $K_{ATP}$  channels containing SUR2 (cardiovascular type) by ketamine might have adverse consequences in clinical practice. Recently, physiologic studies on mice lacking different  $K_{ATP}$  channel subunits have begun to clarify the roles of cardiac and vascular  $K_{ATP}$  channels in cardiovascular pathophysiology. Cardiac  $K_{ATP}$  channel (Kir6.2<sup>-/-</sup>)-deficient mice had a number of cardiac abnormalities during myocardial ischemia or severe stress, include impaired ischemic preconditioning and attenuated electrocardiographic ST changes.<sup>28,29</sup> Impaired vascular smooth muscle function was a feature of Kir6.1-deficient and SUR2-deficient mice and manifested as episodic coronary artery vasospasm and a high rate of sudden death.<sup>6,30</sup> Increased systolic and diastolic blood pressure was also observed in SUR2-deficient mice.<sup>30</sup> Indeed, racemic ketamine, but not the S-(+)-ketamine stereoisomer, was found to block early and late preconditioning in rabbit hearts and inhibit vasorelaxation induced by a  $K_{ATP}$  channel opener.<sup>13–16</sup> In heart, however, mitochondrial rather than sarcolemmal  $K_{ATP}$  channels might play an important role in ischemic and anesthetic preconditioning. Because the molecular identity of the channel has not been established, molecular biologic approaches such as reassociation of cloned channels or gene targeting technique are not yet applicable for the study of mitochondria  $K_{ATP}$  channel function. Recently, Zaugg *et al.*<sup>31</sup> reported that 10  $\mu\text{M}$  R(-)-ketamine, but not S-(+)-ketamine, inhibited diazoxide-induced flavoprotein oxidation of rat myocytes, an index of mitochondrial  $K_{ATP}$  channel activation. Thus, these observations may point to the similarity of ketamine's inhibitory effects on sarcolemmal and mitochondrial  $K_{ATP}$  channels.

It is well established that MgADP simultaneously activates the  $K_{ATP}$  channels strongly *via* SUR and inhibits them weakly *via* the ATP binding site on Kir6.2.<sup>23,24</sup> In addition, recent studies demonstrated that intracellular MgADP could modulate the sensitivity of  $K_{ATP}$  channel activators and inhibitors.<sup>18,19</sup> The current study indicated that ketamine racemate inhibits  $K_{ATP}$  channels *via* SUR subunits and that a physiologic concentration of MgADP (100  $\mu\text{M}$ ) significantly enhanced the inhibitory effects of ketamine racemate on all three types of SUR1/Kir6.2, SUR2A/Kir6.2, and SUR2B/Kir6.2 channels (table 2, fig. 4B). It is very difficult to explain the precise mechanisms of the interaction between MgADP and ketamine racemate, it might be possible to speculate that ketamine racemate attenuates (or abolishes) the MgADP-induced activation mediated by the high-affinity site on SUR, thereby exposing the inhibitory effect of MgADP on Kir6.2 such that the overall inhibitory potency is enhanced. Therefore, it might be possible to estimate that ketamine racemate may inhibit  $K_{ATP}$  channels con-



taining SUR2 when the channels are opened *in vivo* by increased ADP concentrations, such as those occurring during ischemia.

Our study has several limitations. First, although we used the same amount of SUR cDNA and Kir cDNA for transfection, the genomic integration of the various constructs may have been different, and a varying ratio of SUR *versus* Kir may affect electrophysiological findings. Therefore, it might be better for us to establish the level of expression as well as the ratio of SUR *versus* Kir subunits by polymerase chain reaction method and Western blot analyses. However, in our previous study, we confirmed that the sensitivity to ATP, diazoxide, and glibenclamide and the single-channel conductance of all kinds of reassociated  $K_{ATP}$  channels were similar to those of native  $K_{ATP}$  channels.<sup>17</sup> Therefore, we expect that the reassociated  $K_{ATP}$  channels in the current study can be used as experimental models to characterize the function of the native  $K_{ATP}$  channels and that we can draw conclusions from our experimental model. Second, as we discussed above, the threshold concentrations at which ketamine racemate inhibited reassociated  $K_{ATP}$  channels containing SUR2 (10–100  $\mu\text{M}$ ) are close to free plasma concentrations for ketamine (1.4–30  $\mu\text{M}$ ). In contrast, considering that intracellular concentrations of ketamine racemate should be much lower than this range, the concentrations of ketamine racemate we used in the current study should be much higher than the clinically relevant concentrations, suggesting the inhibitory effects of ketamine racemate on sarcolemmal  $K_{ATP}$  channels were observed at very high concentrations in the inside-out patch clamp configurations. Third, although we studied the effects of ketamine racemate on sarcolemmal  $K_{ATP}$  channels using the inside-out patch clamp configurations, there is a possibility that ketamine racemate acts on these channels from outside of the membrane. To confirm this possibility, it is necessary to study the effects of ketamine racemate using outside-out patch clamp configurations. Fourth, although we studied direct effects of ketamine racemate on sarcolemmal  $K_{ATP}$  channel activities, it should be noted that ketamine could affect  $K_{ATP}$  channel activities through alteration in nitric oxide concentrations, for example.<sup>32</sup>

In conclusion, ketamine-induced inhibition of sarcolemmal  $K_{ATP}$  channels is mediated by SUR subunits. These inhibitory effects of ketamine exhibit a high degree of specificity for cardiovascular  $K_{ATP}$  channels, the most critical binding site for ketamine being in the C-terminal set of TMs of SUR2A, and they exhibit at least some degree of stereoselectivity; ketamine racemate was more potent than S-(+)-ketamine. Our results further suggest that MgADP enhances the inhibitory effects of ketamine racemate on sarcolemmal  $K_{ATP}$  channel activity under conditions of metabolic inhibition, for example, during ischemia.

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