Functional Analysis of a Mutant Sulfonylurea Receptor, SUR1-R1420C, That Is Responsible for Persistent Hyperinsulinemic Hypoglycemia of Infancy*

Received for publication, July 21, 2000, and in revised form, September 5, 2000
Published, JBC Papers in Press, September 18, 2000, DOI 10.1074/jbc.M00693200

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The ATP-sensitive potassium (K_{ATP}) channel is crucial for the regulation of insulin secretion from the pancreatic β-cell, and mutations in either the sulfonylurea receptor type 1 (SUR1) or Kir6.2 subunit of this channel can cause persistent hyperinsulinemic hypoglycemia of infancy (PHHI). We analyzed the functional consequences of the PHHI missense mutation R1420C, which lies in the second nucleotide-binding fold (NBF2) of SUR1. Mild tryptic digestion of SUR1 after photoaffinity labeling allowed analysis of the nucleotide-binding properties of NBF1 and NBF2. Labeling of NBF1 with 8-azido-[α-32P]ATP was inhibited by MgATP and MgADP with similar K_i for wild-type SUR1 and SUR1-R1420C. However, the MgATP and MgADP affinities of NBF2 of SUR1-R1420C were about 5-fold lower than those of wild-type SUR1. MgATP and MgADP stabilized 8-azido-ATP binding at NBF1 of wild-type SUR1 by interacting with NBF2, but this cooperative nucleotide binding was not observed for SUR1-R1420C. Studies on macroscopic currents recorded in inside-out membrane patches revealed that the SUR1-R1420C mutation exhibits reduced expression but does not affect inhibition by ATP or tolbutamide or activation by diazoxide. However, co-expression with Kir6.2-R50G, which renders the channel less sensitive to ATP inhibition, revealed that the SUR1-R1420C mutation increases the EC_{50} for MgADP activation from 74 to 197 μM. We suggest that the lower expression of the mutant channel and the reduced affinity of NBF2 for MgADP may lead to a smaller K_{ATP} current in R1420C-PHHI β-cells and thereby to the enhanced insulin secretion. We also propose a new model for nucleotide activation of K_{ATP} channels.

ATP-sensitive potassium (K_{ATP}) channels link the metabolic state of the cell to its membrane potential in many tissues including pancreatic β-cells, heart, brain, and skeletal muscle (1–4). It is believed that metabolic regulation is mediated by changes in ATP and Mg^2+-nucleotides (such as MgADP), which inhibit and activate the channel, respectively. In pancreatic β-cells, metabolically induced changes in K_{ATP} channel activity play a key role in glucose-stimulated insulin secretion. At sub-stimulatory glucose concentrations, K_{ATP} channels are open, and their activity serves to maintain the resting membrane potential at a hyperpolarized level. Elevation of blood glucose concentration increases glucose uptake and metabolism by the β-cell producing changes in cytosolic nucleotide concentrations that result in closure of the K_{ATP} channels. This leads to depolarization of the β-cell membrane potential and thus to activation of voltage-gated calcium channels and Ca^{2+} influx. The resulting rise in the intracellular Ca^{2+} concentration triggers insulin release.

The β-cell K_{ATP} channel is a hetero-octamer composed of pore-forming Kir6.2 subunits and regulatory sulfonylurea receptor (SUR1) subunits that coassemble with 4:4 stoichiometry (5–8). Kir6.2 is a member of the inwardly rectifying potassium channel family (9, 10), whereas SUR1 belongs to the ATP-binding cassette transporter superfamily (11). Like other members of this family, SUR1 has two intracellular nucleotide-binding folds (NBFs), each containing a Walker A and a Walker B motif that is involved in Mg^{2+}-nucleotide binding and hydrolysis. Nucleotides interact with both Kir6.2 and SUR1 subunits; ATP inhibits the channel by binding to Kir6.2 (12–14), whereas Mg^{2+}-nucleotides stimulate channel activity by binding to the NBFs of SUR1 (1, 15–20).

Persistent hyperinsulinemic hypoglycemia of infancy (PHHI) is an autosomal recessive disorder characterized by inappropriate insulin secretion despite severe hypoglycemia. In the absence of clinical treatment, PHHI may be lethal or result in irreversible neurologic sequelae. To date, three mutations responsible for PHHI have been identified in the Kir6.2 gene (21–23) and in numerous mutations in the SUR1 gene (1, 24–30). Some of the SUR1 mutations are nonsense or frame-shift mutations that produce a truncated form of SUR1. Others are missense mutations, many of which are found within the NBFs of SUR1 and impair K_{ATP} channel activity by affecting Mg^{2+}-nucleotide interactions with SUR1 (1, 29). As a consequence, the K_{ATP} channel remains closed even in the absence of glucose, which results in persistent and unregulated insulin secretion.

Recently, we identified a missense SUR1 mutation (R1420C) in Japanese PHHI patients (31). They were siblings from a consanguineous family and homozygous for the mutation, and their clinical characteristics consisted of a mild form of PHHI.  

* This work was supported by Grant-in-Aid for Scientific Research on Priority Areas “ABC Proteins” 10217205 from the Ministry of Education, Science, Sports, and Culture of Japan and by research fellowships of the Japan Society for the Promotion of Science for Young Scientists. The work of the Oxford group was supported by the Wellcome Trust and the British Diabetic Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Verkarre et al. (32) also reported this mutation in a patient with focal adenomatous hyperplasia of pancreatic islets (32). Arginine 1420 is located between the Walker A motif and the SGQQ signature sequence of NBF2. An \(^{86}\)Rb\(^{+}\) efflux study revealed that K\(_{\text{ATP}}\) channels composed of SUR1-R1420C and Kir6.2 are not activated by metabolic inhibition as much as wild-type channels. This may be related to the fact that the expression level of SUR1-R1420C was only about half that of the wild-type channel when it was transiently expressed in COS-7 cells (31). We also reported that MgADP, either by direct binding to NBF2 or by hydrolysis of bound MgATP at NBF2, stabilizes the binding of 8-azido-ATP at NBF1 of wild-type SUR1 (19), and that the R1420C mutation impairs this effect without altering high affinity 8-azido-ATP binding to NBF1 (31). However, it is not clear whether the mutation lowers the affinity of NBF2 for nucleotides or if it affects cooperative interactions between the two NBFs.

In this study, we examined the nucleotide-binding properties of the two NBFs of SUR1-R1420C by photoaffinity labeling experiments and studied the properties of Kir6.2/SUR1-R1420C channels by electrophysiological analysis. Our results suggest that the R1420C mutation increases the EC\(_{50}\) for MgADP-dependent channel activation by reducing the nucleotide-binding affinity of NBF2. This property, together with the lower expression of the mutant channel, may contribute to the pathogenesis of PHHI.

**EXPERIMENTAL PROCEDURES**

**Materials**—8-Azido-[\(^{\alpha}{\text{32P}}\)]ATP and 8-Azido-[\(^{\gamma}{\text{32P}}\)]ATP were purchased from ICM Biomedicals.

**Photoaffinity Labeling of SUR1 with 8-Azido-[\(^{\alpha}{\text{32P}}\)]ATP and 8-Azido-[\(^{\gamma}{\text{32P}}\)]ATP**—Membranes from COS-7 cells expressing mouse SUR1 were prepared as described (171). Crude membranes, containing similar amounts of wild-type SUR1 and SUR1-R1420C as determined by Western blotting, were incubated with \(10 \mu\)M 8-azido-[\(^{\alpha}{\text{32P}}\)]ATP in the presence or absence of 0.1–1000 \(\mu\)M ATP or ADP in 3 \(\mu\)l of TEM buffer (40 mM Tris-Cl (pH 7.5), 0.1 mM EDTA, 1 mM MgSO\(_4\)) containing 2 \(\mu\)l ouabain. Proteins were UV-irradiated for 5 min (at 254 nm, 5.5 milliwatts/cm\(^2\)) on ice. Ice-cold TEM buffer was then added to the sample, and the supernatant was removed after centrifugation (15,000 \(\times\) g, 5 min, 2\(°\)C). Pellets were resuspended in 40 mM Tris-Cl (pH 7.5) buffer containing 0.1 mM EDTA, 250 mM sucrose, and 10 \(\mu\)g/ml trypsin to 10 \(\mu\)g of membrane proteins/\(\mu\)l and incubated for 5 min at 37\(°\)C. 100 \(\mu\)M of radioimmuno precipitation buffer (20 mM Tris-Cl (pH 7.5), 1% Triton X-100, 0.1% SDS, and 1% dodecylmaltoside) containing 100 \(\mu\)g/ml (\(\alpha\)-dodecylamino)benzene sulfonic acid was added to the samples to terminate proteolysis, and membrane proteins were solubilized for 30 min at 4\(°\)C. After centrifugation for 15 min at 15,000 \(\times\) g, trypptic fragments were immunoprecipitated from the supernatant using an antibody raised against NBF1 or NBF2 of hamster SUR1 as described previously (20). Samples were electrophoresed on 12% SDS-polyacrylamide gels and autoradiographed. Bound 8-azido-[\(^{\alpha}{\text{32P}}\)]ATP in NBF1 or NBF2 was measured by scanning with a radioimaging analyzer (BAS2000, Fuji Photo Film Co.).

**Cooperative Nucleotide Binding of Two NBFs—Membranes (20 \(\mu\)g of proteins) were incubated with 10 \(\mu\)M 8-azido-[\(^{\alpha}{\text{32P}}\)]ATP in 3 \(\mu\)l of TEM buffer containing 2 \(\mu\)l ouabain for 3 min at 37\(°\)C. The reactions were stopped by adding ice-cold TEM buffer, and free 8-azido-[\(^{\alpha}{\text{32P}}\)]ATP was removed after centrifugation (15,000 \(\times\) g, 5 min, 2\(°\)C). Pellets were resuspended in 10 \(\mu\)l of TEM buffer containing 2 \(\mu\)l ouabain and 0–1000 \(\mu\)M ATP or ADP. The mixture was UV-irradiated immediately after the resuspension or after postincubation for 15 min at 37\(°\)C. Samples were electrophoresed on 7% SDS-polyacrylamide gel and autoradiographed. Bound 8-azido-[\(^{\alpha}{\text{32P}}\)]ATP in SUR1 was measured by radioimaging analyzer as described above.

**Electrophysiological Analysis of Wild-type and Mutant Kir6.2 Channels**—Wild-type or mutant Kir6.2 was coexpressed with wild-type or mutant SUR1-R1420C by electrophysiological analysis. Our results indicate S.E.

**RESULTS**

**Photoaffinity Labeling of SUR1-R1420C with 8-Azido-[\(^{\alpha}{\text{32P}}\)]ATP and 8-Azido-[\(^{\gamma}{\text{32P}}\)]ATP**—To examine the biochemical properties of SUR1-R1420C, we investigated 8-azido-[\(^{\alpha}{\text{32P}}\)]ATP photoaffinity labeling of NBF1 and NBF2 (20). Crude membranes from COS-7 cells expressing SUR1 or SUR1-R1420C were incubated with 50 \(\mu\)M 8-azido-[\(^{\alpha}{\text{32P}}\)]ATP followed by mild trypsin digestion. Labeled trypptic fragments containing NBF1 or NBF2 of both wild-type and mutant SUR1 were immunoprecipitated with anti-NBF1 (Fig. 1, lanes 1 and 3) or NBF2 antibody (Fig. 1, lanes 2 and 4), respectively. This indicates that both NBFs of SUR1-R1420C can bind 8-azido-ATP, as is found for wild-type SUR1.

We have previously suggested that NBF2 of SUR1 might hydrolyze ATP, because NBF2 of SUR1 is photoaffinity-labeled with 8-azido-[\(^{\alpha}{\text{32P}}\)]ATP but not with 8-azido-[\(^{\gamma}{\text{32P}}\)]ATP (20). We therefore examined whether SUR1-R1420C is photoaffinity-labeled with 8-azido-[\(^{\gamma}{\text{32P}}\)]ATP. Both NBF1 of SUR1 and SUR1-R1420C were photoaffinity-labeled with 8-azido-[\(^{\gamma}{\text{32P}}\)]ATP (Fig. 1, lanes 6 and 7), indicating that NBF1 either does not have ATPase activity or has little ATPase activity under our experimental conditions. In contrast, NBF2s of SUR1 and SUR1-R1420C were not photoaffinity-labeled with 8-azido-[\(^{\gamma}{\text{32P}}\)]ATP (Fig. 1, lanes 6 and 8), although they were photoaffinity-labeled by 8-azido-[\(^{\alpha}{\text{32P}}\)]ATP (Fig. 1, lanes 2 and 4). These results suggest that the \(\gamma\)-phosphate dissociates from 8-azido-[\(^{\gamma}{\text{32P}}\)]ATP bound at NBF2 and thus that NBF2 of SUR1-R1420C...
R1420C might have ATPase activity as is found for wild-type SUR1.

Affinity of NBFs of SUR1-R1420C for ATP and ADP—To characterize the biochemical properties of SUR1-R1420C further, we investigated the affinities of both NBF1 and NBF2 for ATP and ADP. When crude membranes from COS-7 cells expressing SUR1 or SUR1-R1420C were incubated with 50 μM 8-azido-[α-32P]ATP in the presence of ATP or ADP followed by mild trypsin digestion, photoaffinity labeling of tryptic fragments containing either NBF1 or NBF2 was inhibited in a concentration-dependent manner (Fig. 2). This indicates that SUR1 and SUR1-R1420C can bind ATP and ADP. The data were fit by the Hill equation, and the $K_i$ values obtained are shown in Table I. The $K_i$ values of NBF1 of SUR1 for ATP and ADP were $1.6 \pm 0.64$ (n = 3) and $17 \pm 7.9$ μM (n = 3) respectively, and those of SUR1-R1420C were $1.5 \pm 0.26$ (n = 3) and $13 \pm 6.8$ μM (n = 3), respectively. This indicates that the affinity of NBF1 for nucleotides is not significantly different between wild-type SUR1 and SUR1-R1420C and that the affinity for ATP is significantly higher than that for ADP. We have shown previously that the $K_i$ values for ATP and ADP binding to NBF1 of hamster SUR1 are $4.4 \pm 3.7$ and $26 \pm 8.6$ μM, respectively, and those of NBF2 are $60 \pm 26$ μM and $100 \pm 26$ μM, respectively (34). These values are in good agreement with the affinities obtained in this study for mouse SUR1, indicating that there are no significant differences between hamster and mouse SUR1. However, the $K_i$ values of NBF2 of SUR1-R1420C for ATP and ADP (350 ± 36 (n = 3) and $290 \pm 66$ μM (n = 3), respectively) were significantly higher than those of wild-type SUR1 (64 ± 4.7 (n = 3) and $65 \pm 16$ μM (n = 3), respectively). These results demonstrate that the R1420C mutation decreases the affinity of NBF2 for both ATP and ADP.

Cooperative Nucleotide Binding of SUR1-R1420C—To explore the possibility that the impaired cooperative nucleotide binding observed for SUR1-R1420C is due to the low nucleotide-binding affinity of NBF2, we examined the dependence of cooperative binding on nucleotide concentration. As we have shown previously (19), prebound 8-azido-[α-32P]ATP at NBF1 of wild-type SUR1 gradually dissociated during postincubation at 37 °C in the presence of Mg$^{2+}$ without nucleotide, and MgATP or MgADP stabilized 8-azido-ATP binding in a concentration-dependent manner (Fig. 3). This cooperative nucleotide binding was also observed when SUR1 was photoaffinity-labeled with 8-azido-[γ-32P]ATP (data not shown). $E_{C50}$ values for the stabilization effect of MgATP and MgADP were 59 ± 2.9 and 50 ± 18 μM, respectively, for SUR1 and are in good agreement with the binding affinities of MgATP and MgADP at NBF2 (64 ± 4.7 and $65 \pm 16$ μM, respectively) (Table I). This supports the idea that MgATP and MgADP bind to NBF2 to stabilize 8-azido-ATP binding at NBF1. In contrast, neither ATP nor ADP (1 mM) stabilized 8-azido-[32P]ATP binding at NBF1 of SUR1-R1420C despite the fact that 1 mM MgATP and MgADP inhibited photoaffinity labeling of NBF2 (68 ± 3.2 (n = 3) and $76 \pm 2.9$% (n = 3), respectively) as efficiently as that of wild-type SUR1 (74 ± 10 (n = 3) and $73 \pm 1.5$% (n = 3), respectively).
respectively) (Fig. 2). These results suggest that neither MgATP nor MgADP bound at NBF2 can stabilize 8-azido-ATP binding at NBF1 of SUR1-R1420C.

**Electrophysiological Analysis**—To examine the functional properties of the mutant K\textsubscript{ATP} channels, we coexpressed SUR1-R1420C with Kir6.2 in Xenopus oocytes. As observed for the wild-type channel (Kir6.2/SUR1), no significant current was detected for Kir6.2/SUR1-R1420C channels in the cell-attached configuration, but large currents developed following patch excision into nucleotide-free solution. The mean current amplitude at −100 mV was 3.0 ± 1.0 nA (n = 10) for Kir6.2/SUR1 and 1.5 ± 0.6 nA (n = 15) for Kir6.2/SUR1-R1420C channels. The smaller currents observed with the mutant channel are probably due to reduced expression of the mutant SUR (31). A quantitatively similar reduction in SUR1-R1420C protein expression was observed in COS-7 cells, indicating that the lower expression of mutant SUR1 is confined to the oocyte expression system.

**Pharmacological Regulation**—We first examined the effect of the R1420C mutation on the activation of the channel by the potassium channel opener diazoxide (Fig. 4) and its inhibition by the sulfonylurea tolbutamide. Diazoxide was tested in the presence of MgATP because its stimulatory action is dependent upon the presence of intracellular hydrolyzable nucleotides (35). In the presence of 100 μM ATP, both 200 μM diazoxide and 100 μM ADP led to a pronounced increase in both wild-type and mutant K\textsubscript{ATP} currents. Diazoxide increased Kir6.2/SUR1 currents by 694 ± 40% (n = 4) and Kir6.2/SUR1-R1420C currents by 499 ± 126% (n = 7). The sulfonylurea tolbutamide blocked Kir6.2/SUR1 currents by 53 ± 2% (n = 3) and Kir6.2/SUR1-R1420C currents by 68 ± 7% (n = 3). Thus the R1420C mutation does not affect the pharmacological properties of the channel.

**Nucleotide Regulation**—Inhibition of the channel by ATP was not affected by the R1420C mutation. Kir6.2/SUR1 currents were inhibited with an IC\textsubscript{50} of 13 ± 2 μM and a Hill coefficient of 0.99 ± 0.18 (n = 5), whereas Kir6.2/SUR1-R1420C currents were blocked with an IC\textsubscript{50} of 12 ± 2 μM and a Hill coefficient of 0.90 ± 0.14 (n = 6; Fig. 5).

**ADP** produced a mean current increase of 402 ± 82% (n = 3) and 347 ± 52% (n = 6) for Kir6.2/SUR1 and Kir6.2/SUR1-R1420C, respectively, when tested in the presence of 100 μM MgATP. ADP and GDP also activated both mutant and wild-

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**FIG. 3.** Cooperative binding of nucleotides and 8-azido-[α-32P]ATP to SUR1 and SUR1-R1420C. Membrane proteins (10–20 μg) from COS-7 cells expressing wild-type SUR1 or SUR1-R1420C were preincubated with 10 μM 8-azido-[α-32P]ATP for 3 min at 37 °C. Free 8-azido-[α-32P]ATP was then removed by washing the membranes with excess cold buffer. Proteins were UV-irradiated after a postincubation for 15 min at 37 °C in the absence of nucleotide (SUR1, square with cross; SUR1-R1420C, circle with cross), in the presence of ATP (SUR1, open squares; SUR1-R1420C, open circles), or in the presence of ADP (SUR1, closed squares; SUR1-R1420C, closed circles). The amount of photoaffinity-labeled 8-azido-[α-32P]ATP is given as a percentage of that obtained by UV irradiation without postincubation. Experiments were performed in triplicate, and the symbols show the mean value and the S.E.

**FIG. 4.** Diazoxide and nucleotide modulation of Kir6.2/SUR1-R1420C. A, macroscopic currents recorded from a giant patch on an oocyte co-injected with Kir6.2 and SUR1-R1420C mRNA. The holding potential was 0 mV, and the voltage was ramped successively from −110 mV to +100 mV over a 5-s period. Nucleotides and diazoxide (DZ) were added to the intracellular solution as indicated. The dashed line indicates the zero current level. B, mean macroscopic currents recorded from oocytes coexpressing Kir6.2 and either wild-type SUR1 or SUR1-R1420C in the presence of the nucleotides indicated. The mean conductance in the presence of nucleotide (G\textsubscript{i}) is expressed relative to the mean of that measured in control solution before and after removal of nucleotide (G\textsubscript{c}; indicated by the dashed line). Numbers of oocytes are given above the bars.

**FIG. 5.** ATP sensitivity of Kir6.2/SUR1-R1420C. Mean MgATP concentration-response relationships for Kir6.2/SUR1 (filled squares, n = 5) and Kir6.2-SUR1-R1420C (open squares, n = 6) currents are shown. Test solutions were alternated with control solutions, and the slope conductance (G) is expressed as a fraction of the mean of that obtained in control solution before and after exposure to MgATP (G\textsubscript{c}). The line is the best fit of the Hill equation (Equation 1) to the data using the mean values for IC\textsubscript{50} and h given in the text.
The presence of nucleotide (G) control solution lacking nucleotide (Gc) SUR1-R1420C (squares) presence of 100 μM nucleotide (G) that measured in control solution before and after removal of nucleotide (Gc) indicated by the bars. A, relationship between the MgADP concentration and the mean conductance (G) expressed relative to the mean conductance in 56M, which is in good agreement with the M of the nucleotides indicated. The mean conductance in the presence of nucleotide (G) is expressed relative to the mean of that measured in control solution before and after removal of nucleotide (Gc). The line is drawn according to Equation 2 using the parameter values given in the text.

Both Kir6.2-R50G/SUR1 and Kir6.2-R50G/SUR1-R1420C currents were activated by 100 μM MgATP and by 100 μM MgGTP (Fig. 6A), and in both cases, the extent of activation was greater for Kir6.2-R50G/SUR1-R1420C channels. Mutation of the conserved lysine 719 in NBF1 abolishes the stimulatory effect of MgATP (18). As Fig. 6A also shows, Kir6.2-R50G/SUR1-K719A currents were neither activated nor blocked by 100 μM MgATP, demonstrating that, at this ATP concentration, activation of Kir6.2-R50G/SUR1 and Kir6.2-R50G/SUR1-R1420C currents is not partially masked by an inhibitory effect of ATP. At ATP concentrations of 1 mM and above, however, Kir6.2-R50G/SUR1-K719A currents are inhibited. It is therefore not possible to construct the concentration-response curve for MgATP activation.

One possible explanation for the fact that ADP causes greater activation of Kir6.2-R50G/SUR1 and Kir6.2-R50G/SUR1-R1420C channels is that their open probability (p) is less than that of Kir6.2-R50G/SUR1 channels. The capacity for channel activation before the maximal p, is attained would therefore be greater. However, no clear differences in p were observed (Fig. 7). Kir6.2/SUR1 and Kir6.2/SUR1-R1420C channels had p of 0.29 ± 0.09 (n = 4) and 0.32 ± 0.03 (n = 5), respectively. When SUR1 or SUR1-R1420C was coexpressed with Kir6.2-R50G, the p was 0.35 ± 0.07 (n = 6) and 0.34 ± 0.07 (n = 7), respectively. Neither of these values differed significantly from that of Kir6.2/SUR1 channels (p > 0.05).

Whole-cell Studies—To explore the effect of metabolic inhibition on Kir6.2/SUR1 and Kir6.2/SUR1-R1420C channels, we recorded whole-cell KATP currents from intact oocytes and used 3 mM azide as a metabolic poison. In control solution, oocytes expressing both types of channels exhibited very small current amplitudes, similar to those of water-injected oocytes. As shown in Fig. 8, metabolic poisoning produced a large increase in both Kir6.2/SUR1 and Kir6.2/SUR1-R1420C currents. However, the amplitude of the azide-induced current, measured when the current reached a steady-state level (after 10–15 min of incubation), was significantly smaller for the mutant channel (Fig. 8A). The fact that Kir6.2/SUR1-R1420C currents induced by metabolic inhibition are smaller than those of the wild-type channel may reflect, at least in part, a lower level of expression of the mutant channel because in inside-out membrane patches the currents activated on patch excision were also smaller (Fig. 8B).

**DISCUSSION**

We analyzed the functional properties of SUR1 containing a PHH1 missense mutation, R1420C, and demonstrated that the mutation lowers the affinities of NBF2 for ATP and ADP and impairs the ability of MgATP and MgADP to stabilize ATP binding at NBF1. This cooperative nucleotide binding was not observed for SUR1-R1420C even with 1 mM MgATP or MgADP, although 1 mM MgATP and MgADP inhibited the photoaffinity labeling of NBF2 of wild-type SUR1 and SUR1-R1420C with similar efficiency. The affinities of NBF1s for ATP and ADP are similar for wild-type SUR1 and SUR1-R1420C. These results suggest that the R1420C mutation not only decreases the nucleotide-binding affinity of NBF2 but also impedes the transduction of a conformational change at NBF2 and thereby its ability to stabilize ATP binding at NBF1.

When Kir6.2-R50G and SUR1 were coexpressed in *Xenopus* oocytes, the EC50 for ADP activation of Kir6.2-R50G/SUR1 currents was 74 ± 30 μM, which is in good agreement with the

**FIG. 6. Nucleotide activation of Kir6.2/SUR1-R1420C.** A, mean macroscopic currents recorded from oocytes coexpressing Kir6.2-R50G and either wild-type SUR1, SUR1-R1420C, or SUR1-K719A in the presence of 100 μM of the nucleotides indicated. The mean conductance in the presence of nucleotide (G) is expressed relative to the mean of that measured in control solution before and after removal of nucleotide (Gc) indicated by the dashed line). Numbers of oocytes are given above the bars. B, relationship between the MgADP concentration and the mean conductance (G) expressed relative to the mean conductance in 56M, which is in good agreement with the M of the nucleotides indicated. The mean conductance in the presence of nucleotide (G) is expressed relative to the mean of that measured in control solution before and after removal of nucleotide (Gc). The line is drawn according to Equation 2 using the parameter values given in the text.
Our results suggest that the level of expression of Kir6.2/SUR1-R1420C K\textsubscript{ATP} channels in *Xenopus* oocytes is about half that of wild-type K\textsubscript{ATP} channels because mutant currents in excised patches were about half those of wild-type currents. We have previously reported that Rb\textsuperscript{+} efflux from COS-7 cells expressing Kir6.2/SUR1-R1420C K\textsubscript{ATP} channels is about half that of cells expressing wild-type K\textsubscript{ATP} channels when intracellular ATP is depleted by metabolic inhibition with 2-deoxyglucose and oligomycin. This was correlated with a reduced protein level in Western blots (31). Thus the R1420C mutation results in a reduced K\textsubscript{ATP} current whether expressed in oocytes or in mammalian cells. However, this cannot be the only explanation for the ability of the R1420C mutation to cause PHHI because heterozygotes carrying other PHHI mutations that result in a total loss of protein, which would also be expected to produce K\textsubscript{ATP} currents of about half the normal amplitude, do not develop PHHI. It is possible that the reduced affinity of NBF2 for nucleotides contributes to the PHHI phenotype.

We have proposed a model for nucleotide activation of the K\textsubscript{ATP} channel in which channel activation is induced by binding of ATP at NBF1 and of MgADP at NBF2 of SUR1 (19, 37). Recently, we analyzed the properties of NBFs of three subtypes of SUR (SUR1, SUR2A, and SUR2B) and showed that the affinities of both NBFs of SUR1 for ATP and ADP are significantly higher than those of SUR2A (34). Native pancreatic \( \beta \)-cell K\textsubscript{ATP} channels (composed of SUR1 and Kir6.2) open in the absence of extracellular glucose, whereas cardiac muscle K\textsubscript{ATP} channels (SUR2A and Kir6.2) remain closed (2–4). We have proposed that the greater ability of SUR1 to stimulate opening of K\textsubscript{ATP} channels when metabolism falls is linked to the higher nucleotide-binding affinities of NBF1 and/or NBF2. Our finding that the PHHI missense mutation R1420C lowers the affinity of NBF2 for ATP and ADP and increases the EC\textsubscript{50} for ADP activation of channel activity adds further support to the idea that ADP binding to NBF2 of SUR1 is important for K\textsubscript{ATP} channel stimulation.

Recently the three-dimensional structure of the catalytic domain of Rad50 (Rad50cd), a protein involved in repair of DNA double-strand breaks, has been reported (38). Rad50cd shows marked sequence similarity with NBFs of ATP-binding cassette proteins (39), suggesting a common three-dimensional structure and catalytic mechanism. X-ray crystallography revealed that Rad50cd forms a dimer and that two ATP molecules lie at the dimer interface, sandwiched between the Walker A motif of one Rad50cd subunit and the SGQQ signature of the partner subunit. It is proposed that ATP controls Rad50 function in DNA repair by a dimerization and dissociation cycle. Based on the structure of Rad50cd, arginine 1420 of SUR1 would not be expected to interact directly with ATP but instead to influence ATP binding allosterically. The crystallographic structure of Rad50cd also suggested that the PHHI missense mutation G1479R, which lies within NBF2 and abolishes MgADP stimulation of the K\textsubscript{ATP} channel (1), would be
involved in nucleotide binding at NBF1 rather than at NBF2 as shown in Fig. 9.

Based on the present studies and the structure of Rad50cd, we propose a new model for nucleotide activation of KATP channels (Fig. 9). We suggest that channel activation occurs when MgADP is bound to both NBFs of SUR1 (state 6) or when ATP is bound to NBF1 and MgADP to NBF2 (state 3). Currently it is not clear whether ATP binding to both NBFs (state 2) is also able to support channel activation because ATP hydrolysis takes place at NBF2. Although we have not succeeded in detecting ATP hydrolysis at NBF1 (20, 34) because NBF1 is photoaffinity-labeled with 8-azido-[γ-32P]ATP, we cannot exclude the possibility that ATP dissociates from NBF1 after hydrolysis rather than ATP/ADP exchange between state 1 and state 4 as shown in Fig. 9. Because the intracellular concentration of ATP is much higher than that of ADP in the intact cell, SUR1 is likely to exist predominantly in states 2 and 3.

**CONCLUSIONS**

Shyng et al. (29) analyzed the function of several PHHI missense mutations and reported that most mutations reduced the response to stimulation by MgADP, whereas others did not express functional channels. However, some mutations altered channel function only minimally in vitro but were associated with severe clinical diseases. In this study, we have demonstrated that the R1420C PHHI mutation strongly affects the biochemical properties of SUR1; it lowers the affinity of NBF2 for ATP and ADP and abolishes the ability of nucleotide binding at NBF2 to stabilize 8-azido-ATP binding at NBF1. It also decreases the expression of functional KATP channels. However, increase in the EC_{50} for MgADP activation of channel activity was observed only when measured by coexpression with Kir6.2-R50G. These results suggest that some PHHI missense mutations of SUR1 might directly or indirectly affect the interaction with nucleotides even though those mutations apparently alter channel properties only minimally when measured by coexpression with the wild-type Kir6.2. Finally, we suggest a new model for nucleotide activation of the KATP channel.

**Acknowledgments**—We thank Dr. Yoshitake Terano (Osaka City University) for preparing the antibodies against NBF1 and NBF2 of SUR1.

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