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PHARMACOLOGY

Antidiabetic Sulfonylureas and cAMP Cooperatively Activate Epac2A

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Sulfonylureas are widely used drugs for treating insulin deficiency in patients with type 2 diabetes. Sulfonylureas bind to the regulatory subunit of the pancreatic β cell potassium channel that controls insulin secretion. Sulfonylureas also bind to and activate Epac2A, a member of the Epac family of cyclic adenosine monophosphate (cAMP)–binding proteins that promote insulin secretion through activation of the Ras-like guanosine triphosphatase Rap1. Using molecular docking simulation, we identified amino acid residues in one of two cyclic nucleotide–binding domains, cNBD-A, in Epac2A predicted to mediate the interaction with sulfonylureas. We confirmed the importance of the identified residues by site-directed mutagenesis and analysis of the response of the mutants to sulfonylureas using two assays: changes in fluorescence resonance energy transfer (FRET) of an Epac2A-FRET biosensor and direct sulfonylurea-binding experiments. These residues were also required for the sulfonylurea-dependent Rap1 activation by Epac2A. Binding of sulfonylureas to Epac2A depended on the concentration of cAMP and the structures of the drugs. Sulfonylureas and cAMP cooperatively activated Epac2A through binding to cNBD-A and cNBD-B, respectively. Our data suggest that sulfonylureas stabilize Epac2A in its open, active state and provide insight for the development of drugs that target Epac2A.

INTRODUCTION

Adenosine 3′,5′-monophosphate (cAMP) is a second messenger that regulates various biological processes under both physiological and pathological conditions, including metabolism, cardiac muscle and smooth muscle contraction, hormone and neurotransmitter secretion, and learning and memory. cAMP exerts various effects by binding to its targets such as the regulatory subunits of protein kinase A (PKA), cyclic nucleotide–gated ion channels, and members of the Epac family of guanine nucleotide exchange factors (GEFs) (*1*). Epacs (also known as cAMP-GEFs) are cAMP-binding proteins with GEF activity toward Rap, a Ras-like guanosine triphosphatase (*2*–*4*). Epacs are encoded by two genes, *EPAC1* (*RAPGEF3*) and *EPAC2A* (*RAPGEF4*). *EPAC1* is widely expressed in tissues (*2*–*4*). The *EPAC2* locus generates three mRNAs produced by alternative transcriptional start sites and splicing. *EPAC2A* is expressed mainly in the brain and endocrine tissues, *EPAC2B* in the adrenal gland, and *EPAC2C* in the liver (*4*–*6*).

Epacs regulate a wide range of cellular functions in many cell types. For example, in the heart, Epac2A promotes β_1-adrenergic receptor–induced cardiac arrhythmias via sarcoplasmic reticulum Ca^{2+} leak (*7*). In airway smooth muscle, Epac1 and Epac2A stimulate inflammatory responses (*8*). In cortical neurons, Epac2A activation promotes dynamic synapse remodeling and depresses excitatory transmission (*9*). In pancreatic β cells, Epac2A enhances insulin secretion (*10*–*14*).

Epac1 and Epac2A can be considered to contain two main regions: an N-terminal regulatory region and a C-terminal catalytic region. The N-terminal regulatory region of Epac1 contains one cyclic nucleotide–binding domain (cNBD) and a DEP (Dishevelled, Egl-10, and Pleckstrin) domain. The N-terminal regulatory region of Epac2A contains two cNBDs and a DEP domain. The C-terminal catalytic region of Epac1 has a Ras exchange motif (REM) and a CDC25 homology domain (CDC25-HD) with GEF activity. The C-terminal catalytic region of Epac2A includes both of those domains and a Ras association (RA) domain. The cNBD of Epac1 binds cAMP with high affinity, whereas in Epac2A, cAMP binds to the cNBD-A, the first cNBD, with low affinity and to the cNBD-B, the second cNBD, with high affinity (*15*). Crystallographic analysis of Epac2A showed that, in the absence of cAMP, access of Rap1 to catalytic CDC25-HD is sterically blocked by the regulatory region (*16*–*18*). Binding of cAMP to Epac2A triggers a conformational change, moving the regulatory region away from the catalytic region and leading to Rap1 binding and activation (*16*–*19*).

We previously reported that Epac2A is a direct target of sulfonylureas, a class of drugs widely used in diabetes therapy (*20*). Sulfonylureas are well known for binding the regulatory subunit (SUR1) of the adenosine triphosphate (ATP)–sensitive potassium (K_{ATP}) channel in pancreatic β cells. Binding of sulfonylureas to this subunit promotes the closure of the channel, resulting in depolarization of the cells and enhanced response to stimuli that trigger insulin secretion (*21*, *22*). Because of the involvement of Epac2A as a positive regulator of insulin secretion in pancreatic β cells (*10*–*14*), the identification of Epac2A as a target of sulfonylureas suggests an additional mechanism of action. To explore the mechanism by which sulfonylureas interact with Epac2A to influence its activity, we used molecular docking simulation to identify amino acids in Epac2A and regions of the sulfonylureas that were predicted to interact. We verified these predictions by performing site-directed mutagenesis and analyzing their effects on the binding of sulfonylureas to cells expressing the proteins or to cells expressing the mutants in the context of an Epac2A FRET (fluorescence resonance energy transfer) sensor and on activation of Rap1. We found that sulfonylurea bound to the cNBD-A of Epac2A and that the binding of sulfonylureas to Epac2A depended on the cellular cAMP concentration and the structures of the sulfonylureas. Furthermore, sulfonylureas and cAMP bound distinct domains to cooperatively activate Epac2A to stimulate Rap1 activity. Our findings provide a basis for the development

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of novel antidiabetic drugs targeting insulin secretion, as well as insight into the mechanism of Epac2A activation.

RESULTS

Sulfonylureas activate Epac2A but not Epac1

Sulfonylureas, except for gliclazide, bind to and activate Epac2A, as assessed by a reduction in FRET in COS-1 or human embryonic kidney (HEK) 293T cells expressing an Epac2A FRET biosensor (*20*, *23*). Here, we expressed the Epac2A intramolecular FRET sensor in MIN6-K8 β cells, an insulin-secreting pancreatic β cell line (*24*), and investigated the effect of sulfonylureas on FRET in this cellular context. We used MIN6-K8 β cells because they exhibit a good insulin secretory response to activation of cAMP signaling or glucose stimulation. Binding of Epac2A activators to the FRET sensor reduces the interaction between the enhanced cyan fluorescent protein (ECFP)–tagged N-terminal regulatory region and the enhanced yellow fluorescent protein (EYFP)–tagged C-terminal catalytic region, leading to decreased FRET (*20*), which is an indication of Epac2A activation. We verified that treating MIN6-K8 β cells expressing the Epac2A FRET sensor with 8-pCPT-2′-*O*-Me-cAMP-AM (8-pCPT), an Epac-selective cAMP analog, decreased FRET in a dose-dependent manner (Fig. 1A and fig. S1). Treating MIN6-K8 β cells with glibenclamide, glipizide, or acetohexamide at concentrations of 10 μM or higher (Fig. 1B and fig. S2, A to C) or with tolbutamide or chlorpropamide at concentrations above 100 μM (Fig. 1B and fig. S2, D and E) produced a dose-dependent decrease in Epac2A FRET. Glimepiride at 10 μM also decreased Epac2A FRET (Fig. 1B). In contrast, gliclazide did not alter the Epac2A FRET response (Fig. 1B and fig. S2F), consistent with previous observations that gliclazide does not activate Epac2A (*20*, *23*).

Activation of Epacs by sulfonylureas is specific to Epac2A (*23*). We confirmed this specificity in MIN6-K8 β cells expressing the Epac1 FRET biosensor. Although 8-pCPT decreased Epac1 FRET (Fig. 1C), glibenclamide, tolbutamide, or glimepiride failed to reduce the FRET signal (Fig. 1D), indicating that sulfonylureas exhibit appropriate specificity for Epac2A, and not Epac1, in this system. The activation of Epac1 by 8-pCPT, but not by sulfonylureas, excluded an indirect mechanism of sulfonylurea activation of Epac2A by stimulating an increase in cAMP concentration through inhibition of phosphodiesterases. Furthermore, the specificity of binding to Epac2A and the differences in their effective concentrations suggested that the ability of sulfonylureas to activate Epac2A may depend on protein domains that are unique to Epac2A and the structures of the drugs.

Molecular docking simulation predicts amino acid residues that interact with sulfonylureas

We used molecular docking simulation to predict which amino acid residues of Epac2A interact with sulfonylureas. Epac2A differs from Epac1 in that it has two cNBDs and an RA domain (Fig. 2A). Comparison of the amino acid sequences indicated 36% identity between the cNBD of Epac1 and the cNBD-A of Epac2A and 75% identity between the cNBD of Epac1 and the cNBD-B of Epac2A, suggesting that the cNBD-B of Epac2A corresponds to the cNBD of Epac1 and that cNBD-A is unique to Epac2A. When we tried to dock a sulfonylurea onto the full-length inactive form of Epac2A [Protein Data Bank (PDB) ID: 2BYV] (*17*), we could not identify a binding site because the inactive form was completely closed. The crystal structure of full-length Epac2A in the active conformation is not known, but a partial crystal structure lacking cNBD-A has been solved (*18*), and structures of both the inactive form of cNBD-A (*17*) and the active form of cNBD-B bound to cAMP (*18*) are available (Fig. 2B). BLAST analysis showed that cNBD-A has the highest amino acid identity (33%) and similarity (74%) to cNBD-B among the various cAMP-binding proteins reported to date. Therefore, we constructed a three-dimensional model of the active form of cNBD-A on the basis of the structures of the inactive form of cNBD-A (PDB ID: 1O7F) (*16*) and the active form of cNBD-B (PDB ID: 3CF6) (*18*) using an MOE-homology model (MOE) (*25*) and then used an AMBER99 force field (*26*) to refine the structure (Fig. 2C).

One potentially important difference between cNBD-A and cNBD-B is that cNBD-A has a His^{124}, whereas cNBD-B has an alanine at the corresponding residue. Therefore, we constructed two types of cNBD-A models: one with the His^{124} side chain toward the inside of the pocket, and the other with the side chain toward the outside. We used these models of

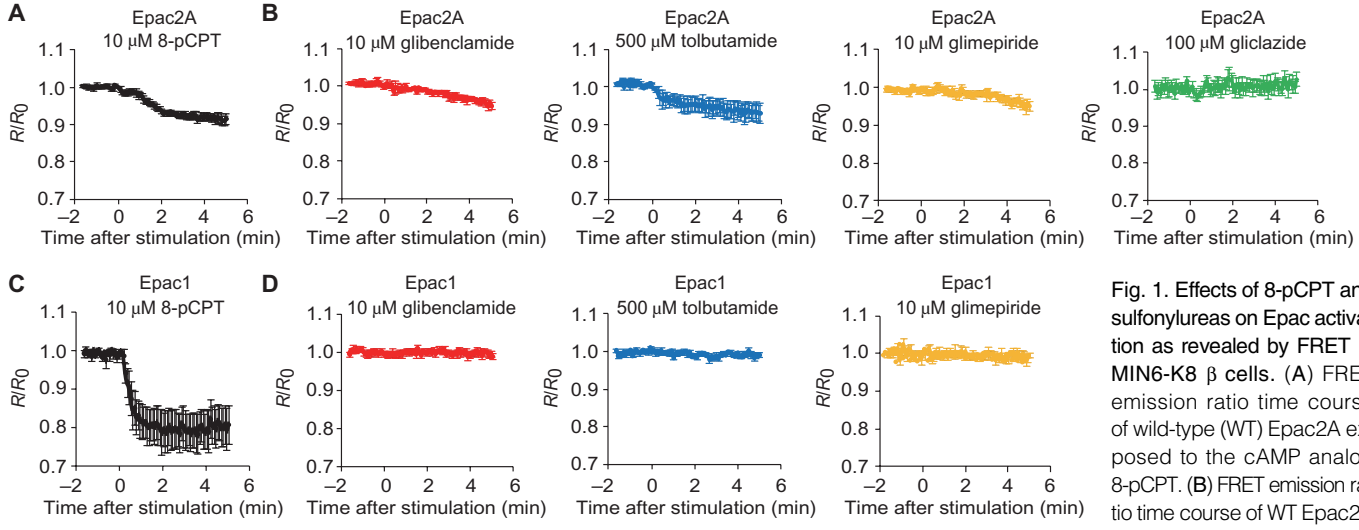


Fig. 1. Effects of 8-pCPT and sulfonylureas on Epac activation as revealed by FRET in MIN6-K8 β cells. (**A**) FRET emission ratio time course of wild-type (WT) Epac2A exposed to the cAMP analog 8-pCPT. (**B**) FRET emission ratio time course of WT Epac2A exposed to the indicated sulfonylurea at the indicated concentration. (**C**) FRET emission ratio time course of WT Epac1 exposed to the cAMP analog. (**D**) FRET emission ratio time course of WT Epac1 exposed to the indicated sulfonylurea at the indicated concentration. FRET change = R/R_0. Data are presented as means ± SEM (n = 4 to 9 experiments for each point).

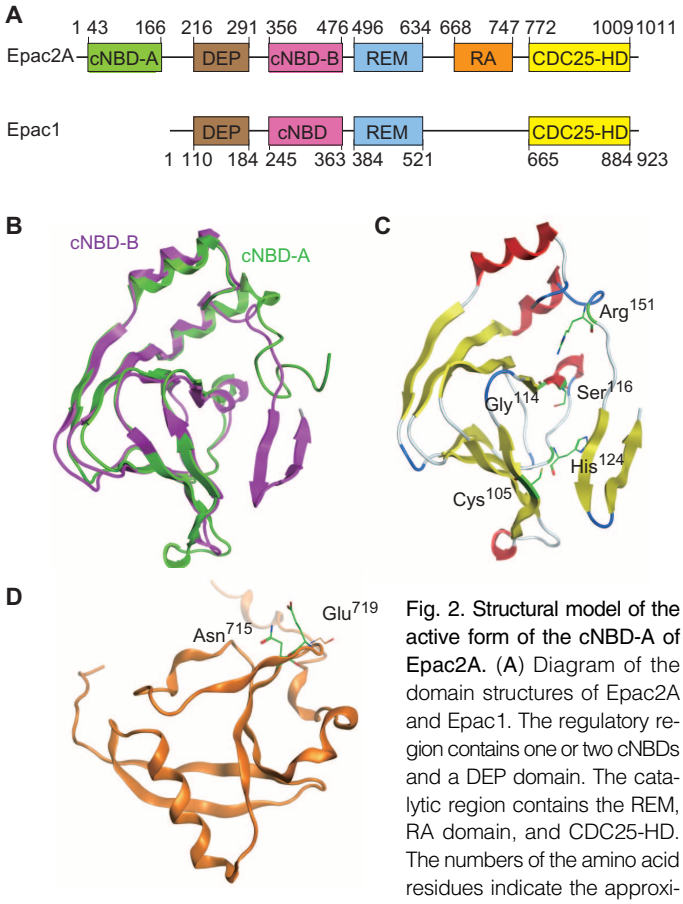


Fig. 2. Structural model of the active form of the cNBD-A of Epac2A. (**A**) Diagram of the domain structures of Epac2A and Epac1. The regulatory region contains one or two cNBDs and a DEP domain. The catalytic region contains the REM, RA domain, and CDC25-HD. The numbers of the amino acid residues indicate the approximate domain boundaries within the primary structures. (**B**) Superimposition of the active form of cNBD-B in Epac2A and the inactive form of cNBD-A in Epac2A. Magenta, active form of cNBD-B in Epac2A (PDB ID: 3CF6); green, inactive form of cNBD-A in Epac2A (PDB ID: 1O7F). (**C**) Structural model of the active form of cNBD-A in Epac2A. We constructed a structural model of the active form of cNBD-A with the inactive form of cNBD-A (amino acid residues 44 to 139, PDB ID: 1O7F) and the active form of cNBD-B (amino acid residues 450 to 477, PDB ID: 3CF6). AMBER99 force field was applied for refinement of the structure. Yellow, β sheet; red, α helix. Labeled residues were predicted to interact with sulfonylureas. (**D**) Structural model of the active form of RA domain in Epac2A (PDB ID: 3CF6). The predicted amino acid residues that interact with sulfonylureas by docking simulation are shown in (C) and (D).

Epac2A in the active conformation to perform molecular docking simulations with cAMP and acetohexamide, chlorpropamide, glibenclamide, gliclazide, glimepiride, glipizide, or tolbutamide. We also used the structural model of the active form of RA domain in Epac2A (PDB ID: 3CF6) to perform molecular docking simulations with cAMP and tolbutamide or glibenclamide (Fig. 2D).

The general structure of sulfonylureas contains a core sulfonylurea region and two side chains, R1 and R2 (Fig. 3A and fig. S3). Two regions of potential interaction were identified, which included several amino acids in the cNBD-A domain and the RA domain. Gliclazide, the sulfonylurea that does not activate Epac2A, showed a single interaction between the core region of the drug and Gly^{114} of the cNBD-A (Fig. 3B). Tolbutamide and chlorpropamide, which required relatively higher concentrations to activate Epac2A, were predicted to interact with the cNBD residues Gly^{114}, Ser^{116}, and His^{124} through hydrogen bonds with the core region of the sulfonylureas (Fig. 3C). The drugs that showed the lowest effective concentrations—glibenclamide, glimepiride, glipizide, and acetohexamide—were predicted to make hydrogen bonds with Gly^{114}, Ser^{116}, and His^{124}, as well as exhibit an additional interaction with Cys^{105} in the cNBD-A (Fig. 3D). Glibenclamide was also predicted to interact with Arg^{151} in the cNBD-A (Fig. 3E), and tolbutamide was predicted to interact with Asn^{715} or Glu^{719} in the RA domain (Fig. 3F). These predicted interactions, along with the calculated affinities (Fig. 3G), suggested that the drugs can be subdivided on the basis of the number of contacts between the core region and the cNBD-A.

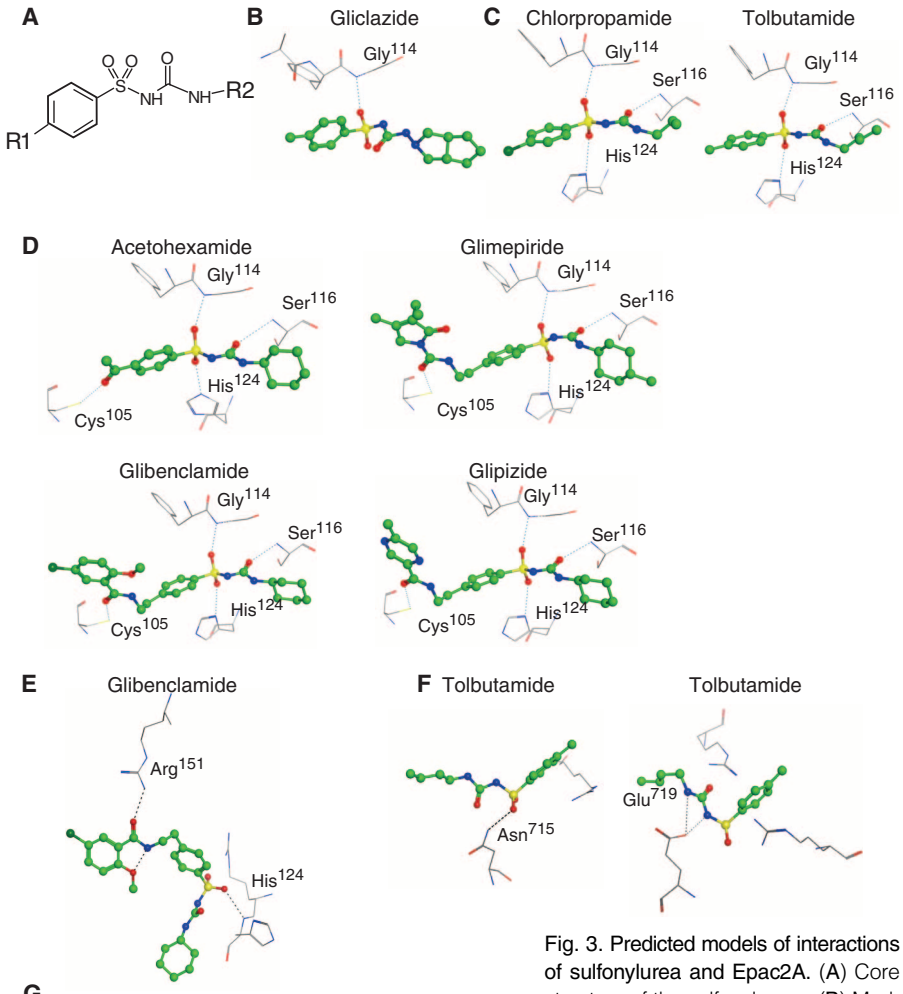
Mutational analysis of Epac2A confirms a sulfonylurea-binding site located in the cNBD-A

To determine whether the amino acid residues in the cNBD-A region identified by molecular docking simulation were involved in sulfonylurea binding, we performed a mutational analysis of Epac2A. We individually mutated each amino acid to alanine in the Epac2A FRET sensor and examined the effects of these mutations on FRET decreases in response to glibenclamide, tolbutamide, or 8-pCPT in MIN6-K8 β cells. Epac2A with mutations G114A, S116A, or H124A did not show decreased FRET in response to glibenclamide or tolbutamide, indicating that these residues were critical to the interaction (Fig. 4A). In contrast, Epac2A C105A FRET was decreased by tolbutamide, but not by glibenclamide, and Epac2A R151A FRET was decreased by both glibenclamide and tolbutamide (Fig. 4A). To confirm that the changes in FRET were not due to differences in the abundance of the mutant compared to the wild-type Epac2A sensor, we verified that the abundance of the H124A mutant was similar to that of the wild type (fig. S4). Collectively, these results suggest that Epac2A Gly^{114}, Ser^{116}, and His^{124} are required for sulfonylurea binding, that Cys^{105} may contribute to the affinity of the interaction, and that the predicted interaction with Arg^{151} is not necessary.

Amino acid residues in the cNBD-A of Epac2A could be required for binding cAMP. Indeed, mutation of Epac2A Gly^{114} to Glu disrupts cAMP binding (*4*, *27*, *28*). Exposure of cells expressing the Epac2A mutants C105A, S116A, H124A, or R151A to 8-pCPT produced a decrease in the FRET signal that was of a similar magnitude as that observed for the wild-type Epac2A FRET sensor (Fig. 4A). However, 8-pCPT produced a greater reduction in FRET of the Epac2A G114A compared to that of wild-type Epac2A FRET sensor (Fig. 4A). Thus, our results indicated that these residues in the cNBD-A of Epac2A are likely not required for cAMP binding but are consistent with a role for Gly^{114}.

Molecular docking simulation predicted that amino acids in the RA domain of Epac2A could influence its interaction with tolbutamide. We examined the effects of mutations in the Epac2A RA domain on cAMP and sulfonylurea binding. We found that 8-pCPT, glibenclamide, or tolbutamide decreased FRET of Epac2A N715A and Epac2A E719A to a degree similar to that of wild-type Epac2A (Fig. 4A), suggesting that the RA domain is not involved in binding these molecules.

To determine if the residues confirmed by site-directed mutagenesis in the cNBD-A of Epac2A were sufficient for Epac-sulfonylurea binding, we mutated the corresponding residues in the Epac1 FRET sensor and tested if this Epac1 mutant bound sulfonylureas. Alignment of the amino acid sequence between the cNBD-A of Epac2A and the cNBD of Epac1 showed that Cys^{105}, Gly^{114}, Ser^{116}, and His^{124} of Epac2A correspond to Thr^{302}, Gly^{311}, Leu^{313}, and Ala^{322} of Epac1 (Fig. 4B). Only Gly^{114} (Epac2A) and Gly^{311} (Epac1) are conserved. Therefore, we constructed a triple-mutant (T302C, L313S, and A322H) Epac1 FRET sensor and expressed this sensor in MIN6-K8 β cells. Treatment of cells with glibenclamide or



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Sulfonylurea	Predicted binding affinity (kcal/mol)
Glimepiride	–9.256
Glibenclamide	–8.963
Glipizide	–8.850
Acetohexamide	–7.588
Tolbutamide	–7.190
Chlorpropamide	–6.920
Gliclazide	–6.486

Fig. 3. Predicted models of interactions of sulfonylurea and Epac2A. (A) Core structure of the sulfonylureas. (B) Model of the interaction between Epac2A cNBD-A and the inactive sulfonylurea. (C) Model of the interaction between Epac2A cNBD-A and sulfonylureas requiring a relatively high concentration to activate Epac2A. (D) Model of sulfonylureas requiring a relatively low concentration to activate Epac2A. (E) Model of the interaction between Epac2A cNBD-A and sulfonylurea. (F) Model of the interaction between Epac2A RA domain and sulfonylurea. (G) Predicted affinities for the sulfonylurea binding to Epac2A. Ball-and-stick indicates sulfonylureas; wire diagram indicates amino acids of Epac2A. Dotted lines indicate predicted interactions. Carbon, nitrogen, oxygen, and sulfur atoms are colored green, blue, red, and yellow, respectively. For tolbutamide, only those interactions subsequently confirmed by mutagenesis are shown. PDB files of models for the interactions between Epac2A and sulfonylureas are provided in the Supplementary Materials.

tolbutamide significantly decreased triple-mutant Epac1 FRET (Fig. 4C), indicating that these residues and Gly^{114} in the cNBD are sufficient for sulfonylureas to bind Epac2A. Triple-mutant Epac1 FRET was only slightly decreased by 8-pCPT (Fig. 4C) as compared to FRET from the wild-type Epac1 FRET sensor, suggesting that Thr^{302}, Leu^{313}, and Ala^{322} in the Epac1 cNBD are involved in binding cAMP.

His^{124} of Epac2A is critical for Rap1 activation by sulfonylureas but not cAMP

Rap1 is activated by sulfonylurea or cAMP through its binding to Epac2A (fig. S5) (*20*, *23*). Comparison of the amino acid sequences revealed an Ala-to-His substitution at amino acid 124 in the cNBD-A of Epac2A relative to the cNBDs of Epac1 or PKA regulatory subunits (Fig. 5A). Alanine at this position in the cNBD of PKA regulatory subunits is required for cAMP binding (*29*). Together with the mutational analysis of Epac2A (Fig. 4A), this suggested that His^{124} could be critical for determining the selectivity of cAMP versus sulfonylurea binding to the cNBD-A of Epac2A. Because the number of pancreatic β cells that can be isolated from *Epac2A* (*Rapgef4*)–null mice is limited, we used *Epac2A* (*Rapgef4*)–deficient mouse clonal pancreatic β cells (*12*) reconstituted with wild-type Epac2A or Epac2A H124A and measured Rap1 activation with a pull-down assay. Cells reconstituted with wild-type Epac2A exhibited a significant increase in activated Rap1 in response to either 8-pCPT or glibenclamide (Fig. 5B), whereas cells reconstituted with the Epac2A H124A mutant activated only in response to 8-pCPT (Fig. 5C). Thus, His^{124} is a critical determinant of the selective binding of sulfonylureas to the Epac2A cNBD-A and the subsequent activation of Rap1.

Sulfonylureas and cAMP synergize to activate Epac2A and Rap1

Previous work suggests that the cNBD-A and cNBD-B of Epac2A adopt a closed confirmation that is opened by cAMP (*17*–*19*). On the basis of our model, in the closed conformation, the sulfonylurea binding site would be inaccessible; therefore, we predicted that sulfonylurea binding to Epac2A depends on the concentration of cAMP. To test for a dependency on cAMP concentration, we performed direct sulfonylurea binding assays with [^{3}H]glibenclamide in COS-1 cells because these cells do not have the SUR1 or endogenous Epac2A and have a low basal concentration of cAMP (*20*), compared to β cells. Wild-type Epac2A did not bind [^{3}H]glibenclamide when expressed in COS-1 cells (Fig. 6A). When we also treated cells with a low concentration of 8-pCPT (1 μM), [^{3}H]glibenclamide efficiently bound to cells expressing wild-type Epac2A (Fig. 6B). However, COS-1 cells expressing Epac2A H124A did not bind [^{3}H]glibenclamide even in the presence of 8-pCPT (Fig. 6C). Thus, sulfonylurea binding to Epac2A depends on the presence of cAMP.

To assess whether cAMP and sulfonylureas acted together to activate Epac2A in pancreatic β cell lines, we examined the combinatorial effects of glibenclamide and 8-pCPT on Epac2A FRET and Rap1 activity. Although high concentrations (>10 μM) of glibenclamide activated Epac2A in these cells (Figs. 4A and 5B), such high concentrations are unlikely to occur in diabetic patients who receive glibenclamide treatment. Treating

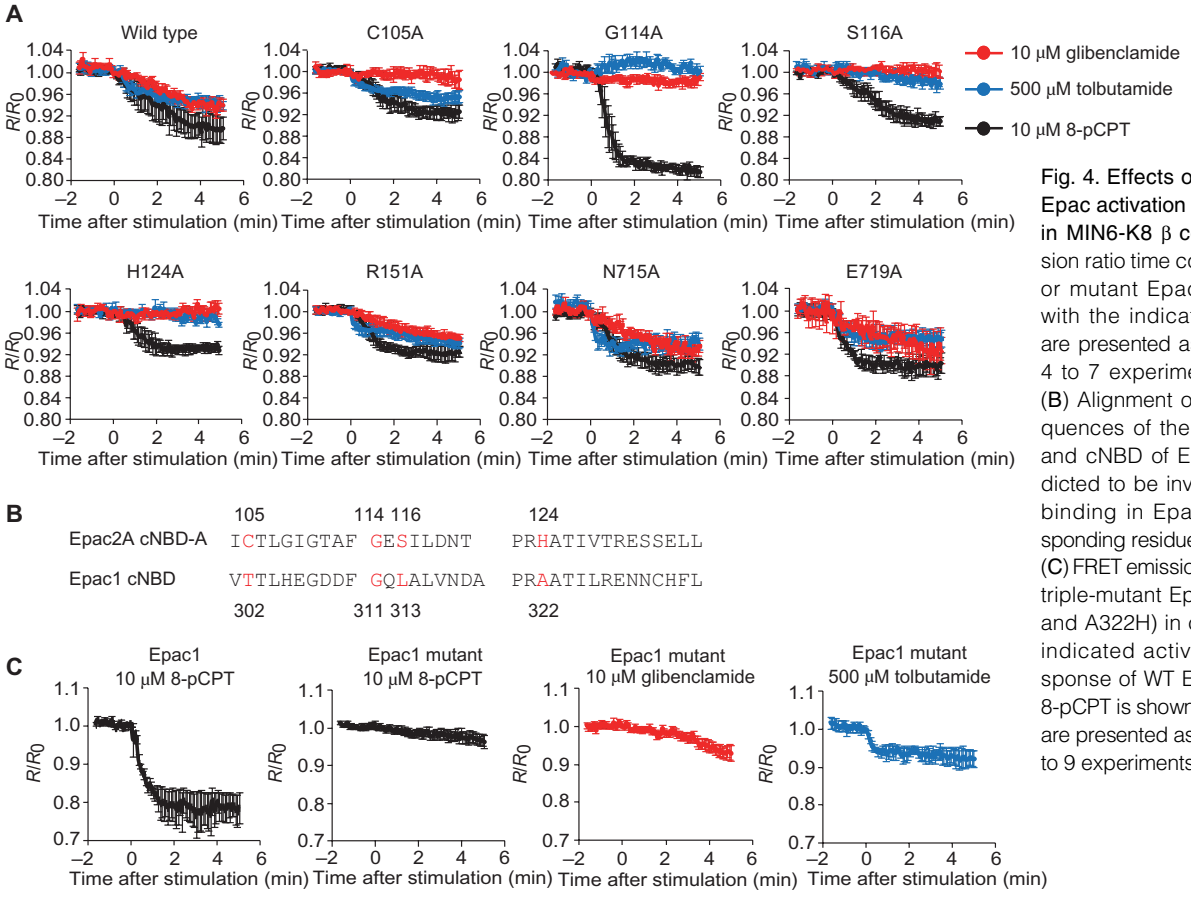
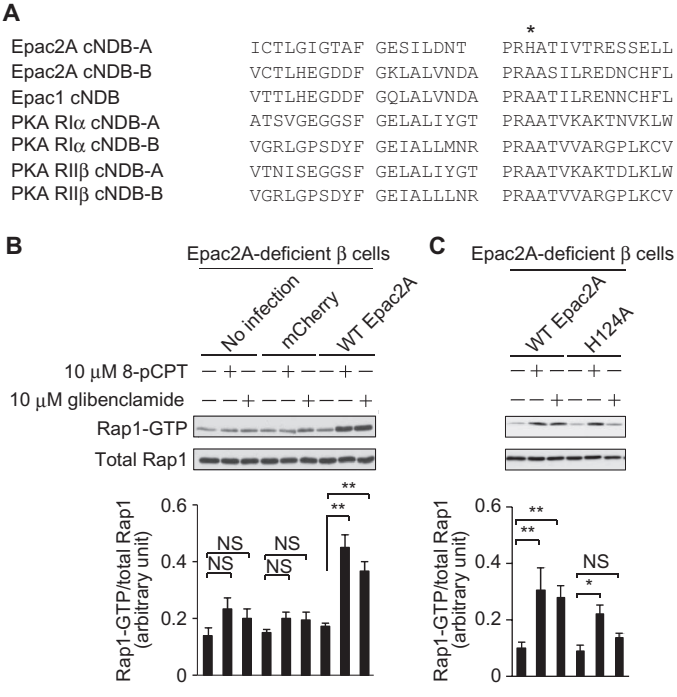


Fig. 4. Effects of Epac mutations on Epac activation as revealed by FRET in MIN6-K8 β cells. (**A**) FRET emission ratio time courses of WT Epac2A or mutant Epac2A in cells treated with the indicated activators. Data are presented as means ± SEM (n = 4 to 7 experiments for each point). (**B**) Alignment of the amino acid sequences of the cNBD-A of Epac2A and cNBD of Epac1. Residues predicted to be involved in sulfonylurea binding in Epac2A and the corresponding residues in Epac1 are in red. (**C**) FRET emission ratio time courses of triple-mutant Epac1 (T302C, L313S, and A322H) in cells treated with the indicated activators. The FRET response of WT Epac1 in response to 8-pCPT is shown for comparison. Data are presented as means ± SEM (n = 4 to 9 experiments for each point).

Fig. 5. Dependence of Rap1 activation by glibenclamide on Epac2A. (**A**) Alignment of the amino acid sequences in the cNBD of Epac2A, Epac1, and PKA regulatory subunits. Asterisk indicates Ala-to-His substitution at position 124 in Epac2A. (**B** and **C**) Effects of glibenclamide and 8-pCPT on Rap1 activation in Epac2A-deficient β cells expressing mCherry (control) or exogenous WT Epac2A or Epac2A H124A mutant (H124A). Quantification of chemiluminescent signals is shown with corresponding bars positioned under the bands. Data are presented as means ± SEM (n = 4 to 7 experiments for each point). *P < 0.05; **P < 0.01; NS, not significant (Dunnett's method).



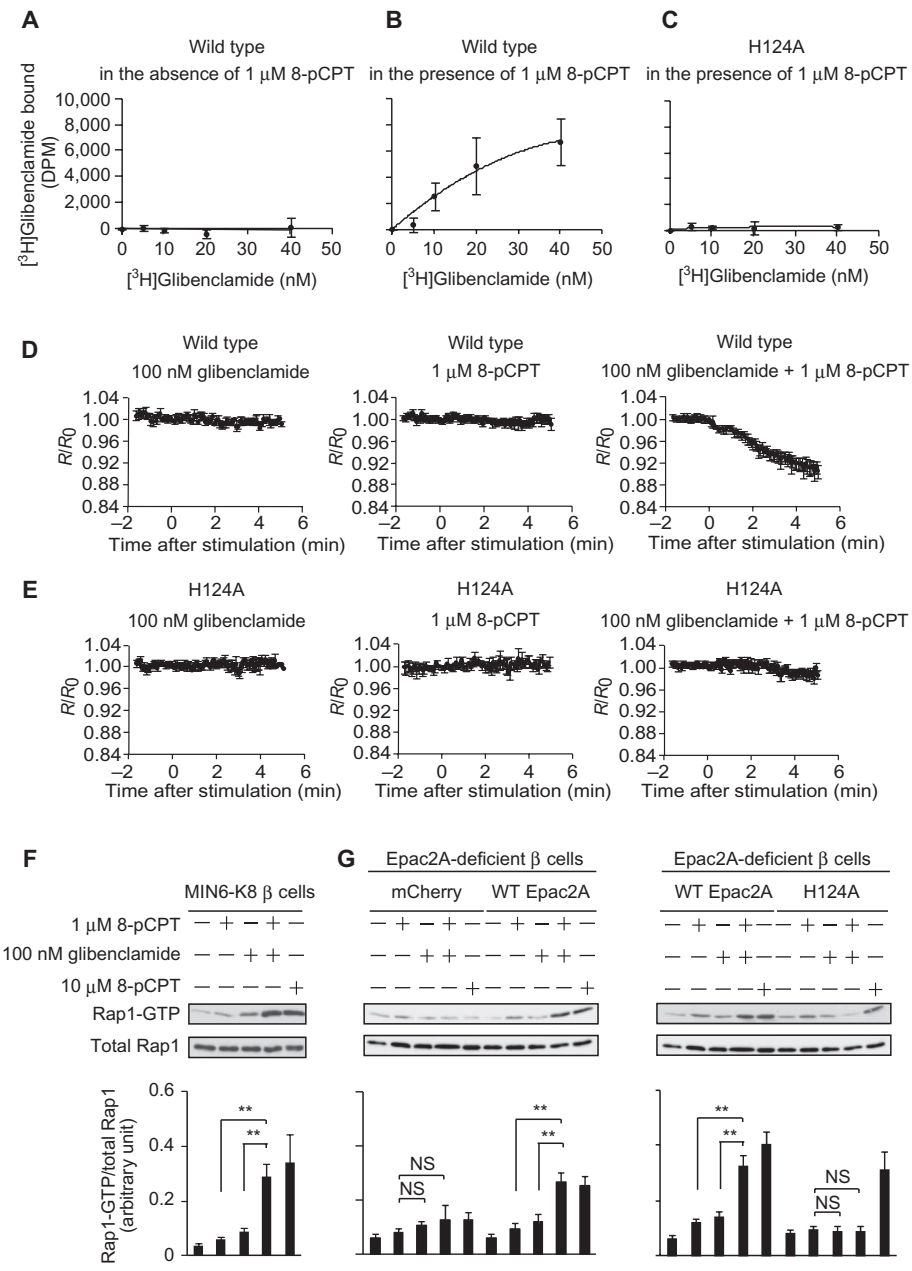


Fig. 6. cAMP-dependent activation of Epac2A and Rap1 by glibenclamide. (**A** to **C**) Graphs showing the direct binding of glibenclamide to Epac2A. [^{3}H]Glibenclamide binding was assessed in COS-1 cells transfected with either WT Epac2A or Epac2A H124A. DPM, disintegration per minute. Data are presented as means ± SEM (n = 4 to 6 experiments for each point). (**D**) FRET emission ratio time courses of WT Epac2A in MIN6-K8 β cells exposed to the indicated compounds. (**E**) FRET emission ratio time courses of Epac2A H124A in MIN6-K8 β cells exposed to the indicated compounds. Data are presented as means ± SEM (n = 5 to 9 experiments for each point). (**F** and **G**) Rap1 activation assays after treatment with the indicated combinations of 8-pCPT and glibenclamide in MIN6-K8 β cells (F) or Epac2A-deficient β cells expressing mCherry (control) or exogenous WT Epac2A or Epac2A H124A (G). Data are presented as means ± SEM (n = 4 to 7 experiments for each point). $^{**}P < 0.01$; NS, not significant (Dunnett's method).

MIN6-K8 β cells with low concentrations of glibenclamide (100 nM) or 8-pCPT (1 μM) alone did not affect Epac2A FRET, whereas the combination of 100 nM glibenclamide and 1 μM 8-pCPT reduced Epac2A FRET (Fig. 6D). Neither 100 nM glibenclamide or 1 μM 8-pCPT alone nor a combination of the two reduced Epac2A H124A FRET (Fig. 6E). Rap1 was synergistically activated by the combination of 100 nM glibenclamide and 1 μM 8-pCPT in MIN6-K8 β cells but not by either reagent alone at these concentrations (Fig. 6F). Similarly, low concentrations of glibenclamide and 8-pCPT synergized to activate Rap1 in Epac2A-deficient β cells reconstituted with wild-type Epac2A, but not in cells with Epac2A H124A (Fig. 6G). Collectively, these findings suggest that sulfonylureas and cAMP bind to different domains of Epac2A and cooperatively activate Rap1.

Binding and activation of Epac2A by sulfonylureas requires endogenous cAMP

Adrenaline inhibits the production of cAMP in β cells by activating G_i-coupled α_2-adrenoceptors (*30*). In MIN6-K8 β cells, 5 μM adrenaline decreased basal cAMP concentration by 67.1% (fig. S6). Adrenaline treatment reversed the decrease in Epac2A FRET induced by glibenclamide or tolbutamide (Fig. 7A), prevented glibenclamide or tolbutamide binding to the Epac2A FRET sensor (Fig. 7B), and blocked Rap1 activation by glibenclamide (Fig. 7C). These results support the conclusion that Epac2A and Rap1 are activated by sulfonylureas in a cAMP-dependent manner.

DISCUSSION

In pancreatic β cells, cAMP promotes the secretion of insulin, a hormone essential for glucose and lipid metabolism in the body (*31*–*33*), by both PKA-dependent and PKA-independent mechanisms (*4*, *10*–*14*, *31*). Sulfonylureas are a class of drugs used to treat diabetes. These drugs bind to the SUR1 regulatory subunit of K_{ATP} channels in pancreatic β cells, promote channel closure, and lead to increased insulin secretion (*21*, *34*–*36*). Sulfonylureas also activate Epac2A and Rap1 signaling to stimulate insulin secretion (*20*, *37*, *38*). We identified the binding sites of sulfonylureas in Epac2A and characterized the interaction of the sulfonylureas and cAMP in the activation of Epac2A. We initially examined two distinct models for the cNBD-A of Epac2A, which differed in the positioning of His^{124}. Cys^{105} seemed important for the binding to the His^{124}-inside model, and Arg^{151} for the His^{124}-outside model. Our mutational analysis suggested that the His^{124}-inside model was more likely correct and was used for the estimation of binding affinities.

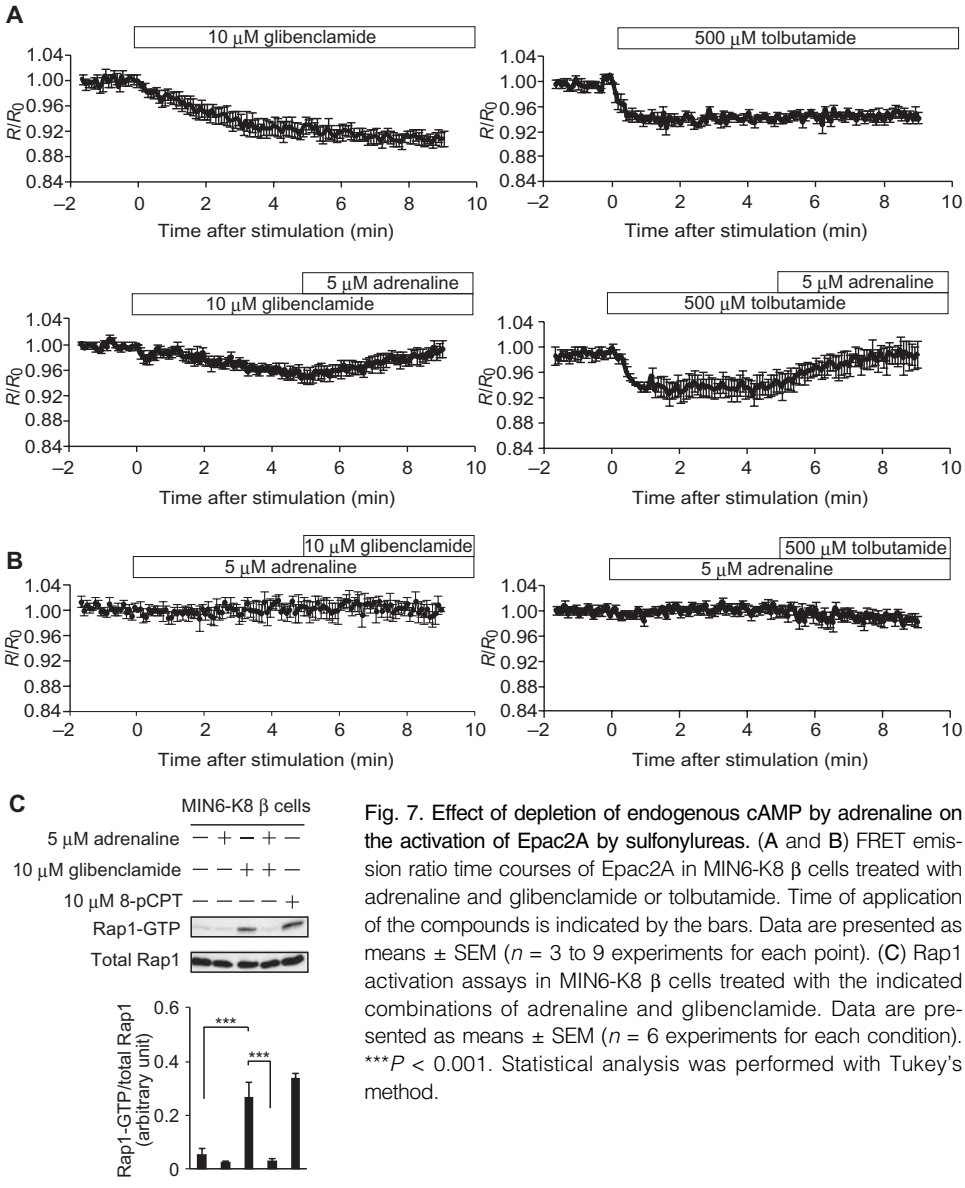


Fig. 7. Effect of depletion of endogenous cAMP by adrenaline on the activation of Epac2A by sulfonylureas. (**A** and **B**) FRET emission ratio time courses of Epac2A in MIN6-K8 β cells treated with adrenaline and glibenclamide or tolbutamide. Time of application of the compounds is indicated by the bars. Data are presented as means ± SEM (n = 3 to 9 experiments for each point). (**C**) Rap1 activation assays in MIN6-K8 β cells treated with the indicated combinations of adrenaline and glibenclamide. Data are presented as means ± SEM (n = 6 experiments for each condition). ***P < 0.001. Statistical analysis was performed with Tukey's method.

We demonstrated that Cys^{105}, Gly^{114}, Ser^{116}, and His^{124} in the cNBD-A participate in sulfonylurea binding. In addition, we showed that the sulfonylurea binding to Epac2A and activation of Rap1 depend on the cAMP concentration, suggesting that these molecules act cooperatively to stimulate insulin release.

The finding that Epac2A is a direct target of sulfonylurea has been controversial (*39*, *40*). One reason for the different results may be that some studies were performed with purified proteins in vitro (*39*, *40*), whereas we analyzed Rap1 activity in cells. Because the plasma membrane is a crucial factor in Epac2A's activation of Rap1 (*41*), activation of Epac2A by sulfonylureas may require the cellular environment.

Similar to our results, high concentrations of the sulfonylureas (glibenclamide, glipizide, acetohexamide, and tolbutamide) activate Epac2A, as assessed by FRET in HEK293T cells (*23*). However, that study found that Arg^{447} in the cNBD-B of Epac2A was involved in sulfonylurea binding. Because the cNBD-A and cNBD-B may be arranged "face to face" (*17*) so that these domains sterically block access of cAMP to each of the cAMP-binding sites (*17*–*19*), the mutation of Arg^{447} might interfere with sulfonylurea binding to cNBD-A, yet retain cAMP binding to cNBD-B.

Our results showed that binding of Epac2A to sulfonylureas depended on the structures of R1 and R2 side chains. The number of amino acids predicted by the molecular docking simulation to participate in the interaction between Epac2A and the drug inversely correlated with the effective concentration of the sulfonylureas for stimulating Epac2A activity (reducing Epac2A FRET). For example, we determined that the interaction of Epac2A with tolbutamide largely depends on Gly^{114}, Ser^{116}, and His^{124}; whereas that with glibenclamide also requires Cys^{105}. Glibenclamide exhibited a lower effective concentration than tolbutamide for binding Epac2A. Thus, we propose that sulfonylureas can be classified into three groups: (i) those that interact with four amino acids (Cys^{105}, Gly^{114}, Ser^{116}, and His^{124}) and activate Epac2A at relatively low concentrations (glibenclamide, glipizide, glimepiride, and acetohexamide); (ii) those that interact with three amino acids (Gly^{114}, Ser^{116}, and His^{124}) and activate Epac2A at relatively high concentrations (tolbutamide and chlorpropamide); and (iii) those that interact with one amino acid (Gly^{114}) and do not activate Epac2A (gliclazide). Calculation of the predicted binding affinities of the various sulfonylureas for Epac2A supports this ordering scheme.

Our data suggest a mechanism for the cooperative activation of Epac2A by sulfonylureas and cAMP. We found that the binding and activation of Epac2A by sulfonylureas but not 8-pCPT requires His^{124} in the cNBD-A. Moreover, in COS-1 cells, binding of glibenclamide to Epac2A required cAMP, which was supplied exogenously as 8-pCPT. Mutation of Gly^{114} to glutamic acid in the cNBD-A of Epac2A disrupts binding to cAMP (*4*, *27*, *28*). In contrast, we found that mutation of Gly^{114} to alanine enhanced binding of the cAMP analog 8-pCPT to Epac2A, measured as an increased reduction in Epac2A G114A FRET relative to that of wild-type Epac2A. The cNBD-A contains both a sulfonylurea-binding site and a low-affinity binding site for cAMP, and the cNBD-B contains a high-affinity binding site for cAMP (*42*) (Fig. 8A). Binding of cAMP to the cNBD-B induces a conformational change, opening the face to face structure, thereby allowing sulfonylureas to bind to the cNBD-A (Fig. 8B). Sulfonylureas could then stabilize Epac2A in its open state. In this model, high concentrations of cAMP would compete with sulfonylureas to bind to cNBD-A through steric hindrance. The effect of mutating Gly^{114} to Ala may destabilize the interaction between the cNBD-A and cNBD-B, thereby increasing the binding of cAMP to both sites.

In conclusion, our data demonstrated that the sulfonylurea-binding site resides in the cNBD-A of Epac2A. The concentration of sulfonylurea required to activate Epac2A depended on the cAMP concentration, as well as the structures

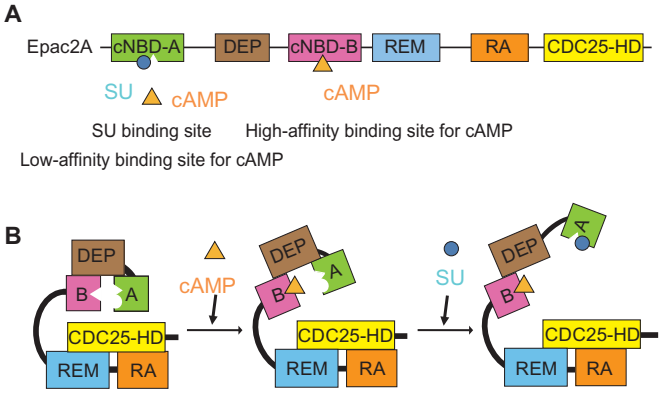


Fig. 8. Proposed mechanism of cooperative Epac2A activation by sulfonylureas and cAMP. (A) Schematic presentation of the binding site of sulfonylurea (SU) and those of cAMP in Epac2A. (B) Cooperative activation of Epac2A by sulfonylurea and cAMP. cAMP binding to the cNBD-B site opens the structure enabling access to the cNBD-A site. Binding to the cNBD-A site enables additional conformational changes, enabling activation of Epac2A.

of sulfonylureas. Binding of cAMP to the cNBD-B and binding of sulfonylurea to the cNBD-A resulted in cooperative activation of Epac2A. The present study provides a basis for the development of novel antidiabetic drugs targeting insulin secretion, as well as insight into the mechanism of Epac2A activation.

MATERIALS AND METHODS

Reagents

Glibenclamide, tolbutamide, chlorpropamide, acetohexamide, and glipizide were purchased from Sigma. Gliclazide was from LKT Laboratories Inc. [^{3}H]Glibenclamide was from PerkinElmer. 8-pCPT was from BioLog Life Science Institute.

Molecular docking simulation and calculation of binding affinities

MOE (CCG Inc.) and MOE-ASEDock (Ryoka Systems Inc.) (*25*) were used in docking studies of sulfonylureas to the active form of cNBD-A of Epac2A as well as in constructing the active form of cNBD-A of Epac2A. We constructed two types of cNBD-A models: one with the His^{124} side chain toward the inside of the pocket, and the other with the side chain toward the outside. We then used ASEDock to dock sulfonylureas against both cNBD-A models. For free energy calculations of sulfonylurea binding, we used the docked structures with His^{124}-inside models. Using NAMD (*43*) for optimization of docked structures, we calculated the free energy of binding using solvated interaction energies (*44*).

Cell culture and transfection

MIN6-K8 β cells, *Epac2A* (*Rapgef4*)–deficient mouse clonal pancreatic β cells, and COS-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum and maintained in a humidified incubator with 95% air and 5% CO_2 at 37°C (*12*, *20*, *24*). Two days before FRET measurements, cells were transiently transfected with plasmids encoding FRET sensor, using Effectene Transfection Reagent (Qiagen).

FRET experiments

FRET experiments in MIN6-K8 β cells transfected with mouse wild-type Epac2A, mutant Epac2A, human wild-type Epac1 (*45*), or mutant Epac1 FRET sensor were performed as described previously (*20*). EYFP/ECFP ratio for Epac2A or EYFP/citrine ratio for Epac1 was normalized to R_0 to describe FRET efficiency changes (FRET change = R/R_0, where R_0 is the ratio at time 0). FRET changes were acquired every 5 s.

Site-directed mutagenesis

Site-directed mutagenesis was carried out with a KOD-Plus-Mutagenesis kit (Toyobo) according to the manufacturer's instructions. Mutations were confirmed with an ABI 3700 PRISM (PerkinElmer Life Sciences) automated sequencer.

Sulfonylurea binding experiments

Sulfonylurea binding experiments were performed as described previously (*20*). Briefly, COS-1 cells transfected with Epac2A complementary DNA (cDNA) were resuspended in assay buffer containing 119 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, and 20 mM Hepes (pH 7.4). Each aliquot was incubated for 1 hour at room temperature with [^{3}H]glibenclamide in the absence or presence of unlabeled glibenclamide at various concentrations. Bound [^{3}H]glibenclamide was separated from free [^{3}H]glibenclamide by rapid vacuum filtration through Whatman GF/C filters (Whatman International). The filters were washed with ice-cold buffer, and the radioactivity was determined with a liquid scintillation counter.

Measurement of Rap1 activity

Pull-down assay for Rap1-GTP (guanosine triphosphate) was performed as described previously (*12*). Dimethyl sulfoxide was used as vehicle. Precise quantification was achieved by densitometric analysis of the immunoreactive bands with the National Institutes of Health Image software. The intensity of the Rap1-GTP signal was normalized to that of total Rap1. Anti-Rap1 antibody was purchased from Millipore (07-916).

Adenovirus-mediated gene transfer

Adenovirus-mediated gene transfer was performed as described previously (*20*). Briefly, recombinant adenovirus carrying mCherry (Ad-mCherry) or Epac2A (Ad-Epac2A) was generated according to the manufacturer's instructions (Invitrogen). Epac2A-deficient β cells were infected with Ad-mCherry or Ad-Epac2A. After 3 days of culture, the infected cells were preincubated with 2.8 mM glucose and then incubated with 2.8 mM glucose plus various stimuli for 15 min. GTP-bound Rap1 was assessed.

Measurement of cAMP

Measurement of cAMP was performed as described previously (*20*). Briefly, MIN6-K8 β cells were seeded at a density of 6×10^4 cells per well (96-well plate) and cultured for 2 days. After 30 min of preincubation with Krebs-Ringer bicarbonate buffer containing 2.8 mM glucose, the cells were treated with vehicle or adrenaline in the same buffer for 15 min. The cells were incubated, and cellular cAMP concentration was determined by HTRF (homogeneous time-resolved fluorescence assay) with the Cisbio cAMP femto 2 kit (Cisbio International) according to the manufacturer's instruction.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/6/298/ra94/DC1
Fig. S1. Concentration-dependent activation of Epac2A by 8-pCPT as revealed by FRET.
Fig. S2. Concentration-dependent activation of Epac2A by sulfonylureas as revealed by FRET.
Fig. S3. Chemical structures of various sulfonylureas.
Fig. S4. Protein abundance of wild-type and mutant Epac2A FRET sensors.
Fig. S5. Concentration-dependent activation of Rap1 by glibenclamide.
Fig. S6. Effect of adrenaline on cellular cAMP concentration.
PDB files (S1 to S8).

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