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A Transmembrane Domain of the Sulfonylurea Receptor Mediates Activation of ATP-Sensitive K^+ Channels by K^+ Channel Openers

NATHALIE D'HAHAN, HÉLÈNE JACQUET, CHRISTOPHE MOREAU, PATRICE CATTY, and MICHEL VIVAUDOU

Laboratoire de Biophysique Moleculaire et Celluraire, Département de Biologie Moléculaire et Structurale, Commissariat à l'Energie Atomique, Grenoble, France

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ABSTRACT

ATP-sensitive K^+ (K_{ATP}) channels are a complex of an ATP-binding cassette transporter, the sulfonylurea receptor (SUR), and an inward rectifier K^+ channel subunit, Kir6.2. The diverse pharmacological responsiveness of K_{ATP} channels from various tissues are thought to arise from distinct SUR isoforms. Thus, when assembled with Kir6.2, the pancreatic β cell isoform SUR1 is activated by the hyperglycemic drug diazoxide but not by hypotensive drugs like cromakalim, whereas the cardiac muscle isoform SUR2A is activated by cromakalim and not by diazoxide. We exploited these differences between SUR1 and SUR2A to pursue a chimeric approach designed to identify the structural determinants of SUR involved in the pharmacological activation of K_{ATP} channels. Wild-type and chimeric SUR were coexpressed with Kir6.2 in *Xenopus* oocytes, and we studied the resulting channels with the patch-clamp technique in the excised inside-out configuration. The third transmembrane domain of SUR is found to be an important determinant of the response to cromakalim, which possibly harbors at least part of its binding site. Contrary to expectations, diazoxide sensitivity could not be linked specifically to the carboxyl-terminal end (nucleotide-binding domain 2) of SUR but appeared to involve complex allosteric interactions between transmembrane and nucleotide-binding domains. In addition to providing direct evidence for the structure-function relationship governing K_{ATP} channel activation by potassium channel-opening drugs, a family of drugs of the highest therapeutic interest, these findings delineate the determinants of ligand specificity within the modular ATP-binding cassette-transporter architecture of SUR.

ATP-sensitive K^+ (K_{ATP}) channels are present in the plasma membrane of muscle cells, neurons, and most other excitable cells, where they serve to adjust the resting membrane potential to the metabolic state of the cell (Ashcroft and Ashcroft, 1990; Isomoto and Kurachi, 1997). These channels are the targets of a number of drugs that can block them, like the sulfonylureas, or open them, like cromakalim, pinacidil, or diazoxide (Gopalakrishnan et al., 1993). These drugs are of great therapeutic interest because they provide a way to pharmacologically adjust the excitability of cells, raising it with blockers and lowering it with openers. Widespread use of potassium channel openers (KCOs) is impaired, however, by their poor tissue specificity and somewhat low affinity (Lawson, 1996). In the search for better drugs, specially in terms of specificity, it appears necessary to obtain a better understanding of the mechanisms of action of these pharmacological agents.

The K_{ATP} channel is a complex of two proteins: the sulfonylurea receptor (SUR; Aguilar-Bryan et al., 1995), which is a member of the ATP-binding cassette (ABC) transporter family, and a smaller protein, Kir6.2 (Inagaki et al., 1995), which belongs to the inward rectifier K^+ channel family. Four Kir6.2 subunits assemble to form a K^+-selective pore that is rendered functional by 4 auxiliary SUR subunits (Clement et al., 1997; Inagaki et al., 1997; Shyng and Nichols, 1997) through direct physical association (Lorenz et al., 1998).

Several isoforms of SUR have been identified. When the isoforms SUR1, SUR2A, or SUR2B are coexpressed with Kir6.2, channels are reconstituted that resemble native K_{ATP} channels from pancreatic β cells, cardiac muscle, and smooth muscle (Inagaki et al., 1995, 1996; Isomoto et al., 1996). The variable tissue-specific properties, particularly the response to openers, of K_{ATP} channels would then arise from the identity of the SUR

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ABBREVIATIONS: SUR, sulfonylurea receptor; KCO, potassium channel opener; K_{ATP} channel, ATP-sensitive potassium channel; TMD, transmembrane domain; NBD, nucleotide-binding domain; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; ABC, ATP-binding cassette.

isoform expressed in that tissue. This observation, as well as more direct functional (Tucker et al., 1997) and biochemical (Schwanstecher et al., 1998) evidence, designates SUR as the primary target of KCOs. Presently, scarce structural data are available on the underlying mechanism of action of KCOs. It is well established that the nucleotide-binding domains of SUR are tightly linked to the sites of action of KCOs because the binding and effect of KCOs require hydrolyzable nucleotides and are compromised by mutations that, in other ABC transporters, impair the ability of these domains to bind and hydrolyze nucleotides (Gribble et al., 1997; Shyng et al., 1997; Schwanstecher et al., 1998). It has also been suggested that the carboxyl terminus of SUR could play an important role in this mechanism because SUR2A and SUR2B differ only in their last 42 amino acids but give rise to channels with very distinct apparent KCO affinity (Isomoto et al., 1996; Schwanstecher et al., 1998).

As an ABC transporter (Croop, 1998), SUR is organized as a modular protein with clearly identifiable transmembrane domains and cytoplasmic nucleotide-binding domains (Aguilar-Bryan et al., 1998). Taking advantage of the differential sensitivity to the openers diazoxide and cromakalim of two SUR isoforms, the pancreatic isoform SUR1 (diazoxide-sensitive, cromakalim-insensitive) and the cardiac isoform SUR2A (diazoxide-insensitive, cromakalim-sensitive), we have followed a chimeric approach to examine whether specific domains of SUR are involved in the activation of K_{ATP} channels by these KCOs.

Materials and Methods

Molecular Biology. Mouse Kir6.2 (GenBank accession no. D50581; Inagaki et al., 1995), hamster SUR1 (GenBank accession no. L40623; Aguilar-Bryan et al., 1995), and rat SUR2A (GenBank accession no. D83598; Inagaki et al., 1996) were subcloned in the *Xenopus* oocyte expression vector pGEMHE (Liman et al., 1992) or its modified versions with enhanced polylinkers, pGH2 and pGH3 (kindly provided by Dr. F. Pagès, Grenoble). Three nonsilent differences were found between the hamster SUR1 cDNA sequence and the published sequence: $G^{3601}C$, $T^{3751}C$, and $A^{4099}G$, which produce the amino acid changes $V^{1201}L$, $C^{1251}R$, and $T^{1367}A$. Because these residues are also Leu, Arg, and Ala in all other rat, mouse, and human SUR1 and SUR2 isoforms, it is likely that these differences were due to errors in the original sequence.

Chimeric cDNA constructs were produced by the splicing by overlap extension polymerase chain reaction (PCR) technique (Horton et al., 1989). Hamster SUR1 and rat SUR2A were used as templates for making all of the chimeras listed in Fig. 4. Successive PCRs with Vent DNA polymerase (New England Biolabs, Beverly, MA) were performed for 25 cycles to produce the junctional cDNA fragments, which had a length of 2 kilobase pairs or less. These fragments were ligated to the flanking 5′ and 3′ remaining sequence elements to produce the final constructs. Plasmid DNAs were amplified, confirmed by restriction analysis and by sequencing of the PCR-derived region, linearized, and transcribed in vitro with the T7 mMessage mMachine kit (Ambion, Austin, TX). cRNAs were electrophoresed on formaldehyde gels, and concentrations were estimated from two dilutions with RNA marker as a standard.

Electrophysiology. *Xenopus laevis* were anesthetized with 3-aminobenzoic acid ethyl ester (1 g/liter water). Part of one ovary was removed, the incision was sutured, and the animal was allowed to recover. Stage V or VI oocytes were defoliculated by an ~60-min incubation at 19°C with 2 mg/ml type A collagenase (Sigma Chemical Co., St. Louis, MO). Selected oocytes were injected the next day with 50 nl of water containing ~2 ng of Kir6.2 cRNA and with ~6 ng of cRNA encoding wild-type or chimeric SURs. They were stored at 19°C in a modified Barth's solution with 1 mM KCl, 0.82 mM $MgSO_4$, 88 mM NaCl, 2.4 mM $NaHCO_3$, 0.41 mM $CaCl_2$, 0.3 mM $Ca(NO_3)_2$, and 16 mM HEPES (pH 7.4) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml gentamycin.

Two to 15 days after injection, oocytes were devitellinized, and exogenous K_{ATP} channels were characterized by the patch-clamp technique in the excised inside-out configuration (Hamill et al., 1981). Experimental methods were similar to those used in our laboratory to record native frog skeletal muscle K_{ATP} channels (Vivaudou and Forestier, 1995; Forestier et al., 1996).

Conditions were designed to optimize recording of K_{ATP} currents and to minimize contributions by endogenous oocyte Cl^- currents. Patch pipettes (2–10 MΩ) contained 154 mM K^+, 146 mM Cl^-, 5 mM Mg^{2+}, and 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 7.1). The cytoplasmic face of the patch was bathed in solutions, all of which contained 174 mM K^+, 40 mM Cl^-, 1 mM EGTA, 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 7.1), and methanesulfonate$^-$ as the remaining anions. When present, Mg^{2+} was 1 mM. ATP (potassium salt; Sigma Chemical Co.), diazoxide [100 mM stock in dimethyl sulfoxide (DMSO); Sigma Chemical Co.], and SR47063 (20 mM stock in DMSO; Sanofi Recherche, Montpellier, France) were added as specified. The concentration of contaminant Mg^{2+} in nominally Mg^{2+}-free solutions was less than 10 μM (Forestier and Vivaudou, 1993). The membrane potential was maintained at −50 mV. Experiments were conducted at room temperature (22–24°C).

Applications of the various solutions to the intracellular face of the patch were performed with a RSC-100 rapid solution changer (Bio-Logic, Claix, France) controlled by in-house software Perf 2.10. Analog signals were filtered at 300 Hz and sampled at 1 kHz. Slow fluctuations of the no-channel-open baseline of the signal were removed by interactive fitting of the baseline with a spline curve and subtraction of this fit from the signal. Acquisition, analysis, and presentation were performed with in-house software Erwin 3.2. ATP dose-response curves were obtained and processed as in Vivaudou and Forestier (1995). Results are displayed as mean ± S.E.M.

Results

Expression of SUR1 and SUR2A/Kir6.2 K_{ATP} Channels in Oocytes. Oocytes coinjected with equimolar quantities of mRNAs encoding Kir6.2 (2 ng) and either SUR1 or SUR2A (6 ng) were found to express a high density of K_{ATP} channels within 2 days. In excised patches with small patch pipettes of ~5-MΩ resistance, macroscopic ATP-inhibitable currents of 100 pA or more at a membrane potential of −50 mV with 150 mM intracellular and extracellular K^+ could be recorded routinely.

SUR1/Kir6.2 and SUR2A/Kir6.2 channels displayed weak inward rectification, had inward current conductances of ~70 picosiemens, were completely blocked by millimolar concentrations of internal ATP, and were inhibited by micromolar doses of glibenclamide (not shown). In our conditions, they displayed remarkably similar sensitivity to block by ATP (Fig. 1). The concentration of ATP causing 50% current inhibition ($K_{1/2}$) was on average 16 μM in both cases, although it could vary between 2 and 200 μM from patch to patch, as observed with native channels (Findlay and Faivre, 1991; Vivaudou and Forestier, 1995).

Pharmacological Properties of Wild-Type K_{ATP} Channels. SUR1/Kir6.2 and SUR2A/Kir6.2 channels were clearly distinguishable on the basis of their pharmacological profiles. In agreement with previous reports (Inagaki et al., 1995, 1996), SUR1/Kir6.2 channels were activated by diazox-

ide but not by SR47063, a cromakalim analog, whereas the opposite was true for SUR2A/Kir6.2 channels (Fig. 2).

Application of 300 μM diazoxide caused a rapidly reversible increase in the SUR1/Kir6.2 currents recorded in 100 μM ATP and 1 mM Mg^{2+}. The magnitude of this effect was variable from patch to patch and decreased during the course of an experiment, as observed with native pancreatic K_{ATP} channels (Kozlowski and Ashford, 1992; Larsson et al., 1993). Taking into account only the result of the first application in every patch (usually performed in the first 5 min after patch excision), diazoxide caused on average a more than 5-fold increase in current.

In oocytes expressing SUR2A and Kir6.2, diazoxide caused a much smaller, 56 ± 15% (n = 17) increase in current, but this could be attributed to the drug vehicle, DMSO, which alone increased current by 48 ± 12% (n = 5).

In contrast, SR47063 (100 μM) consistently activated SUR2A/Kir6.2 channels, causing on average a 19-fold increase in current, but had no significant effect on SUR1/Kir6.2 channels because it produced the same increase (12 ± 10%; n = 17) as its vehicle alone (13 ± 5%; n = 4). Higher concentrations of this hydrophobic drug were not tested because they produced cloudy solutions, indicative of solubility problems.

Magnesium Dependence of Opener Effects. In an attempt to clarify the role of nucleotide hydrolysis on opener action, experiments were conducted on wild-type channels to determine whether the presence of Mg^{2+} affected activation by diazoxide or SR47063. In the presence of 100 μM ATP, channel activity was higher when Mg^{2+} was present, which is consistent with recent data on the activatory role of

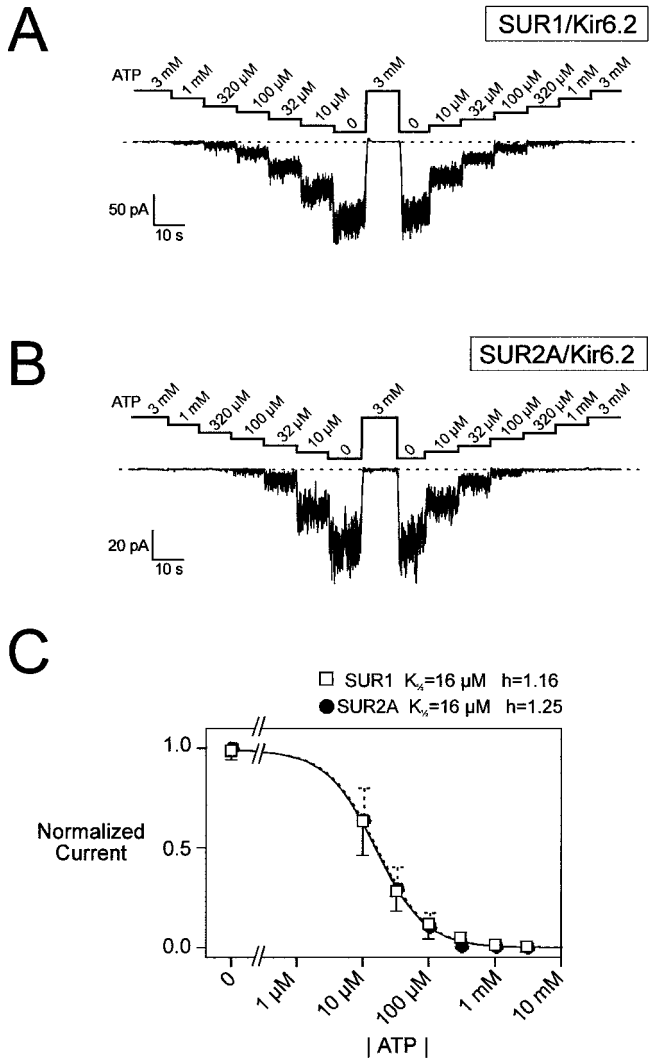

Fig. 1. Dose-dependent inhibition by ATP of SUR1/Kir6.2 and SUR2A/Kir6.2 K_{ATP} channels. A, representative current trace recorded at −50 mV with 1 mM Mg^{2+} from an inside-out patch excised from an oocyte coinjected with Kir6.2 and SUR1. B, Idem with SUR2A, patch 861502B. C, plots of activity of SUR1/Kir6.2 (□) and SUR2A/Kir6.2 (●) channels versus ATP concentration. Each symbol and bar represent the average and S.E.M. of measurements obtained in 12 SUR1 and 9 SUR2A patches using the protocols of A and B. Lines are best fits of the data points to the Hill equation with values of $K_{1/2}$ and h of 15.8 μM and 1.16 (SUR1) and 15.9 μM and 1.24 (SUR2A).

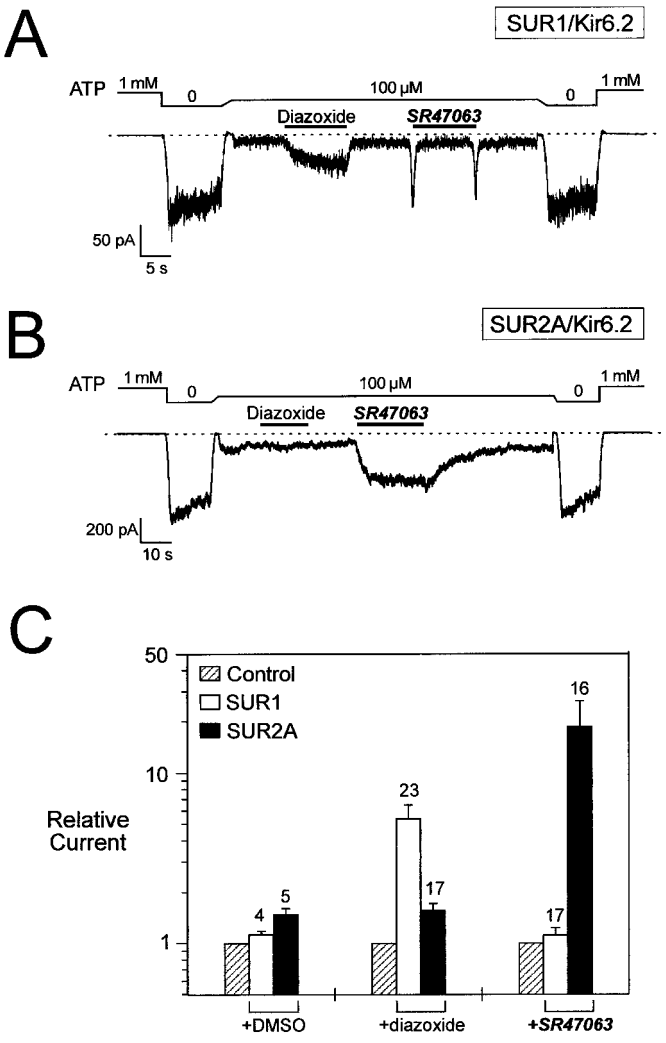

Fig. 2. Differential response to SR47063 and diazoxide of SUR1/Kir6.2 and SUR2A/Kir6.2 K_{ATP} channels. A, representative current trace recorded at −50 mV from an inside-out patch excised from an oocyte coinjected with Kir6.2 and SUR1. Transient currents on drug application and removal in this and other figures are artifacts of the solution-switching procedure due to brief exposure of the patch to ATP-free solution. B, Idem with SUR2A. C, normalized amplitude of currents measured before (Control) and during application of diazoxide, SR47063, or vehicle only (DMSO). Numbers above bars indicate the number of patches tested. Responses were recorded in the same manner as in A and B in the presence of 100 μM ATP and 1 mM Mg^{2+} using 300 μM diazoxide, 100 μM SR47063, and 0.3% DMSO.

MgATP (Gribble et al., 1998a). Figure 3 shows that diazoxide becomes ineffective when Mg^{2+} is removed, an observation already reported for native (e.g., Dunne et al., 1987) and recombinant (Shyng et al., 1997) channels. This was not the case for SR47063, which retained part of its activatory potential in the nominal absence of Mg^{2+}, although its effect was much weaker than that with Mg^{2+} and rapidly reversed on washout of the drug (Fig. 2). To verify that this effect was not a residual effect due to contaminant Mg^{2+}, the experiments represented in Fig. 3B were repeated in the added presence of 5 mM EDTA. Under those conditions, contaminant Mg^{2+} estimated at 8 μM (Forestier and Vivaudou, 1993) would be mostly chelated by EDTA, leaving ~7 nM free Mg^{2+} and ~5 nM MgATP. Still, SR47063 augmented SUR2A/Kir6.2 currents by ~3-fold (2.87 ± 0.61; n = 5), whereas its vehicle alone, 0.5% DMSO, had a negligible effect in those experiments (test/control = 0.92 ± 0.06; n = 4; data not shown).

Design and Expression of Chimeric SUR Receptors. The high sequence homology between SUR1 and SUR2A (67% identity) suggests that they have very similar structures. The clear dichotomy in terms of pharmacological properties of these two highly homologous proteins suggested that a chimeric approach could constitute a viable strategy to pinpoint the regions of SUR responsible for its pharmacological phenotype.

On the basis of hydrophobicity profiles and comparison with other ABC transporters (Tusnády et al., 1997), SUR may be divided in five domains, as shown in Fig. 4: three transmembrane domains (TMDs; TMD0, TMD1, and TMD2) and two cytoplasmic nucleotide-binding domains (NBD1 and NBD2). These last two domains, which incorporate Walker A and Walker B motifs capable of forming an ATP-binding pocket (Walker et al., 1982), are the most conserved regions of ABC transporters and the most homologous regions of SUR1 and SUR2A. In some ABC transporters, distinct pro-

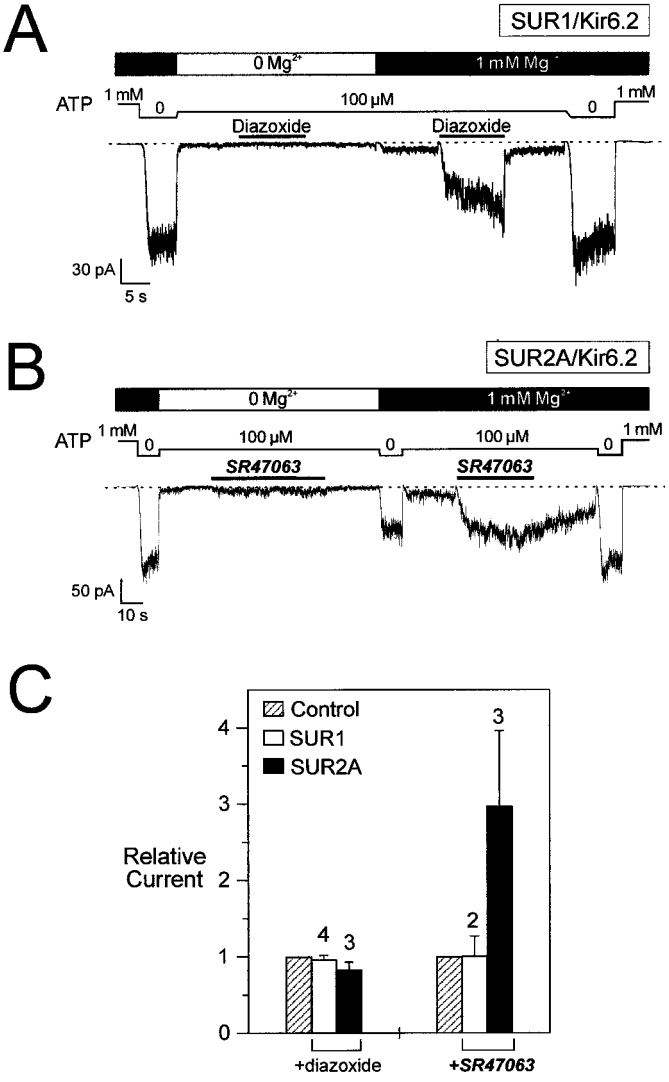

Fig. 3. Magnesium dependence of activation by SR47063 and diazoxide of SUR1/Kir6.2 and SUR2A/Kir6.2 K_{ATP} channels. A, responses to diazoxide (300 μM) before and after the addition of 1 mM Mg^{2+} of currents recorded at −50 mV from an inside-out patch excised from an oocyte coinjected with Kir6.2 and SUR1. B, responses to SR47063 (100 μM) of SUR2A/kir6.2 channels. C, normalized amplitude of currents measured in the absence of Mg^{2+} before (Control) and during application of diazoxide or SR47063. Numbers above bars indicate the number of patches tested. Responses were recorded as in A and B in the presence of 100 μM ATP with no added Mg^{2+}.

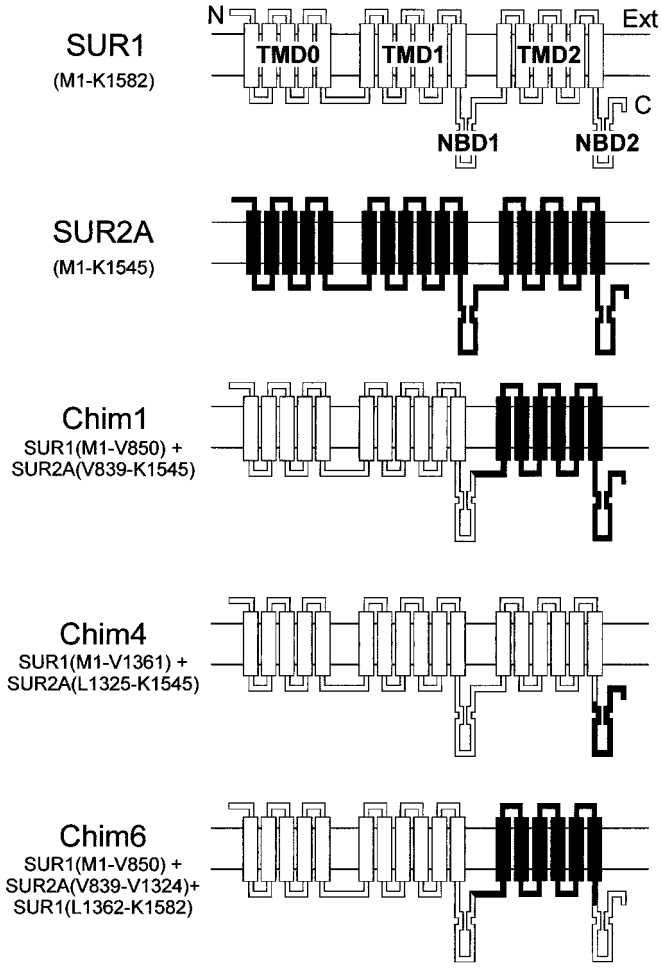

Fig. 4. Nomenclature and topology of chimeric constructs. SUR is represented as a modular transmembrane protein with an extracellular amino-terminal; an intracellular carboxyl-terminal; three transmembrane domains, TMD0, TMD1, and TMD2; and two cytoplasmic nucleotide binding domains, NBD1 and NBD2. Rectangular boxes indicate postulated transmembrane helices (Tusnady et al., 1997). SUR1 and SUR2A sequence elements are drawn in white and black, respectively. The exact amino acid composition of each construct is specified at the left.

teins form each of these domains (Croop, 1998), suggesting that each domain is a structurally and functionally independent module. Therefore, we designed chimeras along the logic of this modular architecture.

The three chimeras presented in this report are arbitrarily named Chim1, Chim4, and Chim6. Chim1 merges in roughly the first half of SUR1 and second half of SUR2A, that is, domains TMD0, TMD1, and NBD1 of SUR1 and TMD2 and NBD2 of SUR2A. Chim4 incorporates domains TMD0, TMD1, TMD2, and NBD1 of SUR1 and NBD2 of SUR2A. Chim6 incorporates domains TMD0, TMD1, NBD1, and NBD2 of SUR1 and TMD2 of SUR2A (Fig. 4).

Oocytes coinjected with cRNAs coding for each of these chimeras (6 ng) and Kir6.2 (2 ng) produced robust ATP-inhibited K^+-selective currents. The underlying chimeric channels had properties identical with those shared by the wild-type channels, including conductance, rectification, and blockade by glibenclamide (data not shown), confirming their structural integrity. Because the wild-type channels showed similar sensitivity to ATP inhibition, it did not come as a surprise that the ATP sensitivity of chimeric channels stayed within the same range as shown in Fig. 5. Chim1 and Chim4/Kir6.2 channels were half-blocked by 11 and 20 μM, respectively, values not significantly different from the 16 μM of SUR1 and SUR2A/Kir6.2 channels.

Effects of KCOs on Chimeric K_{ATP} Channels. The responses to diazoxide and SR47063 of the chimeric channels were tested. Results are illustrated in Fig. 6 and summarized in Fig. 7. Unlike either SUR1 or SUR2A, Chim1 associated with Kir6.2 was activated by both openers. Although a lack of response would have been difficult to interpret, this gain-of-function phenotype suggests that Chim1 acquired diazoxide sensitivity from its SUR1 regions (TMD0, TMD1, and NBD1) and SR47063 sensitivity from its SUR2A regions (TMD2 and NBD2). The response of this chimera to diazoxide was intermediate between that of SUR2A (mainly due to vehicle as discussed above) and that of SUR1, suggesting that domains in both halves of SUR participate in that response. In contrast, the robust response to SR47063 suggests a tight link between this opener and the second half of SUR (TMD2 + NBD2).

To identify precisely which domain was responsible for SR47063 activation, Chim4 and Chim6 were constructed. These are identical with SUR1 except for a single domain contributed by SUR2A: NBD2 for Chim4 and TMD2 for Chim6. Functionally, Chim4/Kir6.2 channels could barely be distinguished from SUR1/Kir6.2 channels. They were activated by diazoxide, although to a lesser extent, but not by SR47063. Chim6/Kir6.2 channels were sensitive to SR47063 like SUR2A/Kir6.2, but their response to diazoxide was significantly weaker than that of SUR1/Kir6.2 channels.

If these results demonstrate the essential role of TMD2 in the action of SR47063, they do not incriminate a single domain as crucial for diazoxide but rather suggest contributions from several domains in both halves of the protein.

Discussion

Identical ATP Sensitivity of SUR1 and SUR2A/Kir6.2 K_{ATP} Channels. In agreement with recently published data (Gribble et al., 1998b), we found that SUR1 and SUR2A/Kir6.2 channels had the same sensitivity to block by intracellular ATP. For both, the concentration of ATP for 50% inhibition ($K_{1/2}$) was 16 μM and the Hill coefficient was slightly above 1, suggesting at least two coupled nucleotide-binding sites. In agreement with this observation, all SUR1/SUR2A chimeras displayed $K_{1/2}$ values that were not significantly different.

For SUR2A/Kir6.2 channels, the $K_{1/2}$ value was below the values of 30 to 150 μM reported by others in transfected cells (Inagaki et al., 1996; Aguilar-Bryan et al., 1998; Okuyama et al., 1998). We do not know the reason for this discrepancy. Even in well-controlled conditions, ATP sensitivity is unstable (Findlay and Faivre, 1991) and could well change with the expression system where the presence and amount of certain channel-regulating factors, such as membrane phospholipids (Fan and Makielski, 1997), remain unpredictable.

Therefore, constituent SUR isoforms cannot be identified dependably on the responses to ATP of K_{ATP} channels. As far

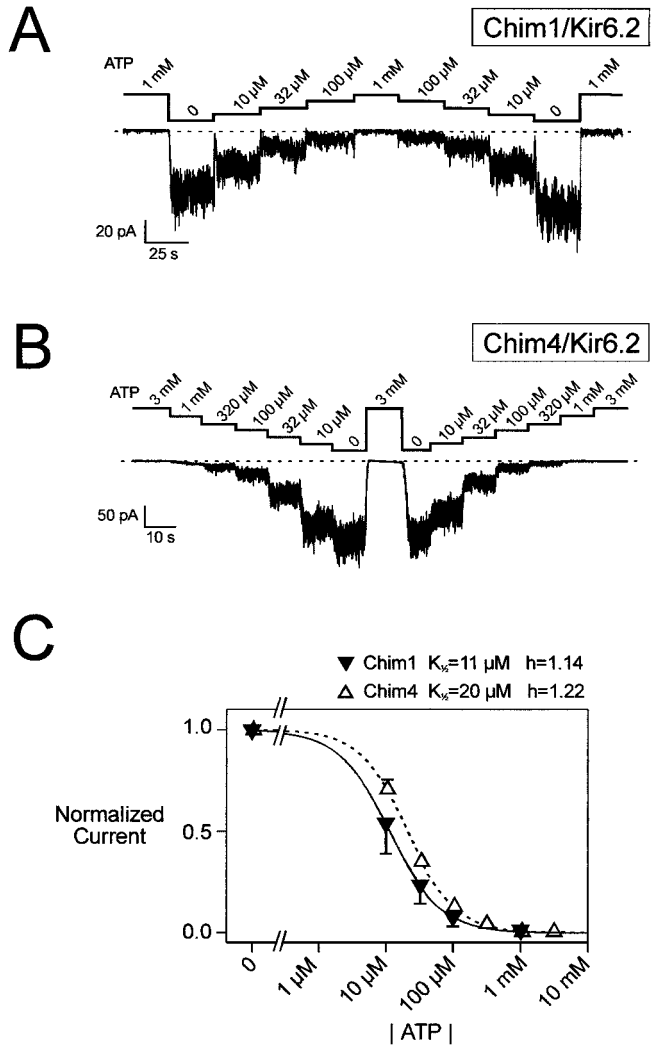

Fig. 5. Dose-dependent inhibition by ATP of chimeric K_{ATP} channels. A, representative current trace recorded at −50 mV with 1 mM Mg^{2+} from an inside-out patch excised from an oocyte coinjected with Kir6.2 and Chim1. B, Idem with Chim4. C, plots of activity of Chim1/Kir6.2 (▼) and Chim4/Kir6.2 (△) channels versus ATP concentration. Each symbol and bar represent the average and S.E.M. of 2 to 13 measurements obtained using the protocols of A and B. Lines are best fits of the data points to the Hill equation with values of $K_{1/2}$ and h of 11.4 μM and 1.14 (Chim1) and 20.0 μM and 1.22 (Chim4).

as we can tell, in *Xenopus* oocytes, SUR1/Kir6.2 and SUR2A/Kir6.2 K_{ATP} channels can be separated with confidence only on the basis of their pharmacological properties.

Essential Role of TMD2 Domain in SR47063 Action. The high sequence homology and clear pharmacological divergence of SUR1 and SUR2A make these proteins perfect candidates for a chimeric approach. The two SUR isoforms used, hamster SUR1 and rat SUR2A, indeed have very homologous primary sequences. Overall, 79% of their amino acids are conserved, with identical amino acids amounting to 67% of the total. Alignment between the two sequences is straightforward over most of their length and provides an ideal basis for the design of chimeric proteins. ABC transporters are organized in characteristic structural domains (Croop, 1998), and chimeras were constructed along the lines of this modular architecture to identify the respective role of domains TMD2 and NBD2.

The three chimeras presented were fully functional and retained all of the properties common to SUR1 and SUR2A, including ATP sensitivity. Their responses to the cromakalim analog SR47063 suggest that the transmembrane region TMD2 of SUR is an essential determinant of the sensitivity to SR47063. The strongest evidence is that SUR1 acquired the SR47063 sensitivity of SUR2A when its TMD2 region was replaced by that of SUR2A (chimera Chim6). This evidence is corroborated by the behavior of the other chimeras, which shows that SR47063 sensitivity is associated with the presence of the TMD2 region of SUR2A. Thus, the TMD2 region could contain a KCO-binding site or an essential part of it. Alternately, it could just provide a link between KCO binding and channel opening. This latter possibility, which would imply that KCOs can bind equally well to SUR1 and SUR2A, is in contradiction with the much smaller binding affinity for SUR1 than SUR2A as estimated by Schwanstecher et al. (1998).

The TMD2 region that makes up a little less than one third of the SUR receptor is the region least conserved between SUR1 and SUR2, with 60% identities compared with more than 80% for the NBD1 and NBD2 domains. It contains four highly hydrophobic stretches of amino acids compatible with at least four and possibly six transmembrane helices (Tusnády et al., 1997). A direct involvement of such a region in the binding of KCOs would be consistent with the hydrophobic nature of KCOs, which could reach their target via the lipid bilayer as has been postulated for the substrates of another ABC transporters, the P-glycoprotein (Sharom, 1997).

Our data indicate that in the absence of Mg^{2+}, SR47063 was still able to produce activation of SUR2A/kir6.2 channels. Although the subunits of the skeletal muscle K_{ATP} channels have not been formally identified, these data are in

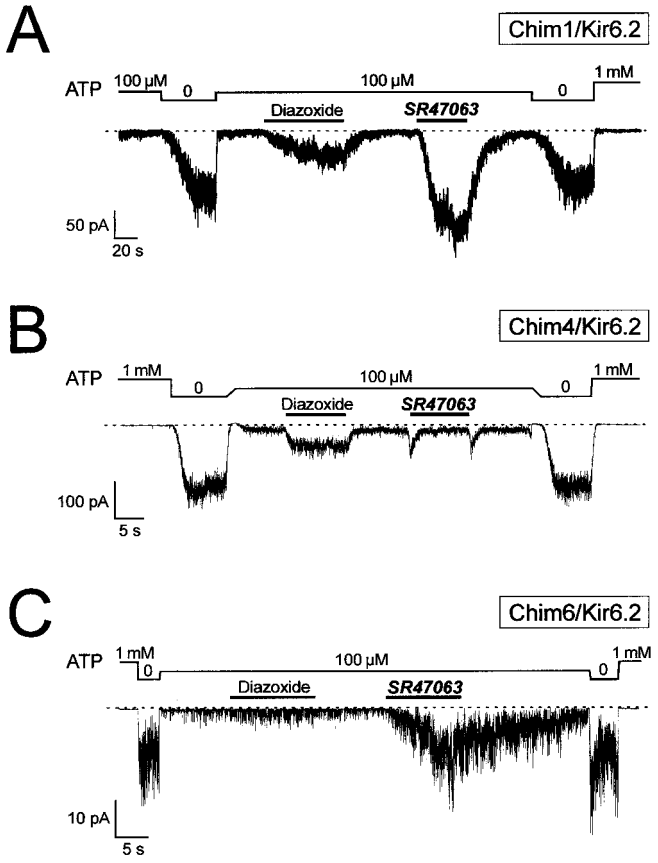

Fig. 6. Pharmacological regulation of chimeric K_{ATP} channels. A, responses to diazoxide (300 μM) and SR47063 (100 μM) of currents recorded at −50 mV from an inside-out patch excised from an oocyte coinjected with Kir6.2 and Chim1. B, Idem with Chim4. C, Idem with Chim6. The differences in the on and off rates of the responses shown in each panel do not reflect different properties of the chimeras but rather reflect the differences in ease of solution exchange arising from patch-to-patch variations in the diffusional distance between patch and pipette tip.

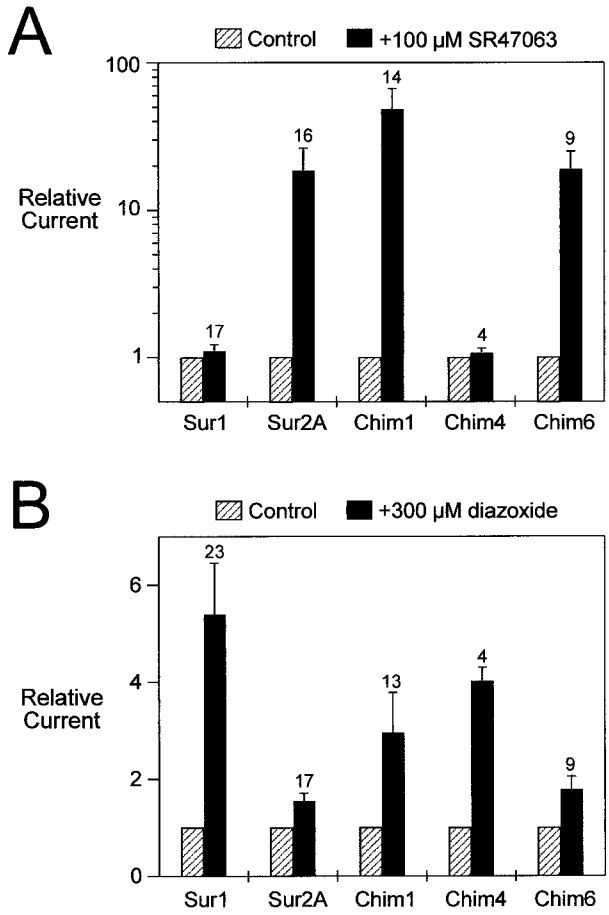

Fig. 7. Effects of SR47063 and diazoxide on wild-type and chimeric K_{ATP} channels. A, normalized amplitude of currents measured at −50 mV in the presence of 100 μM ATP and 1 mM Mg^{2+} before (Control) and during the application of 100 μM SR47063. Numbers above bars indicate the number of patches tested. B, Idem but with 300 μM diazoxide.

agreement with our previous observations on native K_{ATP} channels from skeletal muscle showing little effect of Mg^{2+} on KCO activation (Forestier et al., 1996) and suggesting that KCOs can bind in the absence of both Mg^{2+} and nucleotides (Forestier et al., 1993). Without Mg^{2+}, SUR2A/kir6.2 activation was weaker and reversed much more rapidly on washout of the drug. This could possibly explain why binding of a tritiated KCO to native (Dickinson et al., 1997) or recombinant (Schwanstecher et al., 1998) channels could only be measured when hydrolyzable nucleotides and Mg^{2+} were present. Thus, nucleotide binding and hydrolysis by the NBDs would serve to modulate interaction of KCOs with the TMD2 region.

Role of Carboxyl-Terminal End in Diazoxide Action. The role of the carboxyl-terminal extremity of SUR in diazoxide activation was anticipated in view of the functional differences between the two splice variants SUR2A and SUR2B, which, in the mouse, have only 28 nonidentical amino acids all within the last 42 amino acids. Because diazoxide activates SUR2B/Kir6.2 channels (Isomoto et al., 1996) but not SUR2A/Kir6.2 channels, elementary logic dictates that diazoxide sensitivity resides in the carboxyl-terminal end of SUR. Unfortunately, experimental evidence is at odds with this simple reasoning. The two chimeras Chim1 and Chim4 that have the carboxyl-terminal extremity of the diazoxide-insensitive SUR2A isoform were activated by diazoxide although not to the same degree as SUR1, and the chimera Chim6, which retained the SUR1 carboxyl terminus, was nearly insensitive to diazoxide. Therefore, it does appear that the carboxyl-terminal extremity is one among several determinants of diazoxide action. Indirect estimates of diazoxide binding affinity have shown that despite their functional differences, SUR1 and SUR2A have comparable affinities for diazoxide (Schwanstecher et al., 1998). The site of action of diazoxide would therefore be located within conserved sequence elements, and functional differences among isoforms would be due to variations in efficacy (i.e., in the structures linking channel opening to binding). The present observations do not permit an association of these structures to a specific domain but imply that they are distributed over the whole protein and that their functional role is governed by complex allosteric interactions. On the other hand, the nucleotide binding domains, the most conserved regions of the SUR isoforms, of which the integrity is indispensable for diazoxide activation (Gribble et al., 1997; Shyng et al., 1997), are more likely to be directly involved with the binding than with the efficacy of the opener.

Conclusions. Despite the therapeutic potential of KCOs, the molecular details of their mechanisms of action remain mysterious. We have demonstrated here by a chimeric approach that distinct structures of SUR mediate K_{ATP} channel activation by the openers diazoxide and cromakalim. If a single domain, the last transmembrane spanning region of SUR, was found to be fundamental for cromakalim action, functional determinants of diazoxide action appeared distributed over several domains, and not only over the carboxyl-terminal extremity as previously postulated (Isomoto et al., 1996). This constitutes a first step toward the precise determination of KCO interaction sites that should lead to a better understanding of the structure and mechanisms of SUR and other ABC transporters.

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Send reprint requests to: Dr. Michel Vivaudou, Commissariat à l'Energie Atomique, Département de Biologie Moléculaire et Structurale, Biophysique Moleculaire et Celluraire, 17 rue des Martyrs, 38054, Grenoble, France. E-mail: vivaudou@cea.fr