The function of the ATP-sensitive potassium (K\textsubscript{ATP}) channel relies on the proper coupling between its two subunits: the pore-forming Kir6.2 and the regulator SUR. The conformation of the interface between these two subunits can be monitored using a rhodamine 123 (Rho) protection assay because Rho blocks the intersubunit interface. The activating mutation F132L in SUR1, which causes neonatal diabetes, also rendered the channel resistant to Rho block, suggesting that it stabilized an activated conformation by uncoupling TMD0 from the rest of SUR1. At a nearby residue, the SUR1 mutation E128K impairs trafficking, thereby reducing surface expression and causing hyperinsulinism. To augment channel density at the plasma membrane to investigate the effect of mutating this residue on channel function, we introduced the milder mutation E126A at the matching residue of SUR2A. Mutation E126A induced a Rho-hypersensitive phenotype indicative of a functional uncoupling between SUR1 and Kir6.2. These results suggest that the TMD0-Kir6.2 interface is mobile and that the gating modes of Kir6.2 correlate with distinct positions of TMD0. They further demonstrate that the second intracellular loop of SUR, which contains the two residues studied here, is a key structural element of the TMD0-Kir6.2 interface.

The ATP-sensitive K\textsuperscript{+} (K\textsubscript{ATP})\textsuperscript{4} channel is a complex of two proteins: the inward rectifying potassium channel Kir6 and the sulfonylurea receptor SUR. Four Kir6s form a potassium-selective pore that is inhibited by intracellular ATP. This pore is surrounded by four SURs that allosterically relieve ATP inhibition of Kir6 in response to intracellular MgADP (2, 3). The balance of these two effects causes the channel to open when ATP consumption exceeds supply and to close when cellular energy reserves are replenished. This property allows the K\textsubscript{ATP} channel to function as a metabolic sensor that transduces cellular energy variations into bioelectrical signals. In pancreatic \(\beta\)-cells, the K\textsubscript{ATP} channel is a pivotal element in the cascade that links insulin secretion to glucose concentration. When glycaemia rises, metabolism causes a rise in ATP and a decrease in ADP. This tends to close the channel, causing membrane depolarization, calcium entry, and exocytosis of insulin granules (4).

Because of this pivotal role, the K\textsubscript{ATP} channel is a prime target for pharmacological intervention to correct insulin secretion dysfunction, and there exist a number of molecules, several in clinical use, that are able to block or activate the channel by binding to SUR. The K\textsubscript{ATP} channel is also the source of diseases when genetic mutations alter its proper function. For the pancreatic channel made of the isoforms SUR1 and Kir6.2, mutations in either subunit have been identified that disable the channel, causing hyperinsulinism, or that hyperactivate the channel, causing diabetes (5).

SUR belongs to the ATP-binding cassette protein family. Like other ATP-binding cassette proteins, it is composed of two transmembrane domains: TMD1 and TMD2, and two cytoplasmic nucleotide-binding domains: NBD1 and NBD2 (1). Essential to its function as a channel regulator is the supplementary N-terminal transmembrane domain TMD0 predicted to contain five transmembrane helices (supplemental Fig. S1). TMD0 tightly associates with Kir6.2 and modulates its gating (6, 7). Other domains of SUR also interact with Kir6.2: TMD2-NBD2, which co-immunoprecipitates with Kir6.2 (7), and a cytoplasmic region at the TMD2-NBD2 junction (8), which we identified as essential in SUR-mediated activation of Kir6.2 (9). On the Kir6.2 side of the interaction, it is hard to identify a specific region of association because biochemical evidence is lacking. The outer helix TM1 is a potential region of physical interaction (10), whereas the N terminus is clearly involved in functional coupling with SUR (6, 11, 12). Indeed, the Kir6.2 N terminus can transduce force into gating change as we recently determined (6).
demonstrated by coupling neurotransmitter receptors to Kir6.2 (13).

In this work, we examine the functional coupling between Kir6.2 and SUR1 or the cardiac isoform SUR2A using a rhodamine protection assay (14) that permits us to dynamically probe the conformation of the contact between TM0 of SUR and Kir6.2. We investigate how the SUR-Kir6.2 interface is modified when \( K_{\text{ATP}} \) channel openers, natural and synthetic, bind to SUR. We also studied the effects of mutations of neighboring amino acids, Glu\(^{128} \) and Phe\(^{132} \) (SUR1 numbering), of the second intracellular loop of SUR (supplemental Fig. S1) that have been associated with hyperinsulinism and diabetes, respectively (15, 16). Using the Rho protection assay, we demonstrate that mutations of these residues stabilize different conformations of the SUR-Kir6.2 interface.

**EXPERIMENTAL PROCEDURES**

The experimental conditions were essentially as described previously (14). All of the constructs were derived from mouse Kir6.2 (GenBank\(^{\text{TM}} \) accession number D50581), hamster SUR1 (GenBank\(^{\text{TM}} \) accession number L40623), and rat SUR2A (GenBank\(^{\text{TM}} \) accession number D83598) and subcloned in Xenopus oocyte expression vectors derived from pGEMHE (17). The construct TM0 consisted of residues 2–195 of SUR1 with a FLAG epitope (sequence DYKDDDDK) added at the N-terminal (identical to construct Phe195 in Ref. 7). Mutations were introduced by PCR (QuikChange site-directed mutagenesis kit; Stratagene). For the Kir6.2 C36 construct (18), a premature stop codon was introduced at the correct position to delete last 36 residues of the C terminus. The coding sequence of each clone was verified by sequencing. After amplification and linearization, plasmid DN As were transcribed in \textit{vitro} by using the T7 mMessage mMachine kit (Ambion) to produce cRNA for subsequent oocyte microinjection.

Female \textit{Xenopus laevis} were anesthetized with 3-aminobenzoic acid ethyl ester (1 g/liter of water). Part of one ovary was removed with a minilaparotomy, the incision was sutured, and the animal was allowed to recover. Animal handling conformed with French regulations and were approved by local governmental veterinary services (authorization number 38-08-10). Stage V or VI oocytes were defolliculated by a 60-min incubation at 19 °C with 2 mg/ml type A collagenase (Sigma-Aldrich). Selected oocytes were injected the next day with cRNAs encoding wild-type or truncated Kir6.2 (0.2 or 2 ng) and, where applicable, TM0 (2 ng) or wild-type or modified SURs (6 ng). Injected oocytes were stored at 19 °C in Barth’s solution (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\(_3\))\(_2\), 16 mM HEPES, pH 7.4) supplemented with 100 units/ml penicillin, 100 \( \mu \)g/ml streptomycin, and 100 \( \mu \)g/ml gentamycin. 3–5 days after injection, the oocytes were devitellinized, and recombinant \( K_{\text{ATP}} \) channels were characterized by the patch-clamp technique in the excised inside-out configuration at room temperature (~22 °C). Patch pipettes contained 154 mM K\(^+\), 146 mM Cl\(^-\), 5 mM Mg\(^{2+}\), and 10 mM PIPES-KOH (pH 7.1). The cytoplasmic face of the patch was bathed in solutions that, unless otherwise noted, contained 174 mM K\(^+\), 40 mM Cl\(^-\), 1 mM EGTA, 1 mM Mg\(^{2+}\), 10 mM PIPES-KOH (pH 7.1), and methanesulfonate\(^-\) as the remaining anions. For experiments in 0 Mg\(^{2+}\), Mg\(^{2+}\) was omitted, and EGTA was replaced by EDTA to chelate residual traces of divalent cations. ATP (potassium salt; Sigma-Aldrich), Zn\(^{2+}\) (chloride salt; Fluka), diazoxide (100 mM stock in Me\(_2\)SO; Sigma), Sr47063 (20 mM stock in Me\(_2\)SO; Sanofi-Aventis), P1075 (20 mM stock in Me\(_2\)SO; Leo Pharma), and rhodamine 123 (30 mM stock in ethanol; Sigma-Aldrich) were added as specified. The membrane potential was ~50 mV. Applications of the various solutions to the patch were performed using a customized RSC-100 system (Bio-Logic) (19).

Control, acquisition, and analysis were performed with in-house software. Open probability (\( P_o \)) was determined using pClamp 10 (Molecular Devices). Slow fluctuations of the base line were removed by interactive fitting with a spline curve and subtraction of this fit from the signal.

To quantify the effects of Rho in 0 ATP, where currents are more prone to rundown, whenever possible, current decline before application was extrapolated manually, and this extrapolated value served as the control current (14). However, no obvious differences were noticed between the overall results and the observations limited to patches displaying little or no rundown. The results are displayed as the means ± S.E.

**RESULTS**

\( MgADP \) Binding to \( SUR \) Protects Kir6.2 from Rhodamine Block—\textit{Xenopus} oocytes injected with cRNA of Kir6.2ΔC36 or with a mixture of cRNAs coding SUR2A or SUR1 and Kir6.2-expressed exogenous potassium currents inhibited by ATP. In excised inside-out patches, MgADP (300 \( \mu \)M), the main physiological \( K_{\text{ATP}} \) channel opener, inhibited SUR-less Kir6.2 currents by ~40%, but it did not change SUR + Kir6.2 currents. This indicates that at 300 \( \mu \)M, binding of MgADP to SUR elicits an activation that compensates the inhibitory effect on Kir6.2. Application of Rho (10 \( \mu \)M) in the presence of MgADP triggered different effects depending on the expressed channel. Kir6.2ΔC36 was inhibited more than 80% by Rho (Fig. 1, A and F), in the presence or absence of MgADP. The full \( K_{\text{ATP}} \) channel constituted of SUR1 or SUR2A and Kir6.2 were strongly blocked by Rho (~80%) in control but only weakly in the presence of MgADP (13 ± 4 and 22 ± 5% inhibition for SUR2A and SUR1, respectively; Fig. 1, B, C, and F). These results suggest that, like previously reported for ATP (14), binding of MgADP to SUR induces a conformational change that shields Kir6.2 from the inhibitory action of Rho.

To test the role of Mg\(^{2+}\) in the effect of ADP, experiments were conducted in Mg\(^{2+}\)-free conditions (Fig. 1, D–F). In ADP, removal of Mg\(^{2+}\) did not affect Rho inhibition of Kir6.2ΔC36 currents (92 ± 2% without Mg\(^{2+}\) versus 81 ± 5% in 1 mM Mg\(^{2+}\)). In contrast, protection by ADP of SUR2A + Kir6.2 was considerably reduced in magnesium-free conditions (Rho inhibition of 13 ± 4% in 1 mM Mg\(^{2+}\) and 61 ± 7% without Mg\(^{2+}\)). This effect of Mg\(^{2+}\) requires ADP because Mg\(^{2+}\) alone does not protect against Rho block (14).

Inhibition of SUR2A + Kir6.2 was less in magnesium-free ADP than in the complete absence of nucleotides (61 ± 7% in magnesium-free ADP versus 79.5 ± 1.5% in nucleotide-free control; \( p < 0.02 \)). Magnesium-free ADP unexpectedly produced a slow increase in SUR2A + Kir6.2 currents, as evidenced...
K<sub>ATP</sub> Channel Mutations Alter Interactions between Subunits

![Image of MgADP protects K<sub>ATP</sub> channels from inhibition by rhodamine. A–E, currents recorded in inside-out patches from Xenopus oocytes expressing Kir6.2ΔC36 (A and D), Kir6.2 and SUR2A (B and E), or Kir6.2 and SUR1 (C). Rho (10 μM) was applied in the presence of ADP (300 μM) with 1 mM Mg<sup>2+</sup> (MgADP) or with no added Mg<sup>2+</sup> and 1 mM EDTA to chelate contaminating divalent cations (Mg-free ADP). The closed channel base line was determined by application of 2 mM ATP (designated by ATP). F, average inhibition produced by 10 μM rhodamine 123 in absence of nucleotides (light gray bars), in the presence of 300 μM ADP and 1 mM Mg<sup>2+</sup> (black bars), and in the presence of 300 μM ADP without magnesium (dark gray bars). In this and other figures, the error bars represent the standard errors, and the numbers next to the histogram bars indicate the numbers of patches included in the average. nd, not determined.

The mutation F132L in SUR1 induces a Rho-resistant Phenotype—The mutation F132L in TMD0 of SUR1 is responsible for neonatal diabetes (15). Because it was shown by observable increases in maximal currents. Such an effect has been evoked earlier (20). This combination of incomplete Rho block and ADP-enhanced channel activity gave rise to sizable residual currents after Rho application (Fig. 1E).

In another set of experiments (supplemental Fig. S2), the two NBDs of SUR2A were disabled by mutating the key Walker B aspartate residues of both NBDs to glutamine (14, 21). The resulting mutant SUR2A<sub>D832N,D1469N</sub> is no longer activated by aspartate residues of both NBDs to glutamine (14, 21). The NBDs of SUR2A were disabled by mutating the key Walker B was determined by application of 2 mM ATP (designated by ATP). F, average inhibition produced by 10 μM rhodamine 123 in absence of nucleotides (light gray bars), in the presence of 300 μM ADP and 1 mM Mg<sup>2+</sup> (black bars), and in the presence of 300 μM ADP without magnesium (dark gray bars). In this and other figures, the error bars represent the standard errors, and the numbers next to the histogram bars indicate the numbers of patches included in the average. nd, not determined.

observed in the absence of zinc, 10 μM Rho in the presence of zinc produced an inhibition of only 21 ± 6% (Fig. 2).

Similar experiments were performed with SR47063, a pharmacological opener of cardiac type SUR2A + Kir6.2 channels (22) (Fig. 3). In the absence of nucleotides, a saturating concentration of 100 μM SR47063 had opposite effects on Kir6.2ΔC36 and SUR2A + Kir6.2 currents, inhibiting the former by 45 ± 0.02% and activating the latter by 53 ± 0.2%. In the presence of SR47063, Rho inhibition of Kir6.2ΔC36 remained as high as in control (84 ± 4%), whereas inhibition of SUR2A + Kir6.2 was drastically reduced to 20 ± 3%. These observations suggest that activation by distinct path ways converges toward the same Rho phenotype, which we have shown to reflect a particular conformation of the SUR/Kir6.2 interface.

The Activators Zinc and SR47063 Antagonize Rho Inhibition—Zinc, which is co-secreted with insulin by β-cells, is another physiological opener of the pancreatic K<sub>ATP</sub> channels SUR1 + Kir6.2 that acts from both faces of the membrane (21). Because nucleotides interfere with Rho inhibition, we performed tests in nucleotide-free conditions. EGTA, normally present at 1 mM in all internal solutions, was also removed to avoid zinc chelation. In those conditions, a saturating concentration of cytoplasmic zinc (32 μM) weakly potentiated SUR1 + Kir6.2. Compared with the 69 ± 11% inhibition to alter interaction of TMD0 with Kir6.2 (23), we tested its effect on the SUR1/Kir6.2 interface using the Rho protection assay. The SUR1<sub>F132L</sub> + Kir6.2 channel is characterized by a reduced sensitivity to block by nucleotides (17 ± 2% of inhibition by 100 μM ATP versus 82 ± 2% for wild type). Strikingly, SUR1<sub>F132L</sub> currents were largely unaltered by 10 μM Rho, even in the absence of nucleotides where wild-type SUR1 currents were strongly inhibited (Fig. 4). In nucleotide-free conditions, inhibition by 10 μM Rho was 80 ± 2% for the wild type but only 19 ± 6% for the mutant. In 100 μM ATP, inhibition was relieved similarly for wild-type and mutant SUR1 with values of 9 ± 6 and 7 ± 3%, respectively. This particular Rho phenotype is identical to that observed with TMD0 + Kir6.2ΔC36 (Fig. 5C).

We tested the effect of the mutation on TMD0 alone and found that, unexpectedly, it had completely opposite effects on TMD0 and on SUR1, increasing the Rho sensitivity of the former while reducing that of the latter. TMD0<sub>F132L</sub> + Kir6.2ΔC36 was highly sensitive to Rho inhibition much like Kir6.2ΔC36 alone (Fig. 4C). This observation could be explained simply by the fact that TMD0<sub>F132L</sub> does not associate with Kir6.2ΔC36 and that we are recording the same Kir6.2ΔC36 channels with and without co-expressed TMD0<sub>F132L</sub>. However, TMD0<sub>F132L</sub> does associate with Kir6.2ΔC36 as demonstrated by a previous study (23) that reported evidence—increased surface expression, gating up-regulation, and reduction of ATP inhibition—of its effects on Kir6.2. In the same study, the F132L mutation was shown by co-immunoprecipitation to diminish
the interaction between TMD0 and Kir6.2. This reduced interaction would be consistent with the inability of TMD0F132L to protect from Rho block. It remains that, when probed with the Rho protection assay, the F132L mutation had distinct effects on full-length SUR1 and on the isolated TMD0 domain. One should therefore exercise much caution when extrapolating results from part to whole. In particular, it might not be valid to infer the behavior of full-length SUR from results obtained with TMD0 alone (7, 23).

In conclusion, the mutation F132L in SUR1 produces a Rho-resistant phenotype like the activators MgADP, zinc, or SR47063. This phenotype resembles that of TMD0 alone and is characterized by a reduced Kir6.2 sensitivity to block by ATP. It remains that, when probed with the Rho protection assay, the F132L mutation had distinct effects on full-length SUR1 and on the isolated TMD0 domain. One should therefore exercise much caution when extrapolating results from part to whole. In particular, it might not be valid to infer the behavior of full-length SUR from results obtained with TMD0 alone (7, 23).

In conclusion, the mutation F132L in SUR1 produces a Rho-resistant phenotype like the activators MgADP, zinc, or SR47063. This phenotype resembles that of TMD0 alone and is characterized by a reduced Kir6.2 sensitivity to block by ATP. It remains that, when probed with the Rho protection assay, the F132L mutation had distinct effects on full-length SUR1 and on the isolated TMD0 domain. One should therefore exercise much caution when extrapolating results from part to whole. In particular, it might not be valid to infer the behavior of full-length SUR from results obtained with TMD0 alone (7, 23).

The Mutation E126A in SUR2A Induces a Rho-sensitive Phenotype—Residue Phe\(^{132}\) is located in the predicted second intracellular loop of SUR1 (24). Another residue of this short loop, Glu\(^{128}\), also causes disease when mutated; mutation E128K is responsible for hyperinsulinism because it reduces K\(_{ATP}\) channel activity by interfering with proper trafficking of channels to the plasma membrane (16). Because of the opposite pathological consequences of F132L and E128K mutations despite their proximity, it was interesting to also subject E128K to the Rho protection assay. To circumvent the absence of surface expression of SUR1\(_{E128K}\), we tested first the equivalent E126K mutation and later the less drastic E126A mutation in the cardiac isoform SUR2A. If SUR2A\(_{E126K}\) produced few or no excised patch currents (2.1 \(\pm\) 1.6 pA), SUR2A\(_{E126A}\) gave rise to large currents (895 \(\pm\) 215 pA), although the currents were significantly smaller than wild-type SUR2A (3.6 \(\pm\) 0.3 nA). The SUR2A\(_{E126A}\) channels were less sensitive to ATP inhibition than wild-type SUR2A but more than Kir6.2\(_{C36}\) (IC\(_{50}\) values of 44 \(\pm\) 2, 15 \(\pm\) 1, and 101 \(\pm\) 34 \(\mu\)M, respectively; supplemental Fig. S3). Estimation from single-channel records (supplemental Fig. S4) yielded \(P_{0} = 0.073 \pm 0.02\) (\(n = 7\)) for SUR2A\(_{E126A}\), significantly lower than for wild type and equivalent to Kir6.2\(_{C36}\) (6, 7, 23, 25).

Unlike wild-type SUR2A, the E126A mutant was fully blocked by Rho in the presence or absence of ATP (Fig. 5). In that respect the mutant behaved like SUR-less Kir6.2. Nevertheless, SUR2A\(_{E126A}\) + Kir6.2 was activated by the openers SR47063 (Fig. 5E) and P1075 (supplemental Fig. S5), demonstrating that the E126A mutation did not suppress all coupling between SUR2A and Kir6.2. As for the F132L mutation, we attempted to test the effect of the mutation E126A on TMD0 of SUR2A alone but were unable to record sufficient channel activity from oocytes co-expressing TMD0\(_{E126A}\) + Kir6.2\(_{C36}\), thus precluding any valid characterization.
Because the SUR2A_{E126A}+Kir6.2 channel remained sensitive to openers, we asked whether the opener SR47063 could also protect it from block by Rho. Surprisingly, this was not the case (Fig. 5, E and F), implying that protection by opener and activation by opener follow separate parallel pathways. Similar tests with ADP showed no protection either, although this appeared more logical because ADP did not produce any obvious SUR-mediated activation of E126A mutant channels (Fig. 5D).

**DISCUSSION**

We previously reported (14) that rhodamine 123 is a conformation-dependent $K_{ATP}$ channel inhibitor. Rho targets the Kir6.2 subunit because SUR-less Kir6.2 is blocked by Rho. This block was shown to be independent of the degree of opening of Kir6.2, being equally efficient on fully closed and fully open channels. It was also not correlated to intrinsic $P_o$, being equally efficient on low $P_o$ Kir6.2 and high $P_o$ SUR+Kir6.2 channels in the absence of nucleotides. Although slow in its onset and its reversal, Rho block appears unrelated to rundown and phosphatidylinositol 4,5-bisphosphate because it was not influenced by the presence of MgATP. When co-expressed with TMD0 of SUR, Kir6.2 is no longer affected by Rho, suggesting that Rho binds at the TMD0-Kir6.2 interfacial contact zone and that TMD0 masks the Rho-binding site. When co-expressed with full-length SUR1 or SUR2A, Kir6.2 is blocked by Rho in the absence of nucleotides. In the presence of ATP, it is protected, and we showed that this protection results from ATP binding to SUR and causing TMD0 to move to a position where it masks the Rho-binding site (14).

Because TMD0 is the key interaction domain of SUR (7) and because the Rho protection assay is a unique tool to observe the dynamics of its interaction with Kir6.2, we have used this assay to examine the mechanisms of action of SUR physiological and pharmacological openers and of two TMD0 mutations that are associated with inverse clinical phenotypes.

**$K_{ATP}$ Channel Activators Allosterically Modulate the TMD0-Kir6.2 Interface**—All of the activators tested protected Kir6.2 from Rho block. Two physiological activators were examined: ADP and zinc. Both are known to act by interacting with SUR. With Mg$^{2+}$ as a compulsory co-factor, ADP binds to the NBDs and augments channel activity (26–28). Rho inhibition was drastically reduced in the presence of ADP, and this effect required Mg$^{2+}$ and intact NBDs, suggesting that activation and protection are both mediated by MgADP binding to NBDs. Because protection by ATP does not require Mg$^{2+}$ (14), this result suggests that, unlike ATP, ADP needs Mg$^{2+}$ to interact with NBDs.

Zinc can activate $K_{ATP}$ channels, with a marked preference for SUR1 over SUR2A, from both extracellular and intracellular sides (21). Intracellularly, where we used it, zinc targets as an yet unidentified region of SUR. As with other compounds, experiments with zinc were conducted in absence of ATP that by itself would impart Rho protection. Effects caused by zinc could therefore not be attributed to an indirect action resulting from association of this divalent cation with anionic nucleotides. On the other hand, in absence of nucleotides, there is no electrophysiological evidence of zinc binding to SUR because of the lack of any significant effect on SUR1+Kir6.2 channel activity. Nonetheless, zinc did bind to SUR1 because it clearly protected the channels from Rho block. This demonstrates that the Rho protection assay can detect conformational changes in the absence of visible changes in gating. It furthers confirms that Rho protection is independent of Kir6.2 channel activity (14).

We also tested the pharmacological opener SR47063, an analogue of cromakalim. SR47063 is a prototypical opener of SUR2-based channels that targets the transmembrane regions...
K$_{ATP}$ Channel Mutations Alter Interactions between Subunits

**FIGURE 5.** The SUR2A E126A mutation leads to a constitutive Rho-sensitive conformation, similar to that of SUR-less Kir6.2. A and B, currents recorded in inside-out patches co-expressing Kir6.2 and wild-type SUR2A (A) or the E126A mutant (B). The effect of Rho was tested in nucleotide-free solution and in the presence of ATP (100 μM). C, average inhibition by Rho of Kir6.2ΔC36, SUR2A + Kir6.2, and SUR2A E126A + Kir6.2 measured in the absence (gray bars) or presence (black bars) of 100 μM ATP. D, average currents in the presence of MgADP measured before (gray bars) and after (black bars) Rho application. The currents were normalized to the currents measured in 0 ATP before application of MgADP (shown here as white bars). E, same as previous panel but with the opener SR47063. F, average inhibition by 10 μM Rho in the presence of SR47063 (gray bars) or MgADP (black bars).

of SUR2A (29). SR4703 strongly protected SUR2A+Kir6.2 channels from Rho block.

Rho protection being linked to the conformation of the TMD0-Kir6.2 interface, we can conclude that MgADP, zinc, and SR47063 induce a change of that interface. Because the site of action of zinc is not yet known, this change could be the consequence of direct binding of zinc to TMD0. In contrast, MgADP and SR47063 do not directly interact with TMD0. Their binding, to the NBDs and TMD2 domains, respectively, must therefore induce a global conformational change that is allosterically transmitted to TMD0 and alter the configuration of the interface to mask the Rho inhibitory site.

**Structural Perturbations Induced by Disease-causing Mutations in the Second Intracellular Loop**—Mutations F132L and E128K in short intracellular loop 2 located in TMD0 of SUR1 have opposite consequences. The neonatal diabetes F132L mutation reduces ATP sensitivity by augmenting open probability (23). The resulting channel hyperactivity explains the insufficient secretion of insulin by β-cells. The hyperinsulinism E128K mutation disrupts channel trafficking and reduces channel function through poor surface expression (16). After rescue to the cell surface by a chemical chaperone, the E128K mutant channels are hyperactive with a lower than normal ATP sensitivity like the F132L mutants, although they exhibit an abnormally low $P_o$ (25). The F132L and E128K mutations cause therefore an abnormally high activity through different mechanisms. Pratt et al. (25) speculated that E128K caused functional uncoupling between SUR1 and Kir6.2, thus removing the hypersensitivity to ATP brought about by SUR1 (18). Based on co-immunoprecipitation of TMD0 alone and Kir6.2 showing that F132L impaired the association of TMD0 and Kir6.2, Proks et al. (23) assumed that TMD0 and SUR behaved identically and concluded that this mutation disconnects SUR from Kir6.2. In that case, one would expect a severe trafficking defect because Kir6.2 needs SUR as a chaperone to cross quality control checkpoints (30), but surface expression was normal in *Xenopus* oocytes (23) and only moderately reduced in mammalian cells (25). Such inconsistency is resolved by Rho protection data showing that the mutation does not have the same effects on TMD0 alone (no Rho protection by TMD0 alone (no Rho protection by TMD0+E126A consistent with a loose association with Kir6.2) and on full-length SUR (complete Rho protection by SUR1+E126A indicative of a tight association with Kir6.2) and that it is not warranted to extrapolate data from one to the other.

Because the SUR1+E128K mutant is not present at the surface membrane (16), we introduced the equivalent mutation (E126K) in the SUR2A isoform, which tends to express approximately twice better in our hands. Unfortunately, the surface expression of the SUR2A E126K mutant was too weak for electrophysiological characterization. Mutation to a neutral residue, E126A, was found to yield sufficient currents for our tests. Very recently, Pratt et al. (25) were able to record SUR1+E126K channels by boosting surface expression with sulfonylurea pretreatment of transfected mammalian cells. SUR1+E128K was less sensitive to ATP inhibition than the wild type, had a lower $P_o$, and remained sensitive to activation by MgADP. The same features applied also to SUR2A+E126A.

When probed with Rho, SUR1+E126L and SUR2A+E126A showed opposite phenotypes. The wild-type channels are blocked by Rho in absence of nucleotides and are only weakly affected in the presence of ATP, which confers protection by binding to SUR (14). SUR1+E126L Channels were insensitive to Rho block in all conditions, even in the absence of nucleotides, whereas
**KATP Channel Mutations Alter Interactions between Subunits**

![Diagram showing the effect of KATP channel mutations on interactions between subunits](image)

**FIGURE 6.** The mutations F132L and E126A stabilize distinct conformations of TMD0 with respect to Kir6.2. Schematic interpretation of the rhodamine protection data. The Kir6.2 pore is colored blue when sensitive to block by Rho (red square) and dark gray when insensitive. SUR, with its TMD0 domain interacting with Kir6.2, is light gray. Although not represented here for simplicity, domains of SUR other than TMD0 are likely to interact with Kir6.2. Mutant TMD0s are yellow for F132L and red for E126A. The basic observation (boxed) is that channel block by Rho in control conditions (top) is antagonized by channel activators that bind to SUR (star), like ATP, MgADP, SR47063, and zinc (bottom). Because Rho blocks Kir6.2 alone but not Kir6.2 with TMD0, the Rho-binding site is located on Kir6.2 and can be masked by TMD0, either allosterically or directly as represented here for simplicity. The protection conferred by SUR ligands is therefore hypothesized to involve an allosteric change in blocker accessibility resulting from a conformational switch of the TMD0-Kir6.2 interface. The insensitive phenotype of the F132L mutant suggests that the mutation stabilizes a protective TMD0-Kir6.2 conformation similar to that induced by activators. In contrast, the E126A mutant is stabilized in a Rho-sensitive conformation because it is no longer protected from Rho inhibition in the presence of activators.

SUR2A<sub>E126A</sub> channels were sensitive even when ATP was present. Therefore, SUR1<sub>F132L</sub> + Kir6.2 channels responded to Rho like channels formed by TMD0 alone with Kir6.2 and SUR2A<sub>E126A</sub> + Kir6.2 responded like Kir6.2 alone. However, SUR ligands were still capable of modulating Kir6.2; both ADP and diazoxide still activated SUR1<sub>F132L</sub> channels (supplemental Fig. S6). SR47063 and the pinacidil analogue P1075 still activated SUR2A<sub>E126A</sub> (supplemental Fig. S5). This suggests that although the F132L and E126A mutations lock the TMD0-Kir6.2 interface in distinct conformations where the Rho-binding site is either always masked or always accessible, other regions of SUR are unaffected and can interact with Kir6.2 to modulate its gating. This favors the notion that multiple physical links exist between SUR and Kir6.2 and that each can serve distinct functions, consistent with the postulated implication in coupling of the acidic region linking NBD1 and TMD2 (31) and with our recent finding of the role of a C-terminal region of SUR in SUR-mediated activation of Kir6.2 (9).

**Rho Protection Assay Unveils the Mechanism of Disease-causing Mutations**—Even though the F132L and E128K mutations in SUR1 both tend to reduce channel sensitivity to ATP, their mechanisms of action obviously differ because F132L increases \( P_o \) and E128K decreases \( P_o \). Nonetheless, after detailed investigation, Proks et al. (23) working on F132L and Pratt et al. (25) working on E128K reached identical conclusions: the mutation disrupts the coupling/interaction between TMD0 and Kir6.2. The additional insight obtained by the Rho protection assay appears to resolve this discrepancy as it strengthens the proposed mechanism for E128K while questioning that for F132L.

As a surrogate for the low expressing SUR1<sub>E128K</sub>, we use the mutation E126A in SUR2A. The profiles of SUR1<sub>E128K</sub> and SUR2A<sub>E126A</sub> were qualitatively similar with a reduced surface expression, a low \( P_o \), and a low ATP sensitivity. Results from the Rho protection assay are summarized in Fig. 6. The Rho-sensitive profile of SUR2A<sub>E126A</sub> is identical to that of SUR-less Kir6.2, suggesting that E126A functionally uncouples TMD0 and Kir6.2. The Rho-protected profile of SUR1<sub>F132L</sub> is the same as that of TMD0 alone. It also matches the profile induced by physiological and pharmacological openers. This suggests that the conformation of TMD0 in all these cases: F132L, TMD0 alone, and activators, could be similar. Thus, the channel hyperactivity caused by the F132L mutation would reflect the stabilization of TMD0 in the same activated conformation that is triggered by activators.

One could speculate that compounds that induce structural changes opposite to that caused by the mutations might be able to correct or attenuate the functional consequences of these mutations. The Rho protection assay could serve to identify such compounds.

**ACKNOWLEDGMENTS**—We are grateful to Dr. J. Bryan (Houston, TX) for hamster SUR1, Dr. S. Seino (Chiba, Japan) for mouse Kir6.2 and rat SUR2A, Dr. K. Chan (Cleveland, OH) for the construct TMD0 (SUR1), Dr. P. Gautier (Sanofi-Aventis, Montpellier, France) for SR 47063, and Dr. L. Billerup (Leo Pharma, Ballerup, Denmark) for P1075.

**REFERENCES**

1. Moreau, C., Prost, A. L., Dérand, R., and Vivaudou, M. (2005) J. Mol. Cell. Cardiol. 38, 951–963
2. Nichols, C. G. (2006) Nature 440, 470–476
3. Alekseev, A. E., Hodgson, D. M., Karger, A. B., Park, S., Zingman, L. V., and Terzic, A. (2005) J. Mol. Cell. Cardiol. 38, 895–905
4. Ashcroft, F. M. (2007) Am. J. Physiol. Endocrinol. Metab. 293, E880–E889
5. Flanagan, S. E., Clauin, S., Bellanne-Chantelot, C., de Lonlay, P., Harries, L. W., Glynn, A. L., and Ellard, S. (2009) Hum. Mutat. 30, 170–180
6. Babenko, A. P., and Bryan, J. (2003) J. Biol. Chem. 278, 41577–41580
7. Chan, K. Y., Koh, P. L., Zhang, H., and Logothetis, D. E. (2003) EMBO J. 22, 3833–3843
8. Rainbow, R. D., James, M., Hudman, D., Al Johi, M., Singh, H., Watson, P. L., Ashmore, L., Davies, N. W., Lodwick, D., and Norman, R. I. (2004) Biochem. J. 379, 173–181
9. Dupuis, J. P., Revilloud, J., Moreau, C. J., and Vivaudou, M. (2008) J. Physiol. 586, 3075–3085
10. Schwappach, B., Zerangue, N., Jan, Y. N., and Jan, L. Y. (2000) Neuron 26, 155–167
11. Reimann, F., Tucker, S., Proks, P., and Ashcroft, F. M. (1999) J. Physiol. 518, 325–336
12. Babenko, A. P., and Bryan, J. (2002) J. Biol. Chem. 277, 43997–44004
13. Moreau, C. J., Dupuis, J. P., Revilloud, J., Arumugam, K., and Vivaudou, M. (2008) Nature Nanotech. 3, 620–625
K$_{ATP}$ Channel Mutations Alter Interactions between Subunits