Epac1 and cAMP-dependent Protein Kinase Holoenzyme Have Similar cAMP Affinity, but Their cAMP Domains Have Distinct Structural Features and Cyclic Nucleotide Recognition*

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The cAMP-dependent protein kinase (PKA I and II) and the cAMP-stimulated GDP exchange factors (Epac1 and -2) are major cAMP effectors. The cAMP affinity of the PKA holoenzyme has not been determined previously. We found that cAMP bound to PKA I with a $K_d$ value (2.9 μM) similar to that of Epac1. In contrast, the free regulatory subunit of PKA type I (RI) had $K_d$ values in the low nanomolar range. The cAMP sites of RI therefore appear engineered to respond to physiological cAMP concentrations only when in the holoenzyme form, whereas Epac can respond in its free form. Epac is phylogenetically younger than PKA, and its functional cAMP site has presumably evolved from site B of PKA. A striking feature is the replacement of a conserved Glu in PKA by Gln (Epac1) or Lys (Epac2). We found that such a switch (E326Q) in site B of human RIα led to a 280-fold decreased cAMP affinity. A similar single switch early in Epac evolution could therefore have decreased the high cAMP affinity of the free regulatory subunit sufficiently to allow Epac to respond to physiologically relevant cAMP levels. Molecular dynamics simulations and cAMP analog mapping indicated that the E326Q switch led to flipping of Tyr-373, which normally stacks with the adenine ring of cAMP. Combined molecular dynamics simulation, GRID analysis, and cAMP analog mapping of wild-type and mutated BI and Epac1 revealed additional differences, independent of the Glu/Gln switch, between the Glu/Gln switch, between the binding sites, regarding space (roominess), hydrophobicity/polarity, and side chain flexibility. This helped explain the specificity of current cAMP analogs and, more importantly, lays a foundation for the generation of even more discriminative analogs.

Lower eukaryotes like Saccharomyces cerevisiae have as sole receptor for the signaling molecule cAMP the two cAMP-binding sites (A and B) of the regulatory (R) subunit of the cAMP-dependent protein kinase (PKA). These tandem cAMP binding domains can be traced in all four isoforms (RIα, RIIβ, RIβ, and RIIα) of mammalian PKA (1), in the cGMP-dependent protein kinases (2, 3), the cyclic nucleotide gated ion channels (3–5), and the exchange proteins directly activated by cAMP, Epac1, and Epac2 (6). In PKA conformational changes induced by cAMP binding to both site A and B are required to dissociate the catalytic (C) subunit from the holoenzyme complex (7, 8). In contrast, cAMP binding to a single site of Epac is sufficient to relieve the tonic intrachain inhibition of its GDP exchange activity toward the small GTPase Rap (6, 9). A major issue in cell signaling is how the second messenger cAMP uses the receptors PKA and Epac to coordinate biological effects (10). Comparison of the cAMP affinity of Epac1 and PKA holoenzyme would help predict which of the two cAMP receptors, if present in the same compartment, is likely to be preferentially activated by a slight increase of cAMP. For this the cAMP affinity of PKA holoenzyme, so far unknown, must be determined.

The functional cAMP site in Epac is presumably derived from the B site of PKA because the N-terminal site (A) is functionally deficient in Epac2 and completely lost in Epac1 (11). Despite overall amino acid sequence similarity, important differences exist between the cAMP domains of PKA and Epac. Most strikingly, the Glu interacting with the 2′-OH group of cAMP and conserved in the cAMP domains of all R subunits is replaced by Gln in Epac1 and by Lys in Epac2 (6). The effect of such a switch in PKA has not been studied.

Cyclic nucleotide analogs are able to rapidly and reversibly activate cAMP receptors in intact cells. The first generation of cyclic nucleotide analogs able to discriminate between Epac and PKA (20) has already been used successfully to dissect the contribution of each receptor to physiological cAMP responses (12–15). We wanted to understand the structural basis of the discriminative ability of these analogs and to probe Epac and PKA for useful

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4 The abbreviations used are: R, regulatory subunit of PKA; PKA, cAMP-dependent protein kinase; C, catalytic subunit of PKA; RI, depending on con-
cAMP Domain of Epac1 Compared with Site BI of PKA

differences to design second generation analogs with further improved affinity and specificity. We thus used cAMP analog mapping to probe for subtle effects of the E326Q mutation in the B site of hRIα and of the Q270E mutation in hEpac1. In addition, we performed comparative modeling of the same sites, both in their cAMP-ligated and unliganded state. So far, crystal structure information is not available for cyclic nucleotide liganded Epac or for R subunit with bound cAMP analogs modified in the adenine or ribose moiety. But crystal structures of related R complexes (8, 16, 17) and of unliganded Epac2 (9, 18) are available to aid the modeling. We found that holoenzyme complex formation lowered the cAMP affinity of RI several hundredfold. In fact, the cAMP affinity of RI in holoenzyme was similar to that of Epac1 (Kd = 2.8 μM). The E326Q switch in RI decreased the cAMP affinity of site B 280-fold, and a similar switch could therefore have contributed to a necessary drop in cAMP affinity during the early evolution of the Epac cAMP domain. Modeling and cAMP analog mapping suggested that the E326Q switch led to flipping of Tyr-373 with disrupted stacking interaction with the adenine ring.

**MATERIALS AND METHODS**

**Cyclic Nucleotide Analogs**—The cAMP and cGMP analogs were modified only in the purine ring or in the cyclic phosphate ring, and all 2′-deoxy-cAMP analogs were provided by Dr. G.-G. Genieser, BioLog Life Science Institute, Bremen, Germany. 2′-O-Methyl-cAMP, 2′-O-ethyl-cAMP, 2′-O-butyl-alkyl-cAMP, and 2′-O-isobutyl-cAMP were kindly provided by Drs. B. Jastorff and J. Kruppa, Bio-Organic Unit, University of Bremen, Germany. Detailed synthetic procedures for the analogs with 8-modified 2′-O-alkyl-substituents are published elsewhere (19, 20).

**Mutagenesis, Expression, and Purification of Proteins**—Site-directed mutagenesis of full-length hRIα to create RIG201E, RIG325D, RIG201E,E326Q, and RIG201E,M331N,N332D,R333A and of full-length human Epac1 to create Epac1Q270E was performed using the QuickChange™ kit (Stratagene, La Jolla, CA). The double-stranded plasmids and two corresponding synthetic oligonucleotide primers with the desired mutations were annealed and extended by means of the Pfu DNA polymerase. After temperature cycling, the parental DNA template was digested using DpnI. The mutated DNA was transformed into Epicurian coli× XL1-XL1-Blue supercompetent cells. The correct sequence of the mutated DNA was confirmed by complete sequencing.

Recombinant GST-human RI and GST-human Epac1, as well as the mutant proteins, were expressed in Escherichia coli BL21 cells and purified as described previously (20). The full-length GST-Epac1 plasmid was a gift from Drs. J. de Rooij and J. Bos, and Epac1-(149–318) was a gift from Drs. A. Kraemer and J. de Rooij. A. Wittinghofer (21). The catalytic subunit of PKA was purified to homogeneity by using a procedure described previously (22). Complete sequencing of the mutant proteins, were expressed in Escherichia coli BL21 cells and purified as described previously (20). The full-length GST-Epac1 plasmid was a gift from Drs. J. de Rooij and J. Bos, and Epac1-(149–318) was a gift from Drs. A. Krämer and A. Wittinghofer (21). The catalytic subunit of PKA was purified to homogeneity by using a procedure described previously (22).

**Determination of [3H]cAMP Binding to Epac1, to Free RI Subunit, and to RI Subunit in the PKA Holoenzyme Complex**—Determination of cAMP Analog Affinity for RI and Epac1; and Assessment of PKA Holoenzyme Formation—Size-exclusion chromatography (20) and ultrafiltration (23) were used to determine the binding of [3H]cAMP to Epac1 under strict equilibrium conditions. In addition, a scintillation proximity assay was used (20) to circumvent the cAMP-induced precipitation of full-length Epac at high protein concentration (21). To optimize the signal to noise ratio and to minimize the error of the estimate of the concentration of free (unbound) labeled cAMP, the ratio between the concentrations of cAMP-binding sites and [3H]cAMP was kept between 0.1 and 0.4, whenever possible. The equilibrium binding of [3H]cAMP to the RI subunit in holoenzyme complex with the C subunit of PKA was determined using a slight modification of the methods used for Epac (20, 23). Briefly, pure C subunit (5 μM in excess of the concentration of RI subunit) and cyclic nucleotide-free RI subunit were incubated with various concentrations of [3H]cAMP. A sample of this mixture was either applied to the top of a size-exclusion column pre-equilibrated with the same concentrations of [3H]cAMP and C subunit or in a centrifuge tube (Microcon YM-10) equipped with an ultrafilter (10-kDa cutoff). The amount of bound labeled cAMP was determined either directly in the fractions eluted from the size-exclusion column or deduced indirectly from the level of isotope (free [3H]cAMP) in the ultrafiltrate from the spin tube (23). The formation of holoenzyme complex between the C subunit of PKA and cAMP-saturated RI subunit with silent A site (RIG201E) or silent B site (RIG325D) was determined in the presence of 50 μM cAMP by size-exclusion chromatography, as explained previously (22, 24). Holoenzyme formation was also determined by the ability of cAMP-saturated RIG201E or RIG325D to inhibit the kinase activity of the C subunit in the presence of 50 μM cAMP and with 70 μM Leu-Arg-Ala-Ser-Leu-Gly (Kemptide) as substrate (see Ref. 22 for details).

The relative affinity of cAMP analogs for Epac1 and for the RI subunit was estimated by determination of their ability to displace [3H]cAMP from its binding site in each protein. For routine purposes we used the well validated ammonium sulfate precipitation method (20, 25, 26). For some analogs the results were validated using the equilibrium methods described above. The relative affinity of the analogs relative to cAMP is represented by the Kd' value, i.e., Kd' = Kd_cAMP/Kd_analog. The data obtained for the individual compounds are means from at least three independent measurements (see also the footnote to Table 1).

**MD Simulations**—MD simulations were carried out with the Amber8 suite of programs (27) implementing the Amber 1999 (28) and GAFF (29) force fields. The model of site BI was prepared by removing the first 132 amino acids from bovine RIα (PDB code 1RGS) (16). The model of the cAMP-ligated binding domain of Epac1 was constructed based on the structure of the cAMP-ligated RI (PDB code 1RGS), as reported previously (20). The raw structure of site BI with E326Q mutation was prepared by replacing one carboxyl O of Glu-326 by NH2 in the PDB file of the wild type, and the converse manipulation was done to produce Epac1Q270E from wtEpac1. All crystallographic water molecules were removed. Missing atoms in the crystallographic model were added by using the model building capabilities of WhatIf (30). Parameters for cAMP were generated using Antechamber with AM1-BCC charge fitting for the gaffe force field. Tip3p (31) water molecules were added so as to create a truncated octahedron, with a minimum of a 10-Å water
layer between the octahedron edges and the nearest solute atoms. Each of the two Epac systems includes 2210 solute atoms as follows: 2177 protein atoms and 33 cAMP atoms. Sodium cations were placed with a simple energy minimization algorithm to achieve a neutral system, and 6370 TIP3P water molecules were added to fill the octahedron. The BI systems include a total of 2106 solute atoms and 7300 TIP3P water molecules. Periodic boundary conditions were simulated with an octahedral unit cell. The energy of the systems was minimized for 3500 cycles, using a combination of steepest descent and conjugate gradient algorithms. During the first 1000 steps of energy minimization, the protein, cAMP, and counterion positions were maintained with harmonic restraints (300 kcal mol\(^{-1}\) \times \AA\(^{-2}\)). The restraints were removed for the final 2500 cycles. The system was then heated to 300 K over 200 ps at constant volume with periodic boundaries, applying Langevin temperature controls, SHAKE constraints (32) on hydrogen atoms, and a 2-fs time step. During heating, harmonic constraints (10 kcal mol\(^{-1}\) \times \AA\(^{-2}\)) were imposed on the atomic positions of the solute and of the sodium counterions. Another 100 ps of equilibration of the system was then performed with weak harmonic constraints on the solute (5 kcal mol\(^{-1}\) \times \AA\(^{-2}\)) at constant pressure and coupled to a heat bath with the weak coupling Berendsen algorithm (33) to keep the system at 300 K. SHAKE constraints were applied on bond lengths involving hydrogen atoms, and a 2-fs time step was used. A 12-Å cutoff was applied to the van der Waals and electrostatic interactions. The actual production phase of the simulation was then performed for 5 ns without constraints at constant temperature (with Berendsen temperature control) and pressure with a 2-fs time step and constraints on the bond lengths involving hydrogens (32). The calculations were performed on a Silicon Graphics Altix server equipped with Itanium 2 1.5-GHz CPUs. The four simulations were run on 2 CPUs each and took a total of 2250 CPU hours. Snapshots for subsequent analysis were taken every 2500 dynamics steps during the production phase of the simulations. DS ViewerPro (Accelrys) was used to prepare the figures.

Molecular Modeling, GRID Analysis, and Docking—All calculations of the molecular interaction fields were performed with GREATER, a graphical interface for GRID (34), version 22. In GRID, the probes (test atoms or molecules) are moved along the surface of the receptor (the target protein), and the interaction energies between the receptor and the probe are evaluated using a force field approach, and the values are stored at nodes in a three-dimensional grid encompassing the binding pocket of the receptor. The interaction box around the receptors was defined to enclose the active sites, and its size was 23 × 23 × 21 Å\(^3\). The GRID box dimensions were based on the edges being 6 Å beyond the position of the crystallographic cAMP molecule. The amino acid side chains of the targets were allowed to move during the calculations (directive MOVE = 1). Default values have been used for all other directives including the spacing between grid points = 1 Å (NPLA = 1). Other authors have tried similar calculations with smaller grid size without affecting the results significantly (35). The maximum positive cut-off energy was set to 5 kcal/mol. Eleven probes were chosen for the calculations as follows: 1) water; 2) hydrophobic probe; 3) methyl (CH\(_3\)) group; 4) sp\(^3\) amine NH\(_3\) cation; 5) neutral flat NH\(_2\), e.g. amide; 6) sp\(^2\) carboxyl oxygen atom; 7) alkyl hydroxy OH group; 8) ether oxygen; 9) aromatic thiophen sulfur; 10) organic bromine atom; and 11) metadiminobenzene.

Docking was with the Dock5 software package, applying the anchor first search procedure. The coordinates for BI were used to generate the pre-computed energy grids to be used in Dock. The grid was constructed with a distance of 20 Å around the bound cAMP molecule. The entire cAMP molecule was then used as an anchor for growing the different substituents at the 2'-position of the sugar and at the 6 and 8 positions of the adenine ring. The torsion angles of the partially grown conformers were optimized, and the final docked conformations were ranked using the energy scoring function of Dock.

RESULTS

Holoenzyme Formation Lowered the cAMP Affinity of RI by 3 Orders of Magnitude and Imposed Positive Cooperativity on cAMP Binding—The binding of cAMP to PKA holoenzyme has not been directly determined previously. The equilibrium \(^3\)H\]cAMP binding to holoenzyme was determined by allowing \(^3\)H\]cAMP (0.05–50 μM) and RI (0.025–4 μM) to preincubate with the C subunit (present in 5 μM excess compared with the RI subunit) and to determine macromolecule-associated \(^3\)H\]cAMP by size-exclusion chromatography (20, 36) or membrane filtration (23). The WT holoenzyme bound cAMP with a \(K_A\) of 2.9 μM, i.e. with 3 orders of magnitude lower cAMP affinity than the free RI subunit. The high value of the Hill coefficient (\(h = 1.4\)) indicated positive cooperativity of cAMP binding to the PKA holoenzyme. For comparison, the holoenzyme formed by RIG201E with silent A site or RIG325D with silent B site showed noncooperative binding, as expected for binding to one site.

The cAMP affinity of holoenzyme with one silent site (Fig. 1A) was slightly higher than that of WT holoenzyme (Fig. 1, C and D), but still nearly 3 orders of magnitude lower than for the free RI subunits (Fig. 1, B–D). The binding of \(^3\)H\]cAMP to wtRI, representing the composite of binding to site A and B, showed apparent negative cooperativity (\(h = 0.8\)) (Fig. 1D), but the low \(h\) value could be explained by the binding to two sites (A and B) with slightly different affinity and with no free energy coupling. This was demonstrated by the overlapping binding isotherms for wtRI and for the calculated composite of binding to site A in RIG325D and site B in RIG201E (Fig. 1, C and D).

We considered the possibility that the observed \(^3\)H\]cAMP binding, even in the presence of 5 μM excess of C subunit, could be to the free rather than to the holoenzyme-associated RI subunit. We determined the \(K_A\) value for the interaction of the C subunit with RIG201E, RIG325D, and wtRI in the presence of excess cAMP, and we found it to be less than 1 nM for RIG201E and RIG325D and about 0.2 μM for wtRI under conditions like those used in the present study (not shown; see also Ref. 22). These studies indicated that at most 4% of the wtR subunit and 0.1% of R subunit with one silent site was free at 5 μM excess of C subunit.

We conclude that holoenzyme formation lowered the cAMP affinity of RI by 3 orders of magnitude. This implies that the free R subunit will always be saturated with cAMP in cellulo and...
that therefore only the holoenzyme can respond to physiologically relevant changes in concentrations of cAMP.

Comparison of the cAMP Affinity of Epac1 and Site BI of RI; Role of the Homologous Residues Gln-270 (Epac1) and Glu-326 (RI)—The \( K_d \) value (2.8 \( \mu M \)) for cAMP binding to full-length Epac1 (Fig. 1E) was the same as found previously for the truncated cyclic nucleotide binding domain (20, 37). This value was also similar to the \( K_d \) value of wtRI in the holoenzyme form of PKA (Fig. 1, C and D), suggesting that Epac and the PKA holoenzyme respond to similar ranges of cAMP concentration.

If Epac had retained the high cAMP affinity of the free R subunit during evolution, it would be saturated by cAMP already in the resting state of a cell and thereby unable to respond to an increase of cellular cAMP. Therefore, the high intrinsic cAMP affinity of site B must have decreased early during Epac evolution. We noted that the important Tyr-373 in hRI was replaced by a His at position 317 in hEpac1 but was retained in Epac from Caenorhabditis elegans, which is the least evolved species in whose genome Epac has been identified so far. The important Glu-326 in hRI was replaced by Gln at position 270 of hEpac1 and by Lys in Epac from C. elegans (Fig. 2). We therefore decided to test whether the conservative E326Q mutation in site BI of RI could decrease the affinity for cAMP. We found that this mutation led to a 280-fold decreased affinity of site BI for cAMP, the \( K_d \) being 250 nM (Fig. 1B and Table 1). We noted also that the conserved Epac1 sequence 275NDA277 was different from hRI (Fig. 2), but hRI with M331N,N332D,R333A bound cAMP like wild-type site BI (not shown).

Unless the E326Q switch had been accompanied by other major alterations in Epac1 compared with the R subunit of PKA, reverse mutagenesis, as in Epac1Q270E, should restore the high affinity for cAMP. This was not the case. Only a moderately (2.5-fold) enhanced affinity was observed for either the isolated cAMP domain of Epac1Q270E (36) or the full-length Epac1Q270E (Fig. 1E). Thus, Epac1 bound cAMP with 1200-fold lower affinity than site BI even when it had a Glu in position 270.

**FIGURE 1.** The binding of cAMP to Epac1 and to sites A and B of free and holoenzyme-complexed RI. Effects of the E326Q mutation in site B of human RI and the Q270E mutation in human Epac1. A shows the degree of saturation of site BI of RI with silent A site (RIG201E; ) and site Al of RI with silent B site (RIG325D; ) as a function of concentration of \( [3H]cAMP \). The R subunit (at concentrations from 0.025 to 4 \( \mu M \)) was kept in holoenzyme complex by a 5 \( \mu M \) excess of C subunit of PKA. The binding was determined by equilibrium size-exclusion chromatography as described under "Materials and Methods." The left-hand graphs of B show titration of RIG201E ( ) and RIG325D ( ), but in the absence of C subunit. Note the much higher affinity for \( [3H]cAMP \) in the absence of C subunit. The right-hand side of B shows a titration curve for RI subunit with silent A site whose Glu-326 has been replaced by Gln (GST-RIG201E,E326Q). Bound \( [3H]cAMP \) was determined by either equilibrium size-exclusion chromatography ( ) or by ammonium sulfate precipitation ( ). The R subunit (at concentrations from 0.025 to 4 \( \mu M \)) was kept in holoenzyme complex by a 5 \( \mu M \) excess of C subunit of PKA. The binding was determined by equilibrium size-exclusion chromatography ( ) or by ammonium sulfate precipitation ( ). The right-hand side of B shows a titration curve for RI subunit with silent A site whose Glu-326 has been replaced by Gln (GST-RIG201E,E326Q). Bound \( [3H]cAMP \) was determined by either equilibrium size-exclusion chromatography ( ) or by ammonium sulfate precipitation ( ). D shows Hill plots of the data in B. The Hill coefficients (h) were calculated based on the average of the two experiments.
These data were compatible with a Glu to Gln or Glu to Lys (as in Epac2) switch occurring early in the evolution of Epac from PKA, and having as consequence a dramatic decrease of cAMP affinity. Obviously, additional changes in Epac must have occurred to explain the lack of return to high affinity cAMP affinity. For simplicity we will use the numbering for hRI in this and later figure legends, also when referring to data based on the hRI. For comparison we show the structures of the functional (C-terminal) cAMP site of Epac in C. elegans and the only cAMP site of hEpac. Residues that are conserved in all the sequences are highlighted in yellow.

**FIGURE 2. Sequence alignment of site B of PKA and the cAMP site of Epac from selected species.** The sequence of site B of the R subunit of PKA is shown for Dictostelium discoideum, C. elegans, human RIα, and bovine RIα. Note that human and bovine RIα have identical B site sequence but differ in numbering because of two extra residues in the N-terminal domain of the hRIα. For simplicity we will use the numbering for hRIα in this and later figure legends, also when referring to data based on the hRIα. For comparison we show the structures of the functional (C-terminal) cAMP site of Epac in C. elegans and the only cAMP site of hEpac. Residues that are conserved in all the sequences are highlighted in yellow. Residues that have been mutated in the present study either physically or in silico are in red. The Swiss-Prot data base sequence entries were P05987, P30625, P10644/P00314, P34378, and O95398 in order from top to bottom.

**TABLE 1**

| Compound                  | Epac1 $K_d$ (μM) | Epac1Q270E, L $K_d$ (μM) | Site BI, L $K_d$ (nM) | SITE BIE326Q, $K_d$ (nM) |
|---------------------------|------------------|--------------------------|-----------------------|--------------------------|
| cAMP                      | 2.90 (1.0)       | 1.00 (1.0)                | 0.90 (1.0)            | 250 (1.0)                |
| 8- Hexylamino-cAMP        | 2.07 (1.4)       | 0.85 (1.3)                | 0.38 (2.4)            | 472 (0.5)                |
| 8-AHA-cAMP                | 2.23 (1.3)       | 0.78 (1.41)               | 0.17 (5.3)            | 208 (1.2)                |
| 8-ABA-cAMP                | 18.1 (0.16)      | 4.07 (0.27)               | 0.29 (3.1)            | 192 (1.3)                |
| 8-[N’(Methylanthraniloyl)]aminobutylamino-cAMP | 2.07 (1.4) | 0.82 (1.1) | 532 (0.47) |
| 8-AEA-cAMP                | 26.1 (0.14)      | 13.60 (0.06)              |                       |                          |
| 8-NH2-cAMP                | 0.36 (8.0)       | 0.13 (8.23)               | 0.19 (4.7)            | 109 (2.3)                |
| 8-MIA-cAMP                | 1.20 (2.4)       | 0.36 (3.06)               | 0.32 (2.4)            | 167 (1.5)                |
| 6-Phe-8-cPT-cAMP          | 0.03 (110)       | 0.06 (18)                 | 0.82 (1.1)            | 391 (0.64)               |
| 8-CPT-cAMP                | 0.02 (65)        | 0.11 (9.9)                | 0.82 (1.1)            | 391 (0.64)               |
| 8-OH-cAMP                 | 0.80 (3.6)       | 1.30 (0.69)               | 532 (0.47)            |                          |
| 8-Br-cAMP                 | 0.35 (8.1)       | 0.21 (5.17)               | 1.63 (0.51)           | 490 (0.51)               |
| 8-Pip-cAMP                | 13.81 (0.21)     | 8.76 (0.14)               | 45 (0.02)             | 12500 (0.02)             |
| cGMP (2-NH2, 6-O)         | 37.2 (0.078)     | 17.70 (0.062)             | 75 (0.012)            | 65000 (0.004)            |
| 6-Phe-cAMP                | 1.03 (2.78)      | 2.36 (0.38)               | 812 (0.31)            |                          |
| 6-Monobutylamino-cAMP     | 3.76 (0.77)      | 2.15 (0.51)               | 15.5 (0.058)          | 4550 (0.055)             |
| 2’-O-Me-cAMP              | 24.2 (0.12)      | 84.6 (0.013)              | 346 (0.0026)          | 80000 (0.003)            |
| 2’-Ori-cAMP               | 58 (0.05)        | 141 (0.008)               | 237 (0.0038)          | 60000 (0.004)            |
| 2’-O’P-cAMP               | 116 (0.025)      | 250 (0.004)               | 1800 (0.0005)         |                          |
| 2’-OBU-cAMP               | 116 (0.25)       | 239 (0.0046)              | 1500 (0.0007)         |                          |
| 8-Br-2’-Ome-cAMP          | 3.2 (0.9)        | 11.9 (0.09)               | 2250 (0.0004)         | 60000 (0.0004)           |
| 8-CPT-2’-Ome-cAMP         | 0.63 (4.6)       | 2.24 (0.49)               | 563 (0.0016)          | 125000 (0.002)           |
| 2’-Deoxy cAMP             | 1160 (0.003)     | 2070 (0.0003)             | 3000 (0.0003)         |                          |
| 8-CPT-2’-deoxy cAMP       | 17.1 (0.17)      | 27.5 (0.04)               | 1290 (0.007)          |                          |
| 6-Phe-8-CPT-2’-deoxy cAMP | 6.30 (0.46)      | 8.50 (0.13)               |                      |                          |

*The table lists the $K_d$ value for cAMP and a number of analogs, given either in μM (Epac1 and Epac1Q270E) or in nM (site BI or site BIE326Q). The numbers in parentheses ($K_d = K_{Epac1/CAMP}/K_{Epac1 analog}$) give a measure of the affinity relative to cAMP for each analog. For analogs reported previously (20), the values for wtEpac1 and wtRIα represent an average of those data and new determinations using a fresh batch of analog.*
partly responsible for separating the loop between β-sheets 4 and 5 from the C-terminal α-helix (Fig. 4A). The average amino acid backbone fluctuation, as calculated from the MD simulations, revealed that the C-terminal α-helix and the residues at the apex of the loop joining β-sheets 4 and 5 were the most flexible parts of site BI, and became even more flexible after the E326Q mutation (Fig. 3B). This may be related to the loss of the interaction between Arg-305 and Phe-376 in RIE326Q.

A surface representation of site BI illustrated that the E326Q switch led to a wider orifice of the cAMP binding pocket (Fig. 4, D and E). This fact and the loss of stacking interaction between cAMP and Tyr-373 would enhance the dissociation of bound cAMP and could thereby explain the strong decrease of cAMP affinity caused by the E326Q switch (Fig. 1B).

To probe further for reasons for the lack of return of high affinity cAMP binding of Epac1 upon the switch Q270E (Fig. 1E), we undertook an MD simulation of Epac1Q270E in which His-317 had been replaced by Tyr and the triplet NDA (275–277) by MNR, like in the homologous residues of site BI of hRIα (Fig. 2). We found only a minor approximation of Tyr-317 to either the Glu-270 or the adenine ring of cAMP (Fig. 4F). This indicated that Epac1 has acquired distinct properties that could not be explained by point mutations of residues 270, 275–277, and 317 alone.

The simulation of the binding domain of Epac pointed to a significant displacement of the terminal amphipathic α-helix comprising residues 306–323 as a result of the Q270E switch, regardless of the presence of a His or Tyr at position 317. All these residues faced oppositely of Gln/Glu-270 in the Epac1 model, His-317 being the only polar residue of the helix facing Gln/Glu-270. The displacement of this hydrophobic helix in EpacQ270E could be related to the increased polarity introduced by Glu-270. In contrast, the charge of Glu-326 appears to be better accommodated in site BI through contacts with Tyr-373 and polar residues like Asn-369 in the helix. This difference between Epac and site BI may be one explanation for the lack of high affinity cAMP binding to Epac1 with switched Q270E.

**cAMP Analog Mapping and Docking of Analogs to Epac1 and Site Bo of Rlα**—Cyclic nucleotide analogs were used to probe for fine differences between the cAMP sites of native RI and Epac1, as well as any effect of the reciprocal mutations E326Q in the free RI subunit and Q270E in Epac. The methods were based on competition between the analogs and [3H]cAMP and have been validated for free RI and Epac (20). The analog affinity was determined through competitive displacement of [3H]cAMP from the binding site and is expressed as apparent $K_d$ and as affinity relative to cAMP ($K_d/cAMP$). In an initial series of experiments we found no difference in analog affinity whether using full-length Epac1 or the isolated cAMP domain (Epac1-(149–318)) of Epac1 (not shown). This fact and the similar cAMP affinity of the truncated and full-length Epac1 (Fig. 1E) suggest that the cAMP binding domain of Epac1 has relative autonomy regarding cyclic nucleotide binding characteristics. This supports the validity of the modeling of the cAMP site of Epac1 based only on the residues of the cAMP binding domain itself (Fig. 4). In the case of Epac1Q270E, only the full-length protein was studied.
cAMP Domain of Epac1 Compared with Site BI of PKA

A
B

C

D

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K

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X

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Z
cAMP Domain of Epac1 Compared with Site BI of PKA

Substitutions of cAMP were generally less detrimental for binding to the predicted more roomy and flexible binding site of Epac1 than to site BI (Table 1). In fact, only four (8-hexyl-lamino, 8-AHA-, 8-ABA-, and 8-MA-cAMP) of the 24 analogs tested had higher affinity relative to cAMP (comparison of $K_d$ values) for BI than for Epac1. The strongest discrimination in disfavor of Epac was for 8-ABA-cAMP, which had 19-fold lower relative affinity for Epac1 than for site BI (Table 1). To understand the structural basis for the poor affinity for Epac1, a complete MD simulation was performed with 8-ABA-cAMP docked into the Epac1 site (Fig. 5). This was combined with prediction of regions with high hydrophobicity (green) in Fig. 5) and high affinity for the probe NH$_3^+$ (blue in Fig. 5), based on GRID analysis. GRID analysis allows an evaluation of areas on the surface of the cAMP binding pocket with high affinity for selected chemical probes. The terminal NH$_3^+$ of 8-ABA docked into a hydrophobic region separated by more than 5 Å from a predicted high affinity region for NH$_3^+$ (Fig. 5).

We have proposed previously (19) that 8-NHR-substituted cAMP analogs have enhanced BI affinity because of favorable interaction of the NH with the apical OH of Tyr-373 (in hRIa). If our model showing flipping of Tyr-373 in BIE326Q (Fig. 3) is correct, the 8-NHR-substituted analogs should therefore lose disproportionately more affinity for the E326Q switched site B than other cAMP analogs or cAMP itself. In fact, the 8-NHR analogs consistently had a larger loss of affinity (Table 1). No change in relative affinity was noted for 8-NHR-substituted analogs between Epac1 and Epac1Q270E (Table 1), in accordance with the lack of a suitable tyrosine in the vicinity of the C-8 position of cAMP in Epac.

The commonly used PKA activator 8-CPT-cAMP had 65-fold higher affinity than cAMP for Epac1. The 8-CPT enhanced the Epac1 affinity at least 40-fold also when present as a second substituent, added to 6-Phe-cAMP, 2’-deoxy cAMP or 2’-O-Me-cAMP. To reveal the basis for the Epac preference, we undertook GRID calculations for the meta-diaminobenzene probe, whose size and polarity resemble the CPT substituent. The probe had a large and relevantly positioned area (green in Fig. 6) for favorable interaction with Epac1 (Fig. 6C). It had a smaller area for favorable interaction in Epac1Q270E (Fig. 6D), and no favorable interaction close to the C-8 position for either BI or BIE326Q (Fig. 6, A and B). In line with these predictions, the 8-CPT substituent was less beneficial for binding to Epac1Q270E than Epac1 and had no significant effect on analog affinity for site BI (Table 1). The findings were also in line with the model of Epac and EpacQ270E with bound cAMP. Whereas...
His-317 of Epac1 was too far away to stack with the adenine ring of cAMP, an aromatic 8-substituent of cAMP could stack between His-317 and Phe-268. In EpacQ270E, the His-317 was further separated from Phe-268 as well as from the adenine ring of cAMP (Fig. 4B). The model therefore predicted that the Q270E switch would decrease the ability of Epac to form high affinity interaction with aromatic 8-substituents in the plane of the adenine ring of cAMP. Therefore, the binding data, the GRID analysis, and the structural model all suggest that the Epac1Q270E mutation not only affected the binding site near the 2'-position of cAMP but also modulated the structure in the vicinity of the C-8 position of bound cAMP.

The success of 8-CPT-2'-OMe-cAMP, relative to cAMP, to discriminate between Epac1 and PKA (Table 1) relies in part on the contribution of the 8-CPT group (see above) and in part from the introduction of a methyl group at the 2'-position of cAMP (10, 20). The 2'-O-alkyl-cAMP analogs were believed to owe their discriminative power to the switch from Glu to Gln at the position corresponding to Glu-326 in site BI and Gln-270 in Epac1 (39) (Fig. 2). The analog mapping data did not support this simplistic view. Although 2'-deoxy-cAMP and 2'-O-alkyl-cAMP analogs had lower affinity (relative to cAMP) for Epac1Q270E than for Epac1, they bound to Epac1Q270E with relatively higher affinity than to either site BI or BIE326Q (Table 1). A GRID analysis with an alkyl hydroxyl (O-1) probe detected strong binding preference close to Glu-326 in site BI but less in the corresponding location in Epac1Q270E (Fig. 6). We conclude that Epac1 had less affinity than site BI for 2'-OH, whether a Glu or Gln faces the 2'-OH of cAMP. The methyl probe and dry (hydrophobic) probes did not provide significant differences between the receptors in the region normally facing the 2'-position of bound cAMP (not shown). Likewise, the receptors appeared similar in their ability to accommodate bulky 2'-substituents, because little relative difference was noted for 2'-O-R, whether R was methyl, ethyl, propyl, or butyl (Table 1).

The design of novel improved analogs will be greatly aided if the effects of multiple substitutions can be predicted based on data for mono-substituted compounds. One obvious path is to introduce novel modifications using 2'-OMe-cAMP as the backbone. The 2'-OMe group had a nearly additive effect on Epac1 affinity when combined with 8-CPT substitution, with 8-Br- or even with 6-Phe-8-CPT- (Table 1) (see also Ref. 20). This indicated that the 2' modification did not perturb the Epac1 site or displace the cAMP analog in a way interfering with the accommodation of the mentioned adenine substituents. In contrast, for site BI the combination of substituents tended to be infra-additive (Table 1). We conclude that extensive modifications of the adenine ring of 2'-OMe-cAMP may be possible without sacrificing the affinity advantage of 2'-OMe-cAMP for Epac1 relative to site BI.

Use of cAMP Analogs to Compare Free and PKA Holoenzyme-associated RI and Demonstrate Site A-B (Intrachain) Cooperativity of Binding in PKA Holoenzyme—An important issue was whether the data obtained for the free RI subunits were relevant also for RI in the PKA holoenzyme, which is the form encountered by the analog in the intact cell. Early studies using a large panel of cAMP analogs showed a near perfect correlation between the average affinity (relative to cAMP) of each analog to site A and B of isolated free R subunit and the ability to activate the kinase activity of PKA (40, 41). One would therefore presume that cAMP analogs activating PKA had similar affinity, relative to cAMP, for free R and PKA. Because this has not been demonstrated experimentally, we tested a few selected analogs for the ability to compete with [3H]cAMP for binding to RI in the PKA holoenzyme form. The mapping of PKA holoenzyme is less straightforward than for free R subunit because of lack of useful equilibrium binding methods allowing the dis-
crimination of ligand binding to site A and B. We used therefore a nonequilibrium method to study effect of selected analogs on \([^{3}H]cAMP\) binding to site A and B (Fig. 7, A–D) and supplemented by using other analogs with similar site selectivity and two different equilibrium binding methods (Fig. 7E).

We compared first the ability of 8-AHA-cAMP, unlabeled cAMP, and 8-Pip-cAMP to competitively displace \([^{3}H]cAMP\) from site AI and BI of PKA that was nearly saturated by \([^{3}H]cAMP\) before the addition of unlabeled competitor. The 8-AHA-cAMP competed much more efficiently for binding to site BI (Fig. 7D) than site AI (Fig. 7B), as expected from its site BI preference in free RI (Table 1) (42). 8-Pip-cAMP, which prefers site AI in free RI (42), also did so in the PKA holoenzyme (Fig. 7, B and D). Using a size-exclusion equilibrium binding assay method, a 2-fold excess of the site BI-selective analog 8-ABA-cAMP relative to \([^{3}H]cAMP\) was found to nearly abolish the binding of \([^{3}H]cAMP\) to the B site of RI with silent A site (Fig. 7E, upper inset). A similar excess of the site AI-prefering 6-Phe-cAMP nearly abolished the binding of \([^{3}H]cAMP\) to the A site of RI with silent B site (not shown). We conclude that the relative affinity of key analogs for free and holoenzyme-associated RI was similar, although they bound with 3 orders of magnitude less affinity to RI in the holoenzyme complex, i.e. that the binding sites sensed the analog modifications similarly in the free and holoenzyme-complexed R subunit.

Finally, we used cAMP analogs to probe if the positive cooperativity observed for \([^{3}H]cAMP\) binding to the RI dimer in PKA holoenzyme (Fig. 1, C and D) was because of intrachain site A-B interaction or interchain interaction between similar sites. Only in the first case would an analog preferring site B enhance the binding of \([^{3}H]cAMP\) to site A. Such enhancement of binding could obviously only be studied when \([^{3}H]cAMP\) was present at a subsaturating concentration. We found that the strongly B-site preferring 8-AHA-cAMP nearly abolished the binding of \([^{3}H]cAMP\) to the A site of RI with silent B site (not shown). We conclude that the relative affinity of key analogs for free and holoenzyme-associated RI was similar, although they bound with 3 orders of magnitude less affinity to RI in the holoenzyme complex, i.e. that the binding sites sensed the analog modifications similarly in the free and holoenzyme-complexed R subunit.
of the site A-B cooperativity. In the assay based on gel filtration, this mechanism of enhancement could not operate because the concentration of free \([^3H]cAMP\) is stable \((20)\). Under these conditions we observed that although an analog abolished binding to one site the total binding was decreased by less than 50% \((\text{Fig. 7E, main panel})\), as expected if it competes with \([^3H]cAMP\) for one site and stimulates the binding to the other site. We conclude that cAMP analog binding to one site of RI enhanced the binding of \([^3H]cAMP\) to the other type of site, as expected for positive intrachain cooperativity of cAMP binding to PKA holoenzyme.

**DISCUSSION**

The C-terminal site B of the primitive eukaryotic R subunit of PKA is the presumed ancestor of the functional C-terminal cAMP site of Epac2 and of the single site of Epac1. Epac1 has evolved to have a 3000-fold lower affinity \((K_d = 2.8 \mu M)\) than site BI of the isolated RI \((K_d = 0.9 \text{ nM})\). This may appear surprising because both PKA and Epac are regulated by physiologically relevant cAMP concentrations, typically in the micromolar range. It should be kept in mind, however, that the cAMP molecule encounters the PKA holoenzyme rather than the free unliganded R subunit of PKA in the intact cell \((43)\). In the present study we found that when associated with the C subunit in the PKA holoenzyme complex, the RI had nearly the same \(K_d\) value for cAMP \((2.9 \mu M)\) as Epac1. The physiologically relevant cAMP affinity of Epac1 and PKA holoenzyme may therefore be quite similar. Presumably, closeness to cellular compartments with locally increased cAMP \((44)\) or the kinase substrate availability \((24)\) may decide whether Epac or PKA is activated first in response to a moderate cAMP increase in the intact cell.

The similar cAMP affinity of Epac and PKA holoenzyme explains why both PKA and Epac can respond to changes at the intracellular cAMP level in the micromolar range, but the question remains of how the Epac-binding site differs from the free unliganded R subunit of PKA in the intact cell \((43)\). In the present study we found that when associated with the C subunit in the PKA holoenzyme complex, the RI had nearly the same \(K_d\) value for cAMP \((2.9 \mu M)\) as Epac1. The physiologically relevant cAMP affinity of Epac1 and PKA holoenzyme may therefore be quite similar. Presumably, closeness to cellular compartments with locally increased cAMP \((44)\) or the kinase substrate availability \((24)\) may decide whether Epac or PKA is activated first in response to a moderate cAMP increase in the intact cell.

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binding. The fact that 6-Phe-8-CPT-cAMP bound with 110-fold higher affinity than cAMP to Epac1 illustrates the potential for improvement of binding by bulky substituents. For comparison, the best enhancement observed to date after screening about 170 cAMP analogs for site BI affinity is 4–6-fold (Table 1).\(^5\) Another striking difference is the presence in Epac1, but not in BI, of a large nonpolar region close to the C-8 position of bound cAMP (Fig. 5). It helped explain both the low Epac1 affinity for 8-ABA-cAMP and the high Epac1 affinity for 8-substituents with phenyl moieties, like 8-CPT-cAMP (Table 1; Figs. 5 and 6).

We conclude that even if the cAMP affinities of Epac1 and the PKA holoenzyme were remarkably similar, several important differences existed between the cAMP-binding sites of Epac1 and the RI subunit of PKA that could not be explained by the substitution of the conserved Glu-326 and Tyr-373 residues in hR\(\alpha\) by Gln-270 and His-317 in hEpac1. Some of these differences were disclosed by cyclic nucleotide analog mapping aided by computational analyses and will be expected to facilitate the design of novel cAMP analogs with improved binding site specificity. A distinct finding was that the PKA holoenzyme associated RI subunit-bound cAMP with positive cooperativity, because of intrachain interaction between the A and B sites. Such positive cooperativity was not noted for Epac, which has only one cAMP site (Figs. 1 and 7). The cooperativity would be expected to increase the responsiveness of PKA to a slight change of cAMP in the intact cell.

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