ATP binding without hydrolysis switches sulfonylurea receptor 1 (SUR1) to outward-facing conformations that activate $K_{ATP}$ channels

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Neuroendocrine-type ATP-sensitive $K^+$ ($K_{ATP}$) channels are metabolite sensors coupling membrane potential with metabolism, thereby linking insulin secretion to plasma glucose levels. They are octameric complexes, (SUR1/Kir6.2)$_4$, comprising sulfonylurea receptor 1 (SUR1 or ABCC8) and a $K^+$-selective inward rectifier (Kir6.2 or KCN11). Interactions between nucleotide-, agonist-, and antagonist-binding sites affect channel activity allosterically. Although it is hypothesized that opening these channels requires SUR1-mediated MgATP hydrolysis, we show here that ATP binding to SUR1, without hydrolysis, opens channels when nucleotide antagonism on Kir6.2 is minimized and SUR1 mutants with increased ATP affinities are used. We found that ATP binding is sufficient to switch SUR1 alone between inward- or outward-facing conformations with low or high dissociation constant, $K_{ATP}$ values for the conformation-sensitive channel antagonist [3H]glibenclamide ([3H]GBM), indicating that ATP can act as a pure agonist. Assembly with Kir6.2 reduced SUR1’s $K_D$ for [3H]GBM. This reduction required the Kir N terminus (KnTn), consistent with KnTn occupying a “transport cavity,” thus positioning it to link ATP-induced SUR1 conformational changes to channel gating. Moreover, ATP/GMB site coupling was constrained in WT SUR1/WKIR6.2 channels; ATP-bound channels had a lower $K_D$ for [3H]GBM than ATP-bound SUR1. This constraint was largely eliminated by the Q1179R neonatal diabetes-associated mutation in helix 15, suggesting that a “swapped” helix pair, 15 and 16, is part of a structural pathway connecting the ATP/GMB sites. Our results suggest that ATP binding to SUR1 biases $K_{ATP}$ channels toward open states, consistent with SUR1 variants with lower $K_D$ values causing neonatal diabetes, whereas increased $K_D$ values cause congenital hyperinsulinism.

Neuroendocrine-type ATP-sensitive $K^+$ ($K_{ATP}$)$^2$ channels comprise an ATP-binding cassette (ABC) protein (1), ABCC8/SUR1, and a $K^+$-selective inward rectifier (2), KCNJ11/Kir6.2, assembled as heterooctamers (3–5), (SUR1/Kir6.2)$_4$. In pancreatic β-cells, these channels are metabolite sensors that couple cellular metabolism with membrane electrical activity to link insulin secretion with blood glucose levels. This coupling is critical for normal physiology; loss of channel function is a cause of congenital hyperinsulinism (Ref. 6; for reviews, see Refs. 7 and 8), whereas gain-of-function mutations in Kir6.2 (9) and SUR1 (10) cause neonatal diabetes (ND) (for reviews, see Refs. 11 and 12). Gain of function is one cause of mature-onset diabetes of the young (13), whereas a ABCC8/SUR1 polymorphism, the Ala amino acid allele at p.A1369S, is associated with an increased risk for type 2 diabetes (14).

Channel activity is regulated positively by ATP and ADP binding to SUR1 and negatively by nucleotide binding to Kir6.2 (15–20). Additionally, multiple metabolites, including phosphoinositides (21–23) and long-chain acyl-CoA esters (24–26), and phosphorylation (27–29) positively modulate channel activity. Pharmacologic modulation of SUR1 by channel antagonists, sulfonylureas like glibenclamide (GBM) and glinides like repaglinide (30–36), and by agonists like diazoxide (Refs. 31 and 37–39; for a review, see Ref. 40) is clinically important. These physiologic and pharmacologic modulators all affect channel gating via allosteric interactions in the sense that their binding sites on SUR1 are coupled to and known to be physiologically distinct from one another based on structural studies (41, 42). The available data are consistent with the idea that SUR1 exists in multiple conformations differing in affinity for ligands and ability to activate channel openings.

How nucleotides regulate $K_{ATP}$ channels remains an open question. Early electrophysiological studies (15) showed that although ATP inhibited activity, MgADP, acting through SUR1, activated channel openings (16–18, 20). The finding that SUR1 was in the ABC protein family (1), whose members are ATPases that use the energy of ATP binding and hydrolysis to transport substrates across membranes (43–45), suggested that

ND, neonatal diabetes; AMPPNP, $S’$-adenosyl-$β,γ$-imidotriphosphate; AMP-PCP, $S’$-adenosine $S’$-methylene-triphosphate; ATPγS, $S’$-adenosine $S’$-$O$-(thiotriphosphate); NBD, nucleotide-binding domain; TMD, transmembrane helical domain; $K_{diss}$, dissociation constant for $K^+$; $K_{diss}$, dissociation constant for ATP; $K_{diss}$, allosteric constant; $K_{diss}$, dissociation constant for ADP.

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ATP is a $K_{ATP}$ channel agonist

ATP hydrolysis on SUR1 might generate a conformation that activated channel openings. The idea that a "posthydrolytic," MgADP-bound conformation of SUR1, generated as part of an ABC enzymatic cycle, uniquely activates channel openings was most concisely described by Zingman et al. (46, 47) and Bienegraeber et al. (48) and reviewed in Zingman et al. (49). The idea was supported by experiments with nonhydrolyzable analogs, e.g. AMPPNP, AMPPCP, and ATPγS, and by reports that the action of potassium channel agonists, e.g. diazoxide for SUR1-based channels and a pinacidil analog, P1075, for related SUR2-based channels, required ATP hydrolysis to reach an activating state (50–52).

The need for hydrolysis was questioned by Choi et al. (53), who failed to find the changes in microscopic reversibility and detailed balance expected for strong coupling, and by Ortiz et al. (54, 55), who used $[^3H]GBM$ to show that ATP binding was enough to switch SUR1, in the absence of Kir6.2, between conformations with high and low affinities for the ligand. In other ABC proteins, ATP binding to the nucleotide-binding domains (NBDs) results in NBD dimerization and a reconfiguration of the transmembrane helical domains (TMDs) from "inward- to outward-facing" states. Thus, Ortiz et al. (54) proposed that outward-facing, ATP-bound conformations of SUR1 with lowest affinity for $[^3H]GBM$ and highest affinity for the agonist diazoxide would activate channel openings without the need for hydrolysis. We tested this proposal here using $K_{ATP}$ channels whose Kir6.2 pores are not inhibited by ATP.

In other ABC proteins, hydrolysis of ATP is essential to "reset" the transporter conformation and allow cyclic substrate transport, but the function of the low rate of hydrolysis reported for SUR1 is not yet clear (56, 57). Despite its structural relation to other ABC transporters, SUR1 is usually assumed to be a "regulatory protein," not a transporter. However, the localization of GBM bound in the central cavity of SUR1 by cryo-EM (41, 42) together with earlier studies aimed at defining the GBM-binding site and defining the role(s) of the N terminus, KNtp, of Kir6.2 in GBM binding and channel gating (for a review, see Ref. 40) suggests that the transport idea deserves revisiting. The implication is that SUR1 is a "cryptic," albeit frustrated, peptide transporter whose "substrate" is KNtp. This idea suggests that evolution has linked two fundamental transport mechanisms, ATP-driven substrate transport and ion transport, to couple cellular metabolism with membrane potential, thus effectively marrying the transport functionality of an ATP-binding cassette protein, ABCC8/SUR1, with the potential, thus effectively marrying the transport functionality, to couple cellular metabolism with membrane port mechanisms, ATP-driven substrate transport and ion transport.

We have focused on ATP-driven conformational changes in SUR1, including testing the need for ATP hydrolysis to activate channel openings and defining the properties of different SUR1 conformations. We show that when the inhibitory action of ATP on the Kir6.2 pore is eliminated, ATP$^{4-}$ acts as a classic channel agonist even when potential nucleotide hydrolysis is minimized by limiting Mg$^{2+}$ and/or by substitution of glutamine for the catalytic glutamate, SUR1E1507Q, needed for hydrolysis. We confirm that outward-facing conformations of SUR1 alone, i.e. without Kir6.2, have reduced affinity for $[^3H]GBM$ and show that assembly with full-length Kir6.2 increases the affinity and attenuates the negative allosteric effect of ATP on GBM interactions (58). One ND mutation, SUR1Q1179R, in transmembrane helix 15 near the GBM-binding site markedly increases coupling between the GBM and ATP sites. This suggests that helix 15 and helix 16, which cross over from TMD2 to contact NBD1, are part of a structural pathway or network connecting these sites. MgADP, like MgATP, efficiently switches the conformation of SUR1 alone but has no significant effect on $[^3H]GBM$ binding to wildtype (WT) SUR1 in channels. The results support the hypothesis that ATP acts as an agonist by switching SUR1 from inactive inward-facing to active outward-facing conformations that bias channels toward open states.

Results

ATP activation of SUR1/Kir6.2$^{G334D}$ $K_{ATP}$ channels

To test whether ATP can act directly as a channel agonist, the inhibitory action of ATP binding to the Kir6.2 pore was minimized by coexpressing SUR1 with a mutant pore subunit, Kir6.2$^{G334D}$. This substitution, identified in cases of neonatal diabetes (59), reduces the affinity of Kir6.2 for inhibitory ATP, thus minimizing ATP antagonism (60–62). Fig. 1, A and B, confirm that ATP$^{4-}$ (10 mM) or MgATP (1 mM) reduce the activity of WT SUR1/WT Kir6.2 channels. In contrast, MgATP acts as an agonist on WT SUR1/Kir6.2$^{G334D}$ channels, increasing the product of the open probability ($P_o$) and number (N) of open channels, $N_P_o$, 7–8-fold (Fig. 1C). The different nucleotide concentrations were chosen because SUR1 has a significantly lower $K_D$ for MgATP versus ATP$^{4-}$ as determined by their respective abilities to stabilize conformations that bind MgATP more weakly (54, 55). WT Kir6.2 has estimated $K_D$ values of ~5–20 μM (19, 63, 64) for inhibitory ATP ($\pm $Mg$^{2+}$) and should be nearly saturated in either case. The agonist action is reversible: after washout of MgATP, channel activity rapidly returns to basal levels. The application of ATP$^{4-}$ (10 mM) had no significant effect on channel open probability.

ATP$^{4-}$ activation of SUR1$^{Q1179R}$/Kir6.2$^{G334D}$ $K_{ATP}$ channels

Several ND SUR1 mutant receptors, including SUR1$^{Q1179R}$, have lower $K_P$ values for nucleotides based on the allosteric effect(s) of ATP$^{4-}$ or MgATP on $[^3H]GBM$ binding (54, 55, 65). SUR1$^{Q1179R}$/Kir6.2$^{G334D}$ channels were used to determine whether nucleotide binding, without the Mg$^{2+}$ cofactor needed for ATP hydrolysis (56), was enough to activate channel openings. Fig. 2, A and D, show that the $N_P_o$ of spontaneously active SUR1$^{Q1179R}$/Kir6.2$^{G334D}$ channels is increased significantly by ATP$^{4-}$ (10 mM), consistent with nucleotide binding being enough to stabilize conformations of SUR1 that activate channel openings. Together with Fig. 1C, the results show that, in the absence of antagonism at Kir6.2, MgATP and more importantly ATP$^{4-}$ are channel agonists, albeit agonists with markedly different $K_D$ values.

GTP$^{4-}$ activation of SUR1$^{Q1179R}$/Kir6.2$^{G334D}$ $K_{ATP}$ channels

We tested whether GTP$^{4-}$, without Mg$^{2+}$, was enough to activate SUR1$^{Q1179R}$/Kir6.2$^{G334D}$ channels. Fig. 2, B and E,
show that GTP\(^{4-}\) (10 mM), like ATP\(^{4-}\), significantly increases channel activity. The results suggest that a lower \(K_D\) for nucleotides may underlie the increased activation of the SUR1\(_{E1507Q}\)/Kir6.2\(_{G334D}\) variant associated with type 2 diabetes risk (14) rather than the proposed altered rate of ATP hydrolysis (66, 67).

**ATP\(^{4-}\) activation of SUR1\(_{E1507Q}/\text{Kir6.2}_{G334D}\) K\(_{ATP}\) channels**

To test further whether nucleotide binding is sufficient to select and stabilize SUR1-activating conformers, a glutamine was substituted for the "catalytic" glutamate in NBD2. This substitution strongly reduces ATPase and transport activity in related proteins (68, 69), and Glu → Gln substitutions have been used to trap ATP-bound conformations for structural studies (70–72). Fig. 2, C and F, show that SUR1\(_{E1507Q}/\text{Kir6.2}_{G334D}\) channels, lacking the catalytic glutamate and Mg\(^{2+}\), are significantly activated by ATP\(^{4-}\). The results imply that ATP\(^{4-}\) and GTP\(^{4-}\) are channel agonists whose binding energies are enough to shift the population of SUR1 conformers toward those that increase channel openings.

**ATP\(^{4-}\) supports the action of K\(_{ATP}\) channel agonists**

To assess whether ATP binding is sufficient to support the action of channel openers, the ability of diazoxide to activate SUR1\(_{E1507K}/\text{WT}\) Kir6.2 channels was tested. The SUR1\(_{E1507K}\) mutation was used because congenital hyperinsulinism patients with this Glu → Lys mutation respond to diazoxide (73), and, without the catalytic glutamate, SUR1\(_{E1507K}/\text{WT}\) Kir6.2 channels are expected to have impaired ATP hydrolysis. Fig. 3A shows that when patches from cells expressing SUR1\(_{E1507K}/\text{WT}\) Kir6.2 channels are pulled into nucleotide-free medium, channels are activated as nucleotide antagonism on their WT pores is reduced. Application of ATP\(^{4-}\) rapidly inhibits channel activity as expected, but coapplication of diazoxide shows that ATP\(^{4-}\) does support its agonist action. Fig. 3B confirms that MgATP inhibits channel activity and
supports the action of diazoxide as expected from patient responses. The response to diazoxide is consistent with the positive allosteric coupling between channel opener and nucleotide-binding sites seen with SUR1 alone where diazoxide stabilizes nucleotide-bound conformations (55).

Assembly with Kir6.2 lowers the dissociation constant, $K_G$, of SUR1 for GBM

We asked how assembly with Kir6.2 affects the GBM-binding site and the intramolecular linkage between the ATP- and GBM-binding sites within the receptor. The dissociation constant, $K_G$, values for $[^3]$HGBM binding to receptors and channels are needed to assess the impact of adding nucleotides; thus, $K_G$ values were determined from homologous competition experiments where $[^3]$HGBM binding is competed by increasing concentrations of unlabeled GBM. The results extend earlier studies in isolated membranes (75) and live cells (76) showing that $K_{ATP}$ channels bind $[^3]$HGBM more tightly than SUR1 alone. Binding assays were done in the absence of ATP and Mg$^{2+}$ with 1 mM EDTA added. The thermodynamic cycle used to estimate GBM stabilization of $K_{ATP}$ channels. Binding assays were done without Mg$^{2+}$ and ATP, conditions where cryo-EM studies show SUR1 in inward-facing conformations with GBM bound and a closed Kir6.2 pore (77, 78). Under these conditions, WT channels have approximately a 7.2-fold lower $K_G$ for GBM. The average $K_G$ value, $0.8$ kcal/mol (mean ± S.E., $n = 9$), derived from the respective dissociation constants. The average ΔΔ$G$ value, $−0.8$ kcal/mol, was estimated from the difference in free energies, ΔΔ$G = −1.2 ± 0.1$ kcal/mol (mean ± S.E., $n = 9$), derived from the respective dissociation constants. The average $ΔΔG$ value, $−0.8$ kcal/mol, was estimated from the difference in free energies, ΔΔ$G = −1.2 ± 0.1$ kcal/mol (mean ± S.E., $n = 9$), derived from the respective dissociation constants.

Figure 3. Activation of SUR1E1507K/WT Kir6.2 channels associated with congenital hyperinsulinism by the agonist diazoxide. At the arrows, patches were pulled into nucleotide-free medium, which activates a large number of channels as inhibitory nucleotides leave the pore. Application of ATP$^{4−}$ (10 mM) (A) or MgATP (1 mM) (B) rapidly inhibits channel activity. Concurrent application of diazoxide (340 μM) increases channel activity in both cases. Activation was observed in five of five trials for ATP$^{4−}$ and for MgATP.

Figure 4. Assembly with Kir6.2 increases the affinity of SUR1 for $[^3]$HGBM. A–C, dissociation constants, $K_G$ values, for $[^3]$HGBM were determined by displacement of 0.3 (blue) or 1 (red) nM $[^3]$HGBM by unlabeled GBM. D, the thermodynamic cycle used to estimate GBM stabilization of $K_{ATP}$ channels. Binding assays were done in the absence of ATP and Mg$^{2+}$ with 1 mM EDTA added.
kcal/mol, for all the WT and mutant channels is somewhat lower. The structural changes underlying the increased stability of GBM–channel complexes are not well defined, but previous studies implicate the N terminus of Kir6.2, KNtp, as a major factor in the higher affinity of SUR1/Kir6.2 channels versus SUR1 for GBM (Refs. 75 and 76 and “Discussion”).

Consistent with our earlier results (54, 55) and cryo-EM structures (41, 42, 77, 78), the high affinity of GBM binding strongly selects and stabilizes inward-facing conformations of SUR1 with a $\Delta G^0$ value of approximately $-12.4$ or $-13.6$ kcal/mol for receptors or channels, respectively. The difference in binding energies of SUR1 versus SUR1/Kir6.2 reflects an increased stability of GBM-bound versus unliganded channels.

We used the thermodynamic cycle shown in Fig. 4D to estimate the increased stability. We assumed channels and subunits are in equilibrium and that GBM interacts with different energies with SUR1 alone versus SUR1 in channels (data are summarized in Table 1). The products of the dissociation constants along the two branches forming GBM–channel complexes must be equal. $K_{\text{ch}0}$, the dissociation constant for channels into subunits, is not known but must be low because channels are stable and complexes can be isolated (41, 42, 57, 77–79). The $K_G$ values for WT receptors versus channels gave an estimated value for $\alpha$ of $-0.14$, indicating channels complexed with GBM are $\sim 7$ times more stable than unliganded $K_{\text{ATP}}$ channels. The average stabilization, for all channels versus receptors, is a $4$-fold increase based on the average $\Delta \Delta G$ value.

Assembly with Kir6.2 alters the allosteric coupling between the GBM and ATP-binding sites in WT SUR1

Table 1 shows that assembly with Kir6.2 alters the SUR1 GBM-binding site, increasing its affinity for GBM. The $K_G$ values were used to assess how channel assembly alters the negative allosteric coupling between GBM- and ATP-binding sites. The effects of increasing concentrations of MgATP on the binding of fixed concentrations of $[3H]$GBM were compared for SUR1 alone or SUR1/Kir6.2 channels. The GBM- and ATP-binding sites are physically distinct; thus, a reduction in bound $[3H]$GBM indicates negative allosteric coupling between these two binding sites. Fig. 5 shows stronger negative coupling for SUR1 alone than for SUR1/Kir6.2 channels. In both cases, a significant fraction of bound $[3H]$GBM remains at the highest, saturating concentrations of MgATP, but the fraction remaining is significantly lower for SUR1 alone; assembly with Kir6.2 markedly attenuates the ability of nucleotides to reduce $[3H]$GBM binding to SUR1/Kir6.2 channels versus SUR1 alone (58). Our previously described ternary complex model (54), shown in Fig. 5, inset, was used to interpret the data. In this model, the receptor (R) is presumed to have coupled GBM- and ATP-binding sites. The coupling factor, $\beta$, is a measure of the interaction between sites; $\beta = 1$ equates to no coupling, whereas $\beta > 1$ reflects an increase in the $K_D$ for the binding of one ligand when the opposite ligand-binding site is occupied. In this model, the fraction of bound $[3H]$GBM remaining at saturating concentrations of nucleotide reflects the higher $K_D$ ($= \beta K_G$), i.e. a reduced affinity, of MgATP-bound SUR1 for $[3H]$GBM.

The parameters $K_D$, the dissociation constant for ATP, and $\beta$, the allosteric constant, were estimated in two ways using Equation 1 given under “Experimental procedures.” In both cases, the individual $K_G$ values were fixed (parameters in Table 2). In one case, the two data sets were fit simultaneously (globally). This assumes the $K_D$ for ATP-binding is the same for both SUR1 and channels and yields a single $K_G$ value and best-fit estimates of $\beta$ for SUR1 alone versus channels. In the alternative case, $K_D$ and $\beta$ were estimated independently for each data set. The two estimates are nearly identical (Table 2). The curves in Fig. 5 were generated using the global parameters, but curves generated using the independent parameters are visually the same. In this four-state model, the fraction of bound $[3H]$GBM for SUR1 alone reflects the $\sim 5$-fold lower affinity of SUR1-MgATP–binding complexes for $[3H]$GBM. The data in Table 1 show that channels have $\sim 7$-fold higher affinity for $[3H]$GBM, which is reflected in the larger fraction of bound $[3H]$GBM seen in ATP-bound SUR1/Kir6.2 channels. It is worth noting that

![ATP is a $K_{\text{ATP}}$ channel agonist](image)

**Table 1**

| Sample                  | Mean | CI lower–upper | $p$ value |
|-------------------------|------|----------------|-----------|
| WT SUR1 alone           | 1.8  | 1.4–2.2        | $<0.001$  |
| WT SUR1/WT Kir6.2       | 0.25 | 0.2–0.3        | $<0.001$  |
| WT SUR1/Kir6.2 E1507Q   | 0.4  | 0.3–0.5        | $<0.001$  |
| SUR1 E1507Q/Kir6.2      | 3.9  | 3.2–4.8        | $<0.001$  |
| SUR1 E1507Q/Kir6.2      | 1.1  | 0.7–1.6        | $<0.001$  |
| SUR1 E1507Q/WT Kir6.2   | 1.1  | 0.8–1.5        | $<0.001$  |
| SUR1 Q1179R/Kir6.2      | 1.7  | 1.4–2.0        | $<0.001$  |
| SUR1 Q1179R/Kir6.2      | 0.6  | 0.5–0.9        | $<0.001$  |
| SUR1 Q1179R              | 0.8  | 0.4–1.5        | $<0.001$  |
| SUR1 Q1179R/Kir6.2      | 0.3  | 0.2–0.5        | $<0.001$  |

**Figure 5.** Association with Kir6.2 affects the allosteric properties of WT SUR1. Increasing MgATP reduces the binding of $[3H]$GBM (1 nM). The data are given as specific bound $[3H]$GBM defined as $[3H]$GBM bound in the presence of MgATP divided by the radioactivity bound without nucleotide. The inset shows the four-state ternary complex model. $R$ is defined as SUR1 with ATP bound to NBD1, the noncanonical nucleotide-binding site. ATP can induce NBD dimerization by binding to NBD2 with or without bound GBM. The $K_G$ values from Fig. 4, estimated in the absence of ATP, were used to estimate $\beta$ and $K_G$. The curves were generated using the global-fit parameters in Table 2; data are means with error bars indicating ±S.E., $n = 6$. The dissociation constant for $[3H]$GBM binding to SUR1/Kir6.2 channels versus SUR1 alone (58). Our previously described ternary complex model (54), shown in Fig. 5, inset, was used to interpret the data. In this model, the receptor (R) is presumed to have coupled GBM- and ATP-binding sites. The coupling factor, $\beta$, is a measure of the interaction between sites; $\beta = 1$ equates to no coupling, whereas $\beta > 1$ reflects an increase in the $K_D$ for the binding of one ligand when the opposite ligand-binding site is occupied. In this model, the fraction of bound $[3H]$GBM remaining at saturating concentrations of nucleotide reflects the higher $K_D$ ($= \beta K_G$), i.e. a reduced affinity, of MgATP-bound SUR1 for $[3H]$GBM.

The parameters $K_D$, the dissociation constant for ATP, and $\beta$, the allosteric constant, were estimated in two ways using Equation 1 given under “Experimental procedures.” In both cases, the individual $K_G$ values were fixed (parameters in Table 2). In one case, the two data sets were fit simultaneously (globally). This assumes the $K_D$ for ATP-binding is the same for both SUR1 and channels and yields a single $K_G$ value and best-fit estimates of $\beta$ for SUR1 alone versus channels. In the alternative case, $K_D$ and $\beta$ were estimated independently for each data set. The two estimates are nearly identical (Table 2). The curves in Fig. 5 were generated using the global parameters, but curves generated using the independent parameters are visually the same. In this four-state model, the fraction of bound $[3H]$GBM for SUR1 alone reflects the $\sim 5$-fold lower affinity of SUR1-MgATP–binding complexes for $[3H]$GBM. The data in Table 1 show that channels have $\sim 7$-fold higher affinity for $[3H]$GBM, which is reflected in the larger fraction of bound $[3H]$GBM seen in ATP-bound SUR1/Kir6.2 channels. It is worth noting that...
**ATP is a $K_{ATP}$ channel agonist**

**Table 2**

**Summary of binding parameters from Fig. 5**

Data are given as mean values and 95% confidence intervals (lower–upper values).

| Species                  | $K_G$  | $\beta$ | $K_T$ | $\beta K_G$ | $\beta K_T$ |
|--------------------------|--------|---------|-------|-------------|-------------|
| **Global fits**          |        |         |       |             |             |
| SUR1                     | 1.8 (1.5–2.2) | 5.0 (4.5–5.6) | 34.1 (29.6–40.4) | –9 | –170 |
| SUR1/Kir6.2              | 0.25 (0.19–0.32) | 2.2 (2.0–2.4) | 34.1 (29.6–40.4) | –0.6 | –75 |
| **Independent fits**     |        |         |       |             |             |
| SUR1                     | 1.8 (1.5–2.2) | 5.0 (4.5–5.6) | 34.4 (27.7–42.6) | –9 | –170 |
| SUR1/Kir6.2              | 0.25 (0.19–0.32) | 2.2 (2.0–2.4) | 39.4 (22.3–69.7) | –0.6 | –86 |

this negative allosteric effect is reciprocal; GBM–SUR1, alone or in channels, binds MgATP more weakly (compare $K_T$ versus $\beta K_G$ values in Table 2).

**SUR1$_{Q1179R}$, an ABCC8 neonatal diabetes mutation, affects allosteric coupling**

Assembly with Kir6.2 markedly affects the coupling between the ATP and GBM sites in WT SUR1 (Fig. 5), but there are no informative data available on the structural pathway(s) or amino acid network(s) linking these sites. Analyses of coupling in ABCC8/SUR1 mutants provides one approach to identify amino acids in potential pathways. We tested whether the Gln $\rightarrow$ Arg substitution, SUR1$_{Q1179R}$, might affect coupling. Position 1179 is near the top of helix 15, within 20 nm of the GBM-binding site (41). Helices 15 and 16 are the pair of helices from TMD2 that cross over to interact with NBD1. Intuitively, this “swapped” pair of helices is a potential pathway or link between the ATP- and GBM-binding sites. Fig. 6 compares the reduction of bound [3H]GBM by MgATP from WT SUR1/WT Kir6.2 versus ND mutant SUR1$_{Q1179R}$/WT Kir6.2 channels. The Gln $\rightarrow$ Arg substitution largely reverses the constraint imposed by assembly with Kir6.2 (Fig. 5). In this experiment, the WT control data set includes the channel data shown in Fig. 5. The curves are based on fits to the ternary complex model (Fig. 5, inset) with values for WT channels of $K_G = 0.25$ (0.19–0.32) nM, $\beta = 2.2$ (2.1–2.3), and $K_T = 38$ (27.1–53.6) $\mu$M and for the SUR1$_{Q1179R}$/WT Kir6.2 channels of $K_G = 1.1$ (3.2–4.8) nM, $\beta = 7.3$ (6.3–8.6), and $K_T = 18$ (14.9–21.9) $\mu$M. Values are given as means with 95% confidence intervals (lower-upper values).

**Effects of MgADP on SUR1 versus SUR1/Kir6.2**

MgADP increases the open probability of WT $K_{ATP}$ channels and stabilizes more outward-facing receptor conformations (42); thus, the effect of MgADP on GBM binding was determined for WT SUR1 versus WT SUR1/WT Kir6.2 channels. Ortiz et al. (54) used an excess of MgAMP to minimize conversion of added ADP to ATP by adenylate kinase and reported that MgADP stabilized SUR1 conformations with lower affinity for GBM somewhat better than MgATP. We added hexokinase plus glucose to reduce low levels of contaminant ATP and to convert any generated ATP to ADP. Under these conditions, MgADP was nearly as effective at switching the conformation of WT SUR1 as MgATP (dissociation constant for ADP, $K_{ADP}$, 35–55 (38–107) versus $K_T$ ~38 (24–52) $\mu$M, respectively) with a $\beta$ value of 1.8 (1.6–1.9) but had no significant effect on the GBM interaction with channels (Fig. 7).

**Discussion**

ATP has dual effects on (SUR1/Kir6.2)$_4$ neuroendocrine $K_{ATP}$ channels. Nucleotide interactions with the Kir6.2 pore inhibit channel openings, whereas binding to SUR1, including potential enzymatic activities, activate openings. This study focused on the ATP-binding cassette subunit ABCC8/SUR1, whether enzymatic hydrolysis of ATP by SUR1 is needed for channel activation, identifying which receptor conformation(s) activates channel openings, and the allosteric pathways coupling the SUR1 ATP- and GBM-binding sites.

**ATP binding to SUR1 is sufficient to activate $K_{ATP}$ channels**

Prior results (53–55) and the data in Figs. 2 and 3 show that strong coupling between ATP hydrolysis and channel gating is not required. ATP binding, a key part of any ABC protein enzymatic cycle, is enough to switch SUR1 to activating conformations. Like other ABC proteins, SUR1 interacts with two ATPs with differing affinities (80). We assume that NBD1, with a higher affinity for ATP ($\pm$ Mg$^{2+}$), is occupied and that ATP binding at NBD2 results in NBD dimerization and a repositioning of TMDs 1 and 2 from inward- to more outward-facing conformations as confirmed by cryo-EM structures of $K_{ATP}$
channel openings (Fig. 2 and 3). This ATP-induced conformational change noncompetitively reduces \(^{[3H]}\text{GBM}\) binding to SUR1 alone or in \(K_{\text{ATP}}\) channels (Figs. 5–7). The reduction of bound \(^{[3H]}\text{GBM}\) by ATP is incomplete; at high, saturating concentrations of ATP, the remaining bound fraction reflects a new, low-affinity equilibrium between \(^{[3H]}\text{GBM}\) and ATP-bound SUR1 either alone or in channels. We used a four-state equilibrium model (Fig. 5, inset, and Equation 1) to quantify the allosteric reduction of bound \(^{[3H]}\text{GBM}\) by ATP and to evaluate the effect(s) of pairing SUR1 with Kir6.2 on this allosteric coupling. Equation 1 relates bound \(^{[3H]}\text{GBM}\) to the concentrations of GBM and ATP, their respective dissociation constants (\(K_G\) for \(^{[3H]}\text{GBM}\) and \(K_T\) for nucleotides), and an allosteric coupling constant, \(\beta\). The products, \(\beta K_G\) and \(\beta K_T\), reflect the dissociation constants for ATP-bound and GBM-bound complexes, respectively. As described above, we assume ATP binding at NBD2 drives the conformational change (54, 55). In structural terms, this reflects switching SUR1 from inward-facing, nucleotide-free “apo” or singly liganded states with the greatest affinity for GBM to outward-facing, ATP-bound states with lower affinity.

To apply the four-state model, the dissociation constants for \(^{[3H]}\text{GBM}, K_G\), are determined independently (Fig. 4 and Table 1) and assumed constant when fitting the ATP inhibition data. Determinations of \(K_T\) and \(\beta\) for WT SUR1 alone or in channels (Fig. 5 and Table 2) indicate that the affinities of ATP-bound SUR1 alone or in channels for \(^{[3H]}\text{GBM}\) are \(-5\)–\(-2.2\)-fold weaker than the unliganded molecules, respectively (compare \(K_G\) versus \(\beta K_G\) values in Table 2). Thus, the greater reduction in bound \(^{[3H]}\text{GBM}\) seen for SUR1 alone reflects the \(-5\)-fold weaker affinity of ATP-bound SUR1 for \(^{[3H]}\text{GBM}\). The lesser ATP effect observed for channels reflects the smaller change in affinities. The negative allosteric effects are reciprocal; GBM-bound SUR1 alone or in channels has a corresponding lower affinity for ATP (compare \(K_T\) versus \(\beta K_T\) values in Table 2). The data have two implications. First, the results imply that the attenuation of the agonist action of ATP underlies the inhibitory effect of GBM on WT \(K_{\text{ATP}}\) channels. Basically, ATP binding to SUR1 biases channels toward open states; reducing the affinity of SUR1 for ATP reduces channel activity. Second, the negative allosteric linkage is clinically important; glibenclamide is now a common therapy for the treatment of cases of neonatal diabetes (85), and GBM therapy will attenuate the excess agonist action of ATP on ABCC8/SUR1 mutations with higher affinities for ATP. Thus, GBM therapy attenuates the consequences of neonatal diabetes ABCC8 mutations with higher affinities for ATP by reducing their affinities for nucleotides.

We applied the four-state model to assess how MgADP affects ATP/GBM site coupling with somewhat surprising
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results. In agreement with earlier studies (54), MgADP did stabilize lower-affinity conformers of SUR1, but the effect on full channels was negligible (Fig. 7). The estimated $K_{\text{ADP}}$ for MgADP binding to SUR1 alone was like the $K_4$ for MgATP, but the allosteric factor, $\beta = 1.8$, was low, and the reduction in bound $[^3\text{H}]$GBM was less than for MgATP (Fig. 7). The result suggests that the energy of MgADP binding at both NBD1 and NBD2 may not be enough to stabilize SUR1 conformers in full channels that have a lower $K_4$ for $[^3\text{H}]$GBM; ATP binding to NBD1 may be needed. Structural studies on channels with both GBM and ADP present and/or bound are needed to determine whether GBM prevents dimerization of the NBDs or affects reconfiguration of the TMDs to activating conformations.

A potential structural pathway coupling the SUR1 ATP- and GBM-binding sites

Our results (Fig. 5 and Refs. 54, 55, and 65) and early studies on the effects of nucleotides on $[^3\text{H}]$GBM binding clearly demonstrate negative allosteric effects (51, 58, 86–88). However, essentially nothing is known about possible amino acid networks or structural pathways coupling the ATP-binding sites (NBDs) with the GBM-binding site in SUR1. One obvious candidate is the pair of “crossover” helices (15 and 16) that link NBD1 with TMD2 in SUR1 (57, 77, 78) and other ABC proteins. We tested this possibility using SUR1Q1179R, a well-studied neonatal diabetes mutation (54, 55, 65, 89, 90). The Gln $\rightarrow$ Arg substitution at position 1179, near the top of helix 15 within 20 nm of the GBM-binding site (Fig. 8 and Ref. 41), effectively eliminates the constraint on coupling imposed by assembly with Kir6.2 (Fig. 7) This substitution places a positive charge adjacent to Lys-1180 and approximately one helical turn from Arg-1174. The introduced charge has two effects, increasing the $K_4$ of the “Arg” variant for $[^3\text{H}]$GBM and increasing the allosteric factor $\beta$ significantly. The results are consistent with the swapped transmembrane helices 15 and 16 coupling ATP binding and NBD dimerization with the sulfonilyurea-binding site.

Why does assembly with Kir6.2 increase the affinity of SUR1 for $[^3\text{H}]$GBM?

As noted above, to apply the four-state model, the dissociation constants for $[^3\text{H}]$GBM were determined separately (Fig. 4 and Table 1). The results, although consistent with earlier studies reporting that SUR1 in channels binds $[^3\text{H}]$GBM 5–10-fold more tightly than SUR1 alone (75, 91), are not well understood. The increased affinity is not unique to GBM; repaglinide, a related channel antagonist, has a 100–150-fold higher affinity for SUR1 in channels versus the receptor alone (75, 76). Early mechanistic attempts to account for these differences proposed that KNtp, the N terminus of Kir6.2, contributed in a direct fashion to GBM binding (for reviews, see Refs. 40, 92, and 93). A variety of data support a direct interaction, including the finding that truncations of KNtp eliminate the difference in affinity between channels and receptors (75, 76) but increase channel open probability and reduce the inhibitory action of the sulfonilyurea antagonist tolbutamide (76, 94–96). Photoaffinity labeling with an azido derivative of GBM labels Kir6.2 but not truncated subunits missing KNtp, which implies that KNtp is near GBM (97). Restricting the positioning of KNtp by fusing the Kir and SUR N and C termini, SUR1–Kir6.2 (3–5), or by constructing “triple fusions,” SUR1–(Kir6.2)$_2$, markedly reduces the affinities for MgATP and GBM (3). Additional studies tested the effects of a synthetic 32-amino-acid “KNtp,” showing it reduces the open probability of $\Delta$N32Kir6.2/SUR1 channels but increases channel activity when applied to full-length channels (96). This suggested that SUR1 has a binding site for KNtp, whose occupation in truncated channels partially restores function, but competing with the endogenous KNtp mimics the effects of truncation (96). If KNtp is near GBM, as implied by these early results, the cryo-EM localization of bound GBM (41, 42) suggests that KNtp can access the central cavity and that SUR1 is a cryptic or frustrated peptide transporter. A cryo-EM study by Wu et al. (42) provides some support for this idea, reporting that electron densities near the GBM site may be due to KNtp, although the densities are not strong enough to allow modeling of the Kir N
terminus. How this cryptic transport activity and potential movement(s) of K\textsubscript{\textit{ATP}} during ATP-driven SUR1 conformational changes are coupled to channel gating remains an intriguing open question.

**Summary**

We have shown that ATP acts as a K\textsubscript{\textit{ATP}} channel agonist, switching SUR1 from inward- to outward-facing conformations that can activate channel openings. This is easily demonstrated when the inhibitory effects of ATP on the pore are minimized and ND mutants of SUR1 with higher affinity for ATP are tested. ATP binding is sufficient; strong coupling to ATP hydrolysis is not required. ATP-induced switching has a negative allosteric effect on GBM binding; channel activating, outward-facing conformations have the lowest affinity for GBM as expected for a channel antagonist. The allosteric effects are reciprocal; bound GBM reduces the affinity of SUR1 for ATP; thus, treatment with sulfonylureas will reduce the agonist action of ATP on WT and ND mutant channels and reduce channel openings. The structural links between the NBDs and GBM-binding site are not defined, but results with one ND SUR1 mutant, SUR1\textsubscript{Q1179R}, suggest involvement of helices 15 and 16 in TMD2 that contact NBD1. The biochemical prediction of ternary complexes, i.e., SUR1 liganded with both GBM and ATP, requires confirmation by cryo-EM studies.

**Experimental procedures**

**Molecular biology and cell culture**

WT or mutant hamster Abcc8/SUR1 cDNAs (National Institutes of Health NCBI sequence L40623.1) were cloned into the multiple cloning site of the Tet-On\textsuperscript{®} 3G bicistronic inducible expression vector, pTRE3G-ZsGreen1 (TaKaRa Bio USA, Inc.). The ZsGreen1 marker was replaced by either WT human KCNJ11/Kir6.2 (National Institutes of Health reference sequence NG_012446) or KCNJ11/Kir6.2\textsubscript{G3340} in which aspartic acid was substituted for glycine at position 334. A puromycin resistance cassette was either engineered into the resulting plasmid or cotransfected into HEK 293 Tet-On cells carrying the Tet-On 3G transactivator (TaKaRa, Inc.). Amino acid numbering is based on the hamster sequence (NIH NCBI sequence L40623.1) to facilitate comparison with the numbering in the cryo-EM publications (41, 42, 77, 78). The exception is SUR1\textsubscript{A1369S} where the numbering follows the human reference sequence (National Institutes of Health NCBI accession number AH003589) without incorporation of exon 17. The equivalent numbering in the hamster sequence includes exon 17. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and glutamine. Stable cell lines were selected and subcloned using puromycin (600 μM). Expression was induced by addition of doxycycline (300 μM) and tested for characterization of channel activity from 24 to 72 h. In some experiments, transient transfections were done and induced with doxycycline after 24 h.

**Membrane isolation**

For membrane preparation, doxycycline-induced cells were centrifuged for 10 min at 500 × g at 37 °C and lysed by incubation in ice-cold hypotonic buffer containing 10 mM HEPES, 1 mM EGTA, and a protease inhibitor, HALT (Thermo Fisher), at pH 7.4. After 30 min on ice, the swollen cells were broken by 50 strokes in a tight-fitting Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation at 1,200 × g for 10 min at 4 °C. The supernatant was centrifuged at 100,000 × g at 4 °C for 60 min, and the resulting membrane pellet was resuspended in a buffer containing 5 mM HEPES, 5 mM KCl, and 139 mM NaCl at pH 7.4 (4 °C). Protein concentration was determined by the BCA method using BSA as the standard. The protein concentration was adjusted to 2.0 mg ml\textsuperscript{-1}, and suspensions were frozen at −80 °C.

\textit{[^3]H}GBM homologous displacement experiments without nucleotides

The dissociation constant, \( K_{G1} \), of SUR1 for \[^3\text{H}\]GBM, in the absence of Mg\textsuperscript{2+} and nucleotides, was measured using isolated membranes from cells expressing WT or mutant SUR1 plus Kir6.2. Membranes were suspended in a buffered solution (139 mM NaCl, 5 mM KCl, 1 mM EDTA, and 50 mM HEPES, pH 7.4) with 0.3 or 1 nM \[^3\text{H}\]GBM plus increasing concentrations of unlabeled GBM for 30 min at 37 °C and then analyzed by rapid filtration as described previously (58) to determine total binding. The estimated values for free Mg\textsuperscript{2+}, <10 nM, were calculated using \textit{MAXC} (98) assuming contaminating Ca\textsuperscript{2+} and Mg\textsuperscript{2+} levels as high as 5 μM. Triplicate determinations were made for each GBM concentration per experiment; the data from three or more experiments were analyzed following the procedure described by Swillens (74) for homologous displacement curves. Analysis and global fitting were done with user-written functions in Origin2018 (OriginLab Corp., Northampton, MA) or in Prism 7 (GraphPad Software, Inc., La Jolla, CA). The data were weighted using a factor of (1/cpm)\textsuperscript{2}.

\[^3\text{H}\]GBM binding in nucleotide-containing solutions

\[^3\text{H}\]GBM binding to isolated membranes was analyzed by rapid filtration as described previously (58). The \[^3\text{H}\]GBM concentration, ~1 nM, was determined for each experiment. MgATP-containing solutions (concentrations ranging from 10 nM to 10 mM MgATP) with a calculated free Mg\textsuperscript{2+} concentration of 1 mM were prepared using the BioEqCalc program from Akers and Goldberg (99). Solutions contained 50 mM HEPES, pH 7.4, 5 mM KCl, and varying amounts of Na\textsubscript{ATP}, Mg\textsubscript{ATP}, and Na\textsubscript{Cl} to give the appropriate MgATP concentration at an ionic strength of 0.2 M.

In the experiments using MgADP, hexokinase (1 unit) and glucose (10 mM) were added to scavenge contaminant ATP and ATP generated by adenylate kinase. ATP determinations using luciferase indicate that the ATP levels were <0.1% of added ADP. The solution conditions, pH, ionic strength, and free Mg\textsuperscript{2+} concentrations, for the MgADP experiments were equivalent to those for MgATP described above.

**Model fitting**

The four-state equilibrium model (Fig. 5, inset) described previously (54) was used to estimate the dissociation constant, \( K_T \), for binding of ATP (or \( K_{\text{ADP}} \) for ADP) at NBD2 of SUR1 and the allosteric constant \( \beta \). The dissociation constants (\( K_{G1} \)) for \[^3\text{H}\]GBM were estimated independently as described above and held constant. Following Alper and Gelb (100) and Chris-
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topoulos (101), a transformed binding equation was used with nonlinear least-squares methods to estimate approximately normally distributed values for $pK_T$ and $p\beta$ and to estimate confidence intervals.

$$\tilde{G} = \frac{10^{pK_T}}{10^{pK_T} + 10^{p\beta}}$$

(Eq. 1)

Here, $K_T = 10^{pK_T}$ and $\beta = 10^{p\beta}$.

Electrophysiology

Patch-clamp recordings were done in the inside-out configuration. $K_{\text{ATP}}$ currents were measured at a membrane potential of $-50$ mV (pipette voltage, $+50$ mV); inward currents are shown as downward deflections. Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, March-Hugstetten, Germany) and had a resistance of 6–8 megaohms. Currents were recorded with an EPC-9 patch-clamp amplifier using Patchmaster software (HEKA, Lambrecht, Germany). Single-channel $K_{\text{ATP}}$ channel currents were about $-4$ pA at a holding potential of $-50$ mV, corresponding to a single-channel conductance of 80 picosiemens.

The pipette solution contained 130 mmol/liter KCl, 1.2 mmol/liter MgCl$_2$, 2 mmol/liter CaCl$_2$, 10 mmol/liter EGTA, and 10 mmol/liter HEPES; pH was adjusted to 7.4 with KOH at 25 °C. The magnesium-free bath solution contained 130 mmol/liter KCl, 4.6 mmol/liter CaCl$_2$, 10 mmol/liter EDTA, and 20 mmol/liter HEPES with pH adjusted to 7.2 with KOH at 25 °C. The MgATP bath solution contained 130 mmol/liter KCl, 2 mmol/liter CaCl$_2$, 10 mmol/liter EGTA, 1 mmol/liter Na$_2$ATP, 1.7 mmol/liter MgCl$_2$, and 20 mmol/liter HEPES with pH adjusted to 7.2 with KOH at 25 °C. Analyses to estimate $N_p$ were done offline in IgorPro 7 (WaveMetrics, Inc., Lake Oswego, OR) with user-written software.

Statistics

Data values are given as means ± S.E. ($n =$ number of replications). Estimated parameter values are reported as means ± (lower–higher) 95% confidence intervals. In Fig. 2, the number of channels in patches from randomly selected cells and randomly selected locations varied widely, presumably due to differences in expression levels and/or cell-surface distribution. To determine the significance of the action of nucleotides on SUR1/Kir6.2G334D $K_{\text{ATP}}$ channels, in the absence of Mg$^{2+}$, a nonparametric statistic, $W$, in the Wilcoxon signed-rank test was applied (102). This is a paired-difference test. Patches are chosen randomly, and the $N_p$ values are determined for a fixed time, typically 30–60 s before and 30–60 s at the end of an application of nucleotide. The absolute differences in $N_p$ values, plus versus minus nucleotides, were calculated and ranked small to largest. In all cases, the signed differences were positive; i.e. application of nucleotides always increased the $N_p$. The rank times the difference values were summed to give $W$. Comparison of $W$ with calculated values for small sample numbers or using online Wilcoxon tests (e.g. http://www.vassarstats.net/wilcoxon.html) gave estimates of statistical significance. $p$ values <0.05 were considered significant.

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