Allosteric Inhibition of Epac

COMPUTATIONAL MODELING AND EXPERIMENTAL VALIDATION TO IDENTIFY ALLOSTERIC SITES AND INHIBITORS*

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Background: Epac is a key mediator of cAMP signaling.
Results: We identified the hinge region of the cyclic nucleotide binding domain of Epac1 as a druggable region, in particular, for allosteric inhibitors.
Conclusion: A thiobarbituric acid derivative allosterically inhibits Epac activation.
Significance: Previously unappreciated regions of Epac can be targeted for allosteric regulation of its activity.

Epac, a guanine nucleotide exchange factor for the low molecular weight G protein Rap, is an effector of cAMP signaling and has been implicated to have roles in numerous diseases, including diabetes mellitus, heart failure, and cancer. We used a computational molecular modeling approach to predict potential binding sites for allosteric modulators of Epac and to identify molecules that might bind to these regions. This approach revealed that the conserved hinge region of the cyclic nucleotide-binding domain of Epac1 is a potentially druggable region of the protein. Using a bioluminescence resonance energy transfer-based assay (CAMYEL, cAMP sensor using YFP-Epac-Rluc), we assessed the predicted compounds for their ability to bind Epac and modulate its activity. We identified a thiobarbituric acid derivative, 5376753, that allosterically inhibits Epac activity and used Swiss 3T3 and HEK293 cells to test the ability of this compound to modulate the activity of Epac and PKA, as determined by Rap1 activity and vasodilator-stimulated phosphoprotein phosphorylation, respectively. Compound 5376753 selectively inhibited Epac in biochemical and cell migration studies. These results document the utility of a computational approach to identify a domain for allosteric regulation of Epac and a novel compound that prevents the activation of Epac1 by cAMP.

Increase in the second messenger cAMP occurs following activation of adenyl cyclases, which catalyze the conversion of ATP to cAMP, by Gs-linked G protein-coupled receptor. The effects of cAMP signaling in mammalian cells are mediated by two main intracellular effectors: protein kinase A (PKA) and exchange protein activated by cAMP (Epac). Epac is a guanine nucleotide exchange factor (GEF) that activates the low-molecular weight G proteins Rap1 and Rap2 and has been implicated in the regulation of cAMP-mediated events that include proliferation, apoptosis, differentiation, secretion, migration, and adhesion (1–3). It has been proposed that dysregulation of Epac signaling occurs in a variety of diseases including autism, heart failure, diabetes mellitus, and cancer (4–8). Thus, there is considerable interest in developing compounds that modulate its activity.

The two isoforms of Epac, Epac1 and Epac2, have similar domain structures and modes of activation (see Fig. 1A). Epac has a regulatory region that sterically blocks Rap1 from interacting with the catalytic region. The regulatory region is composed of a DEP (Dishevelled, Egl-10, pleckstrin) domain, which mediates the subcellular localization of Epac through its binding to phosphatidic acid and a cyclic nucleotide-binding domain (CNBD) responsible for its activation (9, 10). Epac2 possesses a second, lower affinity CNBD that is involved in subcellular localization but not the activation of Epac2. The catalytic region contains a CDC25 homology domain (CDC25HD), which interacts with Rap1 and facilitates the exchange of GTP for GDP, a Ras exchange motif, and a Ras association motif (11).

In its auto-inhibited state, the CNBD interacts with the CDC25HD by an ionic latch that sterically prevents interaction with Rap. Initiation of Epac activation by cAMP occurs when it interacts with the phosphate-binding cassette of the CNBD and induces conformational changes that relieve the steric hindrance between the phosphate-binding cassette and the hinge of the CNBD, allowing the “hinge” to rotate (12, 13). This results in the release of the ionic latch due to an increase in the entropic penalty associated with preserving the auto-inhibited state and allowing the CNBD to move away from the CDC25HD (14). Thus, binding of cAMP induces a conformational change in which the regulatory region of Epac moves away from its catalytic region, facilitating an interaction of the cAMP; GEF, guanine nucleotide exchange factor; IFD, induced fit docking; MD, molecular dynamics; RBD, Rap1-GTP binding domain; BRET, bioluminescence resonance energy transfer; VASP, vasodilator-stimulated phosphoprotein.
CDC25HD with Rap and in turn, exchange of GDP for GTP (see Fig. 1B) (15).

In a previous study, we reported on the use of cAMP sensor using YFP-Epac-Rluc (CAMYEL) to identify competitive or allosteric inhibitors of Epac in vitro (16). CAMYEL is a bioluminescence resonance energy transfer (BRET)-based cAMP sensor based on Epac1 that is sandwiched between citrine and Renilla luciferase (17). The inactive state yields a strong BRET signal that results from the transfer of energy from luciferase to citrine. Activation by cAMP induces conformational changes that result in a decrease in the BRET signal as the luciferase moves away from citrine. We verified the results from the CAMYEL assay by conducting studies with Swiss 3T3 cells and monitoring activation of the downstream target of Epac, Rap1b. In the current study, we used molecular modeling and found that the hinge of the CNBD of Epac1 may be a novel region to target with allosteric inhibitors. Subsequent studies demonstrated that a thiobarbituric acid derivative, initially identified in silico docking at that region, prevents Epac activation in vitro and in cells.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Transfections**—Swiss 3T3 and HEK293 cells were grown in 10-cm culture dishes at 37 °C, 5% CO2 in DMEM supplemented with 5% FBS, 1% penicillin, and 1% streptomycin. Transient transfection of pcDNA3 CAMYEL into HEK293 cells was carried out using TransIT LT-1 (Mirus Bio) according to the manufacturer’s instructions. Lysis and BRET measurements were performed 48–72 h after transfection. For Rap1 pulldown assays in HEK293 cells, the cells were split into 10-cm culture dishes, allowed to adhere overnight, transfected the next day with Epac2 plasmid, and serum-starved in DMEM for 24 h before subsequent assays.

**Isolation and Culture of Adult Rat Cardiac Fibroblasts (CFs)**—Ethical approval for the care and use of animals was granted by the University of California at San Diego Institutional Animal Care and Use Committee. CFs were isolated from adult (8–10 weeks), male Sprague-Dawley rats, as described (18). Briefly, the rats were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection. The heart was excised and digested with collagenase II (Worthington Biochemical, Lakewood, NJ) via a modified reverse-Langendorff apparatus. CFs were isolated from cardiac myocytes by gravity separation and grown to confluency in 10-cm culture dishes at 37 °C, 10% CO2 in DMEM supplemented with 10% FBS, 1% penicillin, and 1% streptomycin. Studies with the CFs were undertaken with cells that were passaged no more than two times.

**BRET/FRET Assay**—HEK293 cells expressing CAMYEL were harvested and lysed in CAMYEL assay buffer (40 mM Hepes, pH 7.2, 140 mM KCl, 10 mM NaCl, 1.5 mM MgCl2) with 0.5% Triton X-100 and 1% Complete protease inhibitor mixture (Roche Applied Science) as described (19). After centrifugation at 20,000 × g for 10 min, the supernatant was removed and diluted to the desired volume. 100 µl was added to 96-well white plates and incubated for 5 min at room temperature with the indicated treatments. Inhibitors were added 5 min before those treatments. Coelenterazine was added to a final concentration of 2 µM immediately before assessing BRET. Emission from Renilla luciferase and citrine was measured simultaneously at 465 and 535 nm in a DTX-800 Plate Reader (Beckman Coulter). Apparent activation curves were determined by fitting the data to a sigmoidal dose-response curve.

**Immunoblot Analysis**—Whole-cell lysates were prepared in 150 mM Na2CO3 buffer (pH 11) and homogenized by sonication. Equal amounts of protein (assayed using a dye-binding reagent; Bio-Rad) were separated by SDS/PAGE using 10% polyacrylamide precast gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane with the X-Cell II Blot Module (Invitrogen). Membranes were blocked in PBS Tween (1%) containing 5% bovine serum albumin and incubated with primary antibody 18 h at 4 °C. Bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and ECL reagent (Amersham Biosciences Pharmacia, Pittsburgh, PA). Bands were compared with molecular weight standards to confirm migration of proteins at the appropriate size. Quantification of protein expression densitometry was performed using ImageJ software (NIH, Bethesda, MD). Epac2 (SB1), pVASP Ser-157, and Rap1A/Rap1B antibodies were purchased from Cell Signaling (Danvers, MA). Epac1 (A-5) antibody was purchased from Santa Cruz. β-Tubulin antibody was purchased from Abcam.

**Rap1 Activation Assay**—The Rap1-GTP-binding domain (RBD) of mammalian RafGDS was expressed in Escherichia coli as a GST fusion protein as described (20). The purified GST-RafGDS-RBD was used to detect activated Rap1. 10-cm plates of Swiss 3T3 cells were washed twice with ice-cold PBS and then lysed with 1 ml of Tris lysis buffer (100 mM Tris, pH 7.5, 300 mM NaCl, 50 mM MgCl2, 20% glycerol, 1% Nonidet P-40, 2 mM DTT, 2 mM vanadate, aprotinin, and 5 µg/ml leupeptin) at the indicated times. The lysates were centrifuged for 10 min, and the supernatants were incubated with 10 µg of GST-RBD on glutathione-Sepharose beads at 4 °C for 60 min. The beads were washed three times and subjected to SDS-PAGE and Western blot analysis with either an anti-pVASP mAb or anti-Rap1 pAb. For control of the input amount of Rap1 protein, we analyzed 20 µl of the cell lysates.

**cAMP Radioimmunoassay**—Swiss 3T3 cells were plated in 24-well plates (30,000 cells/well) and serum-starved for 24 h prior to cAMP quantification by radioimmunoassay, as described previously (18). After serum starvation, cells were incubated with 10 µM 5376753 for 30 min prior to treatment with 10 µM forskolin for 5 min. Incubations were terminated by addition of 7.5% trichloroacetic acid. Radioimmunoassay was used to quantify cellular cAMP concentration, which was normalized to total protein content per sample, as determined by a Bradford assay (Bio-Rad).

**CF Migration**—Migration of CFs was assayed by using a modified Boyden chamber method. CFs were seeded at a density of 1 × 10^5 CFs/100 µl onto uncoated Transwell inserts with 8.0-micron size pores (Corning). CFs were then serum-starved for 24 h. Basal migration was assessed by adding serum-free DMEM (600 µl) to the lower chambers. Cells were allowed to migrate for 16 h in presence of indicated compounds and then fixed in 10% formalin and stained with Hema 3 (Fisher Scientific). Cells on the upper surface of the membrane were
mechanically removed with a cotton swab. Cells that migrated were counted from four different fields (0.1 mm²/field).

**Cell Viability Assay**—Swiss 3T3 cells were seeded at 5 × 10⁴ cells/ml in 24-well plates in DMEM with 10% FBS. 24 h later, the cells were treated with the indicated compounds or 1% dimethyl sulfoxide. Viability was assessed 48 h later using the CellTiter-Glo assay according to the manufacturer’s instructions (Promega).

**Mapping of Druggable Hot Spots in the Inactive Epac Structure**—When mutated, certain amino acid residues near a hinge region of Epac can substantially alter its activity (21). To investigate potential druggability there, we used FTMap and small organic molecules to sample the surface of apo-Epac2 to find favorable locations to dock (22, 23). These small molecular probes were then clustered to find “hot spots” and ranked based on their average energy. Cluster locations were visualized in PyMOL (24).

**Computational Modeling of Apo-Epac1 and Virtual Screening for Allosteric Modulators**—I-TASSER was used to generate a three-dimensional model of the apo-Epac1 structure in its inactive state (25, 26). This model and a library of compounds from Chembridge were prepared with the Schrödinger Protein Preparation Wizard and the LigPrep Program, respectively (27, 28). A preliminary screen centered on the Epac hinge was performed; docking poses were generated using Schrödinger Induced Fit Docking (IFD). A library of 450,000 compounds from Chembridge was filtered to yield 101,691 by removing toxic, or reactive functional groups, more than four chiral centers, >10 rotatable bonds, salts or undesired atoms, or properties outside the following ranges: 250 < molecular weight < 460, 50 < polar surface area < 150, and −2 < cLogP < 5.0. Schrödinger IFD was used with the prepared protein and ligands, the latter of which were represented as 169,381 different optimized compound “models” generated with LigPrep to incorporate ligand flexibility (including tautomeric, stereochemical, and ionization variations) (14, 15).

**Apo-Epac1 Molecular Dynamics (MD)**—To explore protein flexibility further, MD was performed with the apo-Epac1 model. The system was first prepared (parameterized and solvated with Xleap) for AMBER MD as described (16). Second, whereas the protein was kept fixed, the water positions were allowed to minimize. Third, the energy of the whole system was minimized. Next, the water was heated from 0 to 300 K during 20 ps of MD at constant volume using the Langevin thermostat. The system was then equilibrated during 100 ps of subsequent MD at constant pressure. After minimization and equilibration, four independent production MD runs were performed on the Gordon Compute Cluster at the San Diego Supercomputer Center, totaling 200 ns. All MD simulations were performed under constant pressure periodic boundary conditions with isotropic position scaling, a reference pressure of 1 atm, and a pressure relaxation time of 2 ps. The system was maintained at a reference temperature of 300 K using the Langevin thermostat with a collision frequency of 2 ps⁻¹ (29). A simple Leapfrog integrator was used to solve Newton’s equations of motion and propagate the trajectory with a time step of 0.002 ps. Long-range electrostatic interactions were handled with the particle-mesh Ewald procedure using the sander Amber module; long-range van der Waals interactions were estimated by a continuum model. Ref. 30 provides further computational details.

**Incorporating Epac Flexibility into Virtual Screening**—The MD trajectories were concatenated and clustered using GROMACS (root mean square deviation cut-off of 0.125 nm) around the Epac1 hinge (Leu-315 and Phe-342) (31). The central member from each of the 30 largest clusters was considered representative of the set of structures and combined to create an “ensemble” to be used in virtual screening. PDB2PQR was used to generate pdbqt files for the ensemble of structures and the library of Chembridge compounds (32, 33). These files were used as inputs for the AutoDock Vina molecular docking program on University of California at San Diego’s National Science Foundation Center for Theoretical Biological Physics (CTBP) Linux cluster. The docking was centered around the apo-Epac1 hinge (residues Leu-315 and Phe-342) for both the IFD described above and these Vina screens. The results were used to determine compound priority for purchase and experimental validation.

**Epac1=5225554 and Epac1=5376753 Poses near the Hinge**—For each of the two most experimentally promising ligands, IFD docking was performed near the hinge, and the top poses for each were compared. Ligand Interaction Diagram screenshots and Protein Data Bank structures were saved in Maestro (34). PyMOL was used to generate docking pose images with these Protein Data Bank files.

**Reagents**—Reagents were purchased as follows: cAMP and cAMP analogs from Biolog (Bremen, Germany), hinge compounds from Chembridge (San Diego, CA), coelenterazine from Nanolight (Pinetop, AZ), forskolin from Abcam Biochemicals (Cambridge, MA), complete protease inhibitors from Roche Applied Science (Indianapolis, IN), phosphatase inhibitor mixture 3 and PDGF-BB from Sigma-Aldrich and pLX304-Epac2 from the DNASeq plasmid repository. cdNA3 CAMYEL was a kind gift from Dr. Paul Sternweis (UT Southwestern). CE3F4 was kindly provided by Frank Lezoualch (INSERM UMR-1048, Toulouse, France).

**Statistical Analysis**—Z’ factor was calculated as described (35)

\[ Z' = 1 - \frac{(3SD_{\text{max}} + 3SD_{\text{min}})/\Delta M}{\text{mean min value}}, \]

where \( \Delta M = (\text{mean max value} - \text{mean min value}) \), and \( SD_{\text{max}} \) and \( SD_{\text{min}} \) are the respective S.D. Calculations and statistics were performed using GraphPad Prism software (version 6.0; GraphPad Software, La Jolla, CA). Numerical values are presented as means ± S.E. All experiments were performed in triplicate or quadruplicate. Analysis of data from experiments with multiple comparisons was done using analysis of variance with Bonferroni’s correction. Values of \( p < 0.05 \) were considered significant.

**RESULTS**

**Identification of the Hinge Region of Epac as a Putative Druggable Region**—We sought to identify novel regions of Epac that might be targeted with small molecules. Prior experimental work investigating the mechanism of Epac activation had shown that upon cAMP binding at the CNBD, subsequent bending at a hinge region is an important component of protein activity (21). This bend is maintained via hydrophobic interac-
tions among three residues: Leu-408, Phe-435, and Leu-439, which are conserved in Epac1 and Epac2 (21). This provided an intriguing possibility for structure-based drug design. Our interest in pursuing small molecule inhibitors with an affinity for the Epac surface near the hinge was further confirmed by the results of FTMap sampling of potential druggable sites, which showed that the fifth largest cluster of probes was located near Leu-408 and Phe-435 on Epac2 (Fig. 1C).

Screening of Compounds That Bind to the Hinge Region of Epac—An I-TASSER generated model of Epac1 was used for MD and virtual screening. First, the model was used for an IFD screen of the Chembridge library. Ligprep-prepared compound “models” introduced ligand variety in the IFD protocol, which also allows for small accommodations in the protein upon binding. To incorporate additional flexibility of the protein in virtual screening efforts, MD was performed with an Epac1 model. The clustered trajectory provided a collection or “ensemble” of structures representative of the most stable conformations that could be used in a virtual screening approach with the Chembridge compound library (36). We used the faster Autodock Vina software to screen the same library against this ensemble of structures (made of the largest 30 clusters from the MD trajectory). There were unique top hits from the two different approaches so both sets were considered for experimental validation.

Based on results of this computational screen, we used the CAMYEL assay to test the 133 compounds that had the highest score (16). The CAMYEL assay has a \( Z' \) factor of 0.725, indicating that it satisfies the requirement of \( >0.5 \) for use in high throughput screening assays. We treated HEK293 cell lysates with CAMYEL in 96-well white plates with either 10 or 100 \( \mu \text{M} \) of each compound for 5 min followed by activation of CAMYEL with 100 \( \mu \text{M} \) cAMP. Due to the high fluorescent background of the compounds at 100 \( \mu \text{M} \), we analyzed the inhibition of Epac by the compounds at 10 \( \mu \text{M} \) (Fig. 2A).

Screening of the 133 compounds revealed four compounds that decreased Epac activation in the CAMYEL assay; we further characterized these compounds for their ability to inhibit Epac1 in vitro and in cells. We have shown that incubation of Swiss 3T3 cells with the adenylyl cyclase activator, forskolin (to increase cellular cAMP) activates PKA and Epac1, as assessed by VASP phosphorylation at Ser-157 and activation of Rap1, respectively (16). Studies with Swiss 3T3 cells indicated that incubation of 100 \( \mu \text{M} \) 5225554, a barbituric acid, for 30 min inhibited forskolin-stimulated Rap1 activation (Epac-mediated signaling) without altering vasodilator-stimulated phosphoprotein (VASP) phosphorylation (PKA-mediated signaling) (Fig. 2, B and C). The other three compounds did not inhibit Epac1 in cells (data not shown). Interestingly, the IFD screen ranked the top experimental compound overall among the highest in the library, further demonstrating that every system is different and that a one-size-fits-all approach to virtual screening likely does not exist (37).

A Thiobarbituric Acid Derivative Is a More Effective Inhibitor of Epac in Cells Than Is the Barbituric Acid Derivative—Having confirmed the inhibitory effect of compound 5225554, we...
sought to determine the importance of the barbituric acidic head group for binding to Epac1. We thus tested similar molecules, which we obtained from Chembridge. One of these, 5376753, a thiobarbituric acid derivative, was even more effective than 5225554 but was not in the top 10 compounds in the IFD screen that identified 5225554. Analysis of the ability of the compound to inhibit Epac1 at different concentrations indicated that whereas 5225554 can inhibit Epac (Fig. 3, A and C), the thiobarbituric derivative 5376753 was more effective at inhibiting the Epac1 in cells (Fig. 3, B and D). The two compounds have IC_{50} values of 71 and 4 μM, respectively, in their inhibition of Epac1 in Swiss 3T3 cells.

To verify that 5376753 inhibits Epac, we tested its effect on the direct activation of Epac by cAMP analogs. Swiss 3T3 cells were treated with the partial agonist 8-CPT-cAMP (CPT) or the Epac-specific activator 8-CPT-2′-O-Me-cAMP (8-Me) alone or in the presence of 10 μM 5376753 (38). In addition to inhibiting Epac activation by the endogenous ligand cAMP, 5376753 also inhibited activation of Epac by CPT and 8-Me (Fig. 4, A and B). We next tested the ability of 5376753 to inhibit PKA-mediated phosphorylation of VASP. Swiss 3T3 cells were treated with forskolin alone or with the indicated concentration of 5376753. 5376753 was not able to inhibit PKA at any of the concentrations tested (Fig. 4C) and did not inhibit cAMP accumulation induced by the activation of adenyl cyclases in Swiss 3T3 cells by 10 μM forskolin (Fig. 4D).

Proposed Ligand Binding of 5225554 and 5376753—The IFD scores for 5225554 and 5376753 in the Epac1 model were −11.3 and −12.2 kcal/mol, respectively. The ligand interaction diagram (Fig. 5A) of the top pose from IFD of 5225554 shows potential interactions with Trp-283 (π-π), Val-337, and Asp-338 (H-bond). Selecting “polar interactions” within PyMoL suggests that there could be another bond between the ligand and Thr-281 (Fig. 5B). For the IFD top pose of 5376753, the ligand interaction diagram (Fig. 5C) is similar but shows one more potential π-π interaction with Trp-283 and the H-bond to Thr-281. Using PyMoL for visualization (Fig. 5D), it appears that Phe-342 is flipped outward −100° to accommodate the 5376753 docked slightly closer to hinge. Showing the polar interactions also highlighted another potential bond with Arg-336.

All residues involved in the potential interactions between Epac1 and these two ligands are conserved between Epac1 and Epac2, but only D338 is also conserved in PKA. This is consistent with our experimental results. Although the top IFD poses of 5225554 and 5376753 were very similar, there were small potential differences in structure. Phe-342 appears to open up away from 5376753 to allow a closer docking of the dichlorophenyl group than in 5225554. Also 5376753 may have an additional π-π bond to this same group, as well as a hydrogen bond to Arg-336 not seen in the predicted pose of 5225554. There may be additional reasons why 5376753 is more active as an inhibitor in cells, but it is encouraging that the computational modeling is in agreement with the experimental results in terms of the rank of the compounds.

We tested similar compounds to determine whether experimental data were consistent with the proposed binding mechanism (Fig. 5E). Substitution of methyl groups for hydrogen on the nitrogen of the acidic head group decreased the ability of this compound to inhibit Epac in cells, indicating the importance of the N-H group for binding to Epac (Fig. 5, F and G), as predicted by the proposed binding mechanism.

5225554 and 5376753 Noncompetitively Inhibit Epac—We next sought to assess the mechanism by which the compounds inhibit Epac activation. We previously showed that the CAMYEL assay can determine if an inhibitor is acting competitively or noncompetitively (16). We compared the inhibition of CAMYEL by 5225554 and 5376753 to the inhibition by the competitive inhibitor 8-CPT-N6-phenyl-cAMP (CPT-N6) and by the uncompetitive inhibitor CE3F4 (39). Treatment of CAMYEL with 10 μM 5225554 or 5376753 reduced the maximal change in BRET induced by cAMP without changing the apparent affinity of cAMP (Fig. 6A). This is similar to the inhibition induced by treatment with 10 μM of the allosteric inhibitor CE3F4 (Fig. 6B). Incubation of CAMYEL with 3 μM of the competitive inhibitor CPT-N6 followed by activation with...
cAMP resulted in a right-shift of the curve without decreasing the maximal change in BRET (Fig. 6C). Thus, 5225554 and 5376753 allosterically inhibit Epac.

The Inhibition of Epac by 5376753 Is Not Due to Loss in Cell Viability—We conducted additional studies to rule out the possibility that the inhibition of Epac might be due to an inhibition of cell viability. We assessed the ability of varying concentrations of 5225554 or 5376753 to induce death of Swiss 3T3 cells and found that <50 μM of both compounds did not significantly decrease viability (Fig. 7). 5376753 inhibits Epac at lower concentrations than does 5225554 while both have the same ability to induce cell death at higher concentrations, indicating that the loss in viability is an off-target effect not related to ability to bind and inhibit Epac.
FIGURE 5. Ligand interactions. Potential interactions between Epac and ligands 5225554 (A and C) and 5376753 (B and D) identified in Maestro (A and B) and PyMoL (C and D). The legend is shown for the interaction diagrams. Poses show residues Leu-315 and Phe-342 near the hinge and ligands in sticks (yellow in B and pink in D). Residues with potential interactions are shown as sticks with proteins as a schematic, and bonds as dotted dark gray lines (B and D). E, the structures of the three different inhibitors are shown. F, Rap1 activation and VASP phosphorylation were assessed by Western blotting of Swiss 3T3 cells that were untreated (Un), incubated with compounds A (5225554), B (5376753), or C (6333795) (all at 10 μM) for 30 min, and/or stimulated with forskolin (Fsk, 10 μM) for 5 min after incubation with the compounds. G, quantification of activated Rap1 from F (n = 4). *, p < 0.05; **, p < 0.01.
We tested whether 5376753 inhibits biological functions mediated by Epac, in particular, a cAMP-promoted increase in cell migration of CFs. Small increases in cAMP induced by low levels of forskolin (1 μM) stimulate migration of CFs in an Epac-dependent manner (41). We assessed the ability of 5376753 to inhibit migration of primary rat CFs induced by forskolin (1 μM) or 8-Me (50 μM) and found that migration induced by both treatments was blocked by 10 μM 5376753 (Fig. 8A). To verify that this effect resulted from inhibition of Epac and not global inhibition of migration, we assessed whether 5376753 altered the migration of CFs induced by PDGF and found that 5376753 did not block 10 ng/ml PDGF-BB-promoted migration (Fig. 8B) (42). Thus, 5376753 selectively inhibits Epac-mediated migration in fibroblasts.

**DISCUSSION**

Previous work has shown that movement of the hinge region of the CNBD is a key determinant in the activation of Epac. Studies of an F435G Epac2 mutant (Phe-435 is a conserved residue in the hinge) showed that it had GEF activity similar to that of the cAMP-activated wild-type protein but was also capable of partially activating Rap1 in the absence of cAMP (21). Conversely, an Epac F435W mutation with a bulkier residue in that position inhibits the bending of the hinge and catalytic activity of Epac. Together, those results suggest that Phe-435 allows for GEF activity after binding of cAMP and conformational changes that transition Epac into an active state; a bulkier residue does not permit this essential movement of the hinge region. This mechanism implies that binding of small molecules to this region have the potential to prevent Epac activation.

Having identified the hinge of the CNBD as a possible druggable region, we computationally and experimentally screened compounds for their ability to bind to this region. Using an in vitro BRET-based assay that we have validated for the identification of competitive or allosteric modulators of Epac activity (16), in the current study, we assessed the activity of allosteric modulators of Epac activity identified by the computational homology model of Epac1. We found that compound 5225554, a barbituric acid derivative, identified by the computational screen is a novel, allosteric inhibitor of Epac. We verified that 5225554 inhibits Epac1 in Swiss3T3 cells by assessing its inhibition of Rap1 activation induced by forskolin.

The current results showed that compound 5376753, the thiobarbituric acid modification of 5225554, has a greater ability to decrease the conformational changes of Epac necessary for its activation and to inhibit Epac signaling in cells. This greater inhibition may be attributable to increased π stacking between Trp-283 and the dichlorophenyl group that is lacking in 5225554. Substitution of the hydrogens on the nitrogen atoms of the thiobarbituric acid head group with methyls reduced the ability of the compound to inhibit Epac. This result is in agreement with the proposed binding of this compound, in which a hydrogen bond forms between the N-H group of the thiobarbituric acid and Thr-281 of Epac1. Thiobarbituric acid derivatives are more lipophilic than barbituric acids and thus, 5376753 may also be more cell-permeable (43).
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In summary, this study identified 5376753 as a novel non-competitive inhibitor of Epac1. The hinge region of Epac1 and Epac2 are highly conserved compared with PKA. Preliminary data indicate that 537653 may also inhibit Epac2 as well (data not shown). The hinge region of the CNBD is highly conserved between both Epac isoforms but differs from that of the CNBD of the PKA regulatory subunit. We verified that 5376753 does not decrease PKA-mediated VASP phosphorylation nor does it decrease cAMP accumulation induced by adenylyl cyclase activation, thus further implying that 5376753 is an Epac-selective inhibitor. It is intriguing to hypothesize that because targeting the hinge of Epac with small molecules leads to its inhibition, perhaps targeting the hinge region of PKA might also be a site to inhibit PKA signaling as well.

GEFs are not considered “classical” drug targets due to their lack of binding pockets for small molecules but as shown here, we discovered that the hinge region of the CNBD has potential as a possible druggable region of Epac (45). It will be of interest to determine whether other compounds that interact with the hinge region of Epac can be allosteric activators as well as allosteric inhibitors.

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