cAMP Analog Mapping of Epac1 and cAMP Kinase

DISCRIMINATING ANALOGS DEMONSTRATE THAT Epac AND cAMP KINASE ACT SYNERGISTICALLY TO PROMOTE PC-12 CELL NEURITE EXTENSION*

Received for publication, March 3, 2003, and in revised form, June 15, 2003
Published, JBC Papers in Press, June 20, 2003, DOI 10.1074/jbc.M302179200

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Little is known about the relative role of cAMP-dependent protein kinase (cAPK) and guanine exchange factor directly activated by cAMP (Epac) as mediators of cAMP action. We tested cAMP analogs for ability to selectively activate Epac1 or cAPK and discriminate between the binding sites of Epac and of cAPK and cAPKII. We found that commonly used cAMP analogs, like 8-Br-cAMP and 8-pCPT-cAMP, activate Epac and cAPK equally as well as cAMP, i.e. were full agonists. In contrast, 6-modified cAMP analogs, like N6-benzoyl-cAMP, were inefficient Epac activators and full cAPK activators. Analogs modified in the 2'-position of the ribose induced stronger Epac1 activation than cAMP but were only partial agonists for cAPK. 2'-O-Alkyl substitution of cAMP improved Epac/cAPK binding selectivity 10–100-fold. Phenylthio substituents in position 8, particularly with MeO- or Cl- in p-position, enhanced the Epac/cAPK selectivity even more. The combination of 8-pCPT- and 2'-O-methyl substitutions improved the Epac/cAPK binding selectivity about three orders of magnitude. The cAPK selectivity of 6-substituted cAMP analogs, the preferential inhibition of cAPK by moderate concentrations of Rp-cAMPS analogs, and the Epac selectivity of 8-pCPT-2'-O-methyl-cAMP was also demonstrated in intact cells. Using these compounds to selectively modulate Epac and cAPK in PC-12 cells, we observed that analogs selectively activating Epac synergized strongly with cAPK specific analogs to induce neurite outgrowth. We therefore conclude that cAMP-induced neurite outgrowth is mediated by both Epac and cAPK.

With the exception of cyclic nucleotide gated channels in specialized cells like olfactory neurons (1), the only known direct cAMP effector in mammalian cells was, until recently, the cAMP-dependent protein kinase (cAPK), whose mechanism of activation and structure has been studied in detail (2–4).

It has been questioned whether cAPK is the sole mediator of cAMP action (5, 6). The discovery (7, 8) of Rap guanine nucleotide exchange factors directly activated by cAMP (Epac1 and Epac2) raised the possibility that effects hitherto attributed to activation of cAPK were in fact mediated by Epac. Epac1 has an N-terminal DEP (dishevelled, Egl-10, pleckstrin) domain, involved in membrane docking (9) and cell adhesion (10), a cAMP binding domain (CNBD), a Ras exchange motif, and a guanine nucleotide exchange factor homology domain (see Fig. 1A). Epac2 has been crystallized recently (11). It has an additional CNBD (8), which is dispensable for cAMP-induced Rap activation (9, 12).

There is increasing but still modest knowledge about the biological consequences of cAMP activation of endogenous Epac in intact cells. Epac appears not to mediate modulation of extracellular signal-regulated kinase activity by cAMP (13) but may mediate the stimulation by cAMP of exocytosis (14, 15) and the calcitonin-induced H,K-ATPase activation in kidney cells (16) and may modulate integrin-mediated cell adhesion (17). Less is known about how cAMP signaling through cAPK and Epac might be integrated, but there is evidence that overexpressed Epac1 can activate Akt/protein kinase B, whereas stimulation of cAPK inhibits Akt/protein kinase B (18), suggesting that Epac and cAPK serve opposite functions. The aim of the present study was to develop and identify cyclic nucleotide analogs that could help distinguish the roles of cAPK and Epac1 in intact cells. We obtained first a detailed map of the cAMP binding site of Epac1 using more than 50 analogs, many of which are novel. We studied next the ability of selected analogs to activate Epac and cAPK in vitro. We found that 2'-O-alkyl-modified cAMP analogs were only partial agonists of cAPK activation, while being stronger than cAMP itself as activators of Epac. On the other hand, several 6-modified cAMP analogs were full cAPK agonists and poor agonists of Epac activation, even if they bound well to Epac.

BI and RII, regulatory subunit of cAPK isozyme I and II, respectively; Epac, exchange protein directly activated by cAMP; Epac1, Epac full-length protein; CNBD, cyclic nucleotide binding domain; NGF, nerve growth factor; GST, glutathione S-transferase; CREB, cAMP-response element-binding protein; cPuMP, cyclic purine monophosphate; Sp- and Rp-cAMPS, axial and equatorial diastereoisomers, respectively, of cAMPS; DCI-cBIMP, dicyclic-cyclic benzimidazole monophosphate; 6-Bnz-cAMP, N6-benzoyl-cAMP; 6-MB-cAMP, N6-monobutyryl-cAMP. The following abbreviations were used to designate substituents: Pip, pipеридино; pCPT, p-фарц-феноксицил-тио; AHA, аминохециламин; Phe, фенил; Me, метил; PT, фенилхинол; HPT, гидроксифенилтио; MABA, 4-[N-гексанцил]-аминоэтиламин. This paper is available on line at http://www.jbc.org

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Intact cell studies confirmed the cAPK specificity of 6-modified analogs and the Epac specificity of 2'-O-Me-cAMP analogs like 8-pCPT-2'-O-Me-cAMP. Using these analogs as tools we showed that Epac acts synergistically with NGF to promote neurite extension in PC-12 rat pheochromocytoma cells, a model for neuronal differentiation (19, 20). Activation of Epac showed that Epac acted synergistically with NGF to promote neurite extension in PC-12 rat pheochromocytoma cells, a model for neuronal differentiation (19, 20). Activation of Epac showed that Epac acted synergistically with NGF to promote neurite extension in PC-12 rat pheochromocytoma cells, a model for neuronal differentiation (19, 20).

EXPERIMENTAL PROCEDURES

Cyclic Nucleotide Analogs—The cAMP and cGMP analogs modified only in the purine ring or the cyclic phosphate ring and all 2'-deoxy cAMP analogs were provided by BIOLOG Life Science Institute, Bremen, Germany. 2'-O-Ethyl-, 2'-O-propyl-, 2'-O-butyl-, and 2'-O-isobutyl-cAMP were kindly provided by Drs. B. Jastorff and J. Kruppa, Bio-Organic Unit, University of Bremen, Bremen, Germany. 8-Br-2'-O-Methyladenosine was phosphorylated and cyclized in a one pot reaction to 8-Br-2'-O-Me-cAMP as described earlier (21). 8-pCPT-2'-O-Me-cAMP was synthesized by nucleophilic substitution (22) of 8-Br-2'-O-Me-cAMP. Other 8-substituted 2'-O-Me-cAMP analogs were synthesized essentially as described for 8-pCPT-2'-O-Me-cAMP. 8-pCPT-2'-O-Me-cAMP benzyl ester was prepared from 8-pCPT-2'-O-Me-cAMP by alkylation with benzyl bromide (23). All analogs, including the axial and equatorial stereoisomers of 8-pCPT-2'-O-Me-cAMP, were purified by reversed phase high pressure liquid chromatography (YMC ODS-120A; 10 μm) and isolated to purity >98%. The structure of each analog was confirmed by UV-visible and electrospray ionization-mass spectrometry analysis (8-Br-2'-O-Me-cAMP and 8-pCPT-2'-O-Me-cAMP also by 1H NMR and 31P NMR).

Epac and cAPK Purification—Recombinant GST-hEpac1fl, GST-hEpac12–329, GST-hEpac1149–318, GST-hRIIα, and His-hRIIα were expressed in E. coli BL21 cells. The GST fusion proteins were adsorbed to GST-agarose and either eluted with glutathione, or the GST cleaved off in situ with thrombin (7, 24). Epac149–
**Table I**

Affinity of purine base-modified cAMP analogs for Epac1 and site A and B of RI and RII

The table shows the affinity of cAMP analogs relative to cAMP (i.e., $K_i$, which is $K_{i\text{cAMP}}/K_{i\text{cAMP}}$) analog for Epac1 and site A and B of RI (AI and BI) and RII (AII and BII). The data were obtained by competitive displacement, as detailed under “Experimental Procedures.” The results represent the mean of three determinations, ranging ± 15% from the average.

| Compound                  | $K_i$, Epac1 | $K_i$, RI | $K_i$, RII |
|---------------------------|--------------|----------|-----------|
| cAMP                      | 1.0          | 1.0      | 1.0       |
| 2-NH$_2$-cAMP             | 0.13         | 0.043    | 0.35      |
| 8-Pip-cAMP                | 0.21         | 2.1      | 0.060     |
| 8-Br-cAMP                 | 0.81         | 1.3      | 1.0       |
| 8-pCPT-cAMP               | 0.65         | 3.9      | 1.7       |
| 8-AHA-T-cAMP              | 1.3          | 0.055    | 4.1       |
| 6-Phe-cAMP                | 2.8          | 11       | 0.38      |
| 6-Bz-cAMP                 | 1.3          | 4.0      | 0.26      |
| 6-MB-cAMP                 | 0.77         | 3.6      | 0.083     |
| cIMP (6 = O)              | 0.28         | 0.12     | 0.025     |
| cAMP (2-NH$_2$, 6 = O)    | 0.078        | 0.0011   | 0.012     |
| 6-Phe-8-pCPT-cAMP         | 110          | 8.1      | 0.90      |

**Table II**

Mapping of Epac1, RIs, and RIIAs with cAMP analogs modified in their cyclic phosphate or ribose moiety

The table shows the affinities for Epac1, RI (sites AI and BI), and RIIAs (AII and BII) of cAMP analogs with sulfur (S- or Rp- diastereomomer) substitution in the cyclic phosphate ring or with modification in the 2 position of the ribose ring. Several analogs had additional substitutions in the purine ring. Other details are described in the legend to Table I.

| Compound                  | $K_i$, Epac1 | $K_i$, RI | $K_i$, RII |
|---------------------------|--------------|----------|-----------|
| Sp-cAMPS                  | 0.085        | 0.18     | 0.034     |
| Sp-8-pCPT-cAMPS           | 5.2          | 1.6      | 0.087     |
| Sp-5,8-DCl-bIMPS          | 0.69         | 0.022    | 0.13      |
| Rp-cAMPS                  | 0.0083       | 0.0008   | 0.00027   |
| Rp-8-Br-cAMPS             | 0.098        | 0.00008  | 0.000071  |
| Rp-8-pCPT-cAMPS           | 0.36         | 0.015    | 0.0068    |
| 2′-dcAMP                  | 0.0025       | 0.000065 | 0.00028   |
| 8-pCPT-2′-dcAMP           | 0.17         | 0.002    | 0.00069   |
| 6-Phe-8-pCPT-2′-dcAMP     | 0.46         | 0.031    | 0.011     |
| 2′-O-Me-cAMP              | 0.12         | 0.0090   | 0.0026    |
| 2′-O-Et-cAMP              | 0.050        | 0.0049   | 0.0038    |
| 2′-O-Pr-cAMP              | 0.025        | 0.0011   | 0.00051   |
| 2′-O-Bu-cAMP              | 0.025        | 0.0010   | 0.00063   |
| 8-Br-2′-O-Me-cAMP         | 0.90         | 0.0037   | 0.00047   |
| 8-PT-2′-O-Me-cAMP         | 3.4          | 0.018    | 0.00052   |
| 8-pCPT-2′-O-Me-cAMP       | 4.6          | 0.043    | 0.0016    |
| 8-pHPT-2′-O-Me-cAMP       | 6.9          | 0.025    | 0.00060   |
| 8-pMeOPT-2′-O-Me-cAMP     | 7.1          | 0.025    | 0.00060   |

318 was a gift from Drs. A. Krämer and A. Wittinghofer (25). The proteins were further purified by size exclusion fast protein liquid chromatography as described (24). The catalytic subunit of cAPK was prepared as described (24).

**Determination of $[\text{3H}]$cAMP Binding to the R Subunits of cAPK and to Epac1**—An extensively validated ammonium sulfate precipitation method was used to assay $[\text{3H}]$cAMP bound to the RI and RII subunits of cAPK (26). To determine $[\text{3H}]$cAMP bound to the more rapidly exchanging binding site of Epac we used cold (−10 °C) 98% saturated ammonium sulfate. Unbound isotope was removed by immediate filtering and rinsing with 1 ml of the ammonium sulfate solution.

A size-exclusion chromatography method (27) was used to assay the binding of $[\text{3H}]$cAMP to Epac under strict equilibrium conditions. The buffer (Buffer A; 15 mM Hepes, pH 7.2, with 1 mM Na$_2$PO$_4$, 130 mM KCl, 2 mM MgCH$_2$COO$_2$, 2 mM glutathione, 0.1 mM β-mercaptoethanol, 0.3 mM EGTA, 1 mM EDTA, 0.2 mM AMP, and 0.2 mg/ml soyaean trypsin inhibitor) had near physiological pH and ionic strength. To assay for $[\text{3H}]$cAMP without interference from cAMP-induced Epac precipitation GST-Epac was attached to OSH-coated Flashplates (PerkinElmer Life Sciences). To determine $[\text{3H}]$cAMP binding, the plates were incubated with Buffer A (0.1 ml) and various concentrations of $[\text{3H}]$cAMP and analyzed at (25 °C) in a TopCount NXT scintillation counter (Packard, Meriden, CT).

**Determination of the cAMP Analog Binding Affinity for Sites A and B of RI and RII and for Epac1**—The equilibrium inhibition constant of binding ($K_i$) of cAMP analog was determined by competitive displacement of $[\text{3H}]$cAMP binding to R or Epac. The analog affinity relative to cAMP ($K_i$ analog) is $K_i$/cAMP K/analog. The determination of $K_i$ analog for Epac was, in principle, as described previously (28) and validated for RI and RII.

**In Vitro Assay of Epac-induced Rap Activation and cAMP-dependent Protein Kinase Activity—**CAMP analogs were tested for in vitro Rap1 or Rap2 activation by determination of their effect on the rate of cAMP-induced fluorescent GDP analog (3′-O-(N-methylanthraniloyl)-GDP) exchange from Rap (9). The determination of cAMP analog effects on protein kinase activity was routinely by incubation for 40 min at 37 °C in Buffer A with 1 mM [γ-32P]ATP, using kemptide as substrate (24).

**Assay of cAMP Analog Actions in Intact Cells**—The activation of Rap was determined by GST pull-down assay as described (29), except that NaF was omitted, and 5 mM Mg(CH$_2$COO)$_2$ added in lysis and wash buffers. The amount of immobilized Raf-GDS-RBD-GST protein (where GDS is GDP dissociation inhibitor and RBD is Ras binding domain) used to capture Rap1-GTP was in excess to ensure that variation in pipetting error of the slurry would not affect the pull down of Rap-GTP. Phospho-CREB was determined by Western blotting using the Ab5322 (a kind gift from Dr. M. Montminy, Salk Institute, La Jolla, CA) and compared with the amount of total CREB using a non-discriminating CREB antibody (number 9192; www.cellsignal.com). Primary dog thyroid cells were cultured and assessed for rounding as described earlier (5, 30). Rat pheochromocytoma PC-12 cells were cultured in RPMI with 10% horse serum and 5% fetal calf serum, before being stimulated with cAMP analog or NGF for 3 to 72 h, and fixed for evaluation by phase and differential interference contrast microscopy (31).

**Molecular Modeling**—The structures of the CNBDs A and B of RII (Protein Data Bank accession code 1RGS) (4) were used as templates to construct a structural model of the CNBD of Epac1. The model obtained using the Homology module of InsightII 2000 (MSI found at www.msi.
com) was similar to that obtained by the Swiss-Pdb Viewer in conjunction with SWISS-MODEL (32). Final optimization of the structure of the complexes was performed using Discover (MSI). The programs InsightII (MSI) and WebLab Viewer (MSI) were used to prepare the figures.

RESULTS

Binding of $[^3H]cAMP$ and cAMP Analogs to Full-length Epac1, the CNBD Fragment of Epac, and Sites AI, BI, AII, and BII of cAPK—The isolated CNBD (Epac149–318) of Epac is well expressed and does not aggregate in the presence of cAMP, unlike Epacfl and Epac149–881 (25). To know whether the binding properties of CNBD are relevant for full-length Epac, we compared their affinity for cAMP. The isolated CNBD (Epac149–318) of Epac1 bound $[^3H]cAMP$ with an apparent $K_D$ of 2.9 µM (Fig. 1, B and C) at close to physiological pH (7.2) and ionic strength, as determined by the time-honored size exclusion gel chromatography method (27). To prevent aggregation, we anchored GST-Epacfl and GST-Epac149–881 to GSH-coated plates with intrinsic scintillant and determined $[^3H]cAMP$ binding by scintillation proximity assay. GST-Epacfl and GST-Epac149–881 bound $[^3H]cAMP$ with an apparent $K_D$ of 2.8 µM (Fig. 1D).

We conclude that the CNBD of Epac has the same affinity for cAMP whether in a 170-residue peptide (Epac149–318) or included in the full-length Epac molecule. Cyclic AMP analog mapping of the binding sites of Epac1, RI and RII, was undertaken to probe for differences between the Epac and R subunit binding sites. We showed first that the estimated $K_I$ of cAMP analogs for binding to Epac149–318 was similar whether assayed by the routine ammonium sulfate precipitation method or the equilibrium binding chromatography assay (Fig. 2).

Mapping data for analogs modified only in the adenine moiety are shown in Table I. We noted that the commonly used cAPK activators 8-Br-cAMP, 8-AHA-cAMP, and particularly 8-pCPT-cAMP had higher affinity than cAMP itself for Epac. No analog was severely restricted from binding to Epac, whereas several had very low $K_I$ for binding to one or more of the R subunits.
the binding sites of RIα (AI, BI) or RIβ (AII, BII). All the R subunit binding sites discriminated better than Epac against cGMP (Table I). The modestly (12-fold) decreased $K_i$ of cGMP for Epac was because of a combined effect of the introduction of 2-NH$_2$ and 6-O into the cAMP molecule (Table I). The $S$- and $R$-diastereoisomers of cAMPS bound to Epac with a relative affinity similar to that for the cAPK sites (Table II). The presumed cAPK-specific agonists $S$-$p$-$8$-pCPT-cAMPS and $Sp$-$5,6$-DCI-cBIMPs (33, 34) bound to Epac with an affinity similar to that of cAMP itself (Table II).

The loss of the 2'-OH of cAMP was more detrimental for binding to cAPK than to Epac, because both 2'-deoxy- and 2'-O-alkyl-cAMP had higher $K_i$ for Epac than for RI or RII (Table II). A more detailed survey of 8-substituted analogs of 2'-O-Me-cAMP indicated that the highest affinity was achieved when the S-phenyl ring was substituted in the para-position with a polar group, like Cl, HO, O-methyl (Table II), or F (not shown).

**Comparison of cAMP Analogs as Modulators of cAPK and Epac Activity in Vitro and in Intact Cells**—It is not known whether cAMP analogs modified in the 2'-position are full or partial agonists for cAPK. We found that 2'-O-Me-cAMP, 8-Br-2'-O-Me-cAMP, and 8-pCPT-2'-O-Me-cAMP failed to activate cAPK completely in vitro (Figs. 3-5). This indicates that 2'-O-Me analogs of cAMP are only partial agonists with respect to activation of cAPK. At near physiological concentration (300–600 nM) of R subunit (24) 8-Br-2'-O-Me-cAMP (1 mM) achieved less than 25% activation of either isozyme.

The 2'-O-Me-cAMP analogs were next tested for ability to stimulate the Epac1-catalyzed dissociation of the GDP-Rap complex. The compounds 8-pCPT-2'-O-Me-cAMP, 8-phPT-2'-O-Me-cAMP, and 8-pMePT-2'-O-Me-cAMP all stimulated the dissociation of GDP more strongly than cAMP or 8-Br-cAMP (Fig. 6A and data not shown). We conclude that 2'-O-Me-cAMP analogs super-activate Epac.

All cAMP analogs modified in the 6-position of the adenine ring, including cPuMP, 6-Cl-cPuMP, cIMP, cGMP, 6-MB-cAMP, 6-Bnz-cAMP, and to a lesser extent 6-Phe-cAMP, were only partial Epac agonists (data for 6-Bnz-cAMP are shown in Fig. 6A). Furthermore, the partial stimulation was observed at a higher concentration than expected from the analog affinity for free Epac (not shown).

In fibroblasts with enforced expression of Rap1 and Epac1, the analogs 6-MB-cAMP and 6-Bnz-cAMP stimulated strongly the phosphorylation of the cAPK substrate CREB but failed to activate Rap1 (not shown). Preferential stimulation by 6-modified cAMP analog of CREB phosphorylation was observed also in primary dog thyrocytes. In these cells rounding is a specific reaction to cAPK (5). Cell rounding was induced by 6-MB-cAMP (Fig. 7A) and 6-Bnz-cAMP (not shown) without Rap1 activation (Fig. 7A). In contrast, 8-pCPT-cAMP and the adenylylate cyclase activator forskolin induced Rap activation, CREB phosphorylation, and cell rounding, whereas 8-pCPT-2'-O-Me-cAMP only induced Rap activation (Fig. 7A). We conclude that 6-modified cAMP analogs, notably 6-Bnz-cAMP and 6-MB-cAMP, may be useful to preferentially activate cAPK in intact cells.

It was of interest to know whetherRp-cAMPS analogs could be used to inhibit Epac, and we used Rp-8-p-CPT-cAMPS to test this hypothesis, because this compound had a high affinity for Epac (Table II). Rp-8-p-CPT-cAMPS is an inhibitor of both cAPKI and cAPKII in intact cells (33, 35). It was a very weak Epac agonist *in vitro* (Fig. 6B) and did not activate Rap in intact thyrocytes (Fig. 7A). Rp-8-p-CPT-cAMPS had a weak ability to counteract 8-Br-cAMP-induced Rap activation *in vitro* (Fig. 6B) although it could inhibit 8-pCPT-cAMP-induced Rap activation in thyrocytes when present at high concentration.

The compound was a stronger inhibitor of CREB phosphorylation and rounding than Rap activation in thyrocytes (Fig. 7, B and C). This suggests that Rp-8-pCPT-cAMPS would have to be modified to act as a specific Epac antagonist in intact cells. Rp-8-Br-cAMPS, which inhibits cAPKI in intact cells (35), was a weak partial Epac agonist *in vitro* (Fig. 6B) and failed to inhibit either basal (Fig. 7A) or 8-pCPT-cAMP-induced (not shown) Rap activation in intact thyroid cells or fibroblasts. We conclude that presently available Rp-cAMPS analogs are unable to preferentially inhibit Epac and may be more potent antagonists of cAPK than Epac.

**Use of cAMP Analogs to Elucidate the Roles of cAPK and Epac in Mediating cAMP-induced PC-12 Cell Neurite Extensions and Rap1 Activation**—It is uncertain whether Epac or cAPK is responsible for the cAMP-induced Rap1 activation in PC-12 cells (20). The Epac activator 8-pCPT-2'-O-Me-cAMP activated Rap1 without stimulating CREB phosphorylation. The cAPK activator 6-Bnz-cAMP stimulated CREB phosphorylation but did not significantly activate Rap1 (Fig. 7D). We conclude that Epac stimulation can activate Rap1 in the absence of cAPK activation.

Neurite extensions were induced by the cAPK activator 6-Bnz-cAMP, the Epac activator 8-pCPT-2'-O-Me-cAMP, or NGF (Fig. 8). Cells incubated with 8-pCPT-2'-O-Me-cAMP had increased sensitivity to 6-Bnz-cAMP. Cells incubated with NGF were also sensitized to 6-Bnz-cAMP. In the combined presence of NGF and 8-pCPT-2'-O-Me-cAMP the cells responded strongly to 6-Bnz-cAMP at concentrations (0.02–0.05 mM; see Fig. 9B) that hardly had any effect when given alone.
This suggested that Epac, and to some extent NGF, had a permissive and sensitizing effect on cAPK-stimulated neurite extension.

To ensure that 6-Bnz-cAMP and not 8-pCPT-2'-O-Me-cAMP acted via cAPK also with respect to neurite extensions, we tested them in the presence of the ATP site-directed cAPK inhibitor H-89 (36, 37) and a combination of R\textsuperscript{p}-cAMPS and R\textsuperscript{p}-8-Br-cAMPS. These R\textsuperscript{p} analogs have lower affinity for Epac than R\textsuperscript{p}-8-pCPT-cAMPS (Table II) and should therefore be more cAPK selective. Furthermore, when combined, they inhibit both cAPKI and cAPKII, because R\textsuperscript{p}-8-Br-cAMPS is an inhibitor of cAPKI and R\textsuperscript{p}-cAMPS of cAPKII in intact cells (35).

The cAPK antagonists inhibited the action of 6-Bnz-cAMP much more strongly than the action of 8-pCPT-2'-O-Me-cAMP and failed to affect the NGF action (Fig. 9A). This suggested that Epac, and to some extent NGF, had a permissive and sensitizing effect on cAPK-stimulated neurite extension.

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Fig. 9. Demonstration of specificity of and synergy in promotion of PC-12 cell extensions by cAPK-activating and Epac-activating cAMP analogs. PC-12 cells were treated for 5 h (A–D) or 1.5 h (E) with various cyclic nucleotide analogs in the absence or presence of NGF and analyzed for the presence of extensions >20 μm (panels A, B, D, and E) or 15 μm (panel C). Panel A, cells were incubated with various concentrations of 6-Bnz-cAMP alone (○), with 0.5 mM Rp-cAMPS (□) or 0.5 mM Rp-8-Br-cAMPS (△), or with 0.6 mM 8-pCPT-2′-O-Me-cAMP (▲). Panel B, cells were treated with various concentrations of 6-Bnz-cAMP and either 50 ng/ml NGF (●) or NGF + 0.6 mM 8-pCPT-2′-O-Me-cAMP (△). Panel C shows the effect of the cAPK inhibitors H-89 (0.6 μM), Rp-cAMPS (0.5 mM), and Rp-8-Br-cAMPS (0.5 mM) on neurite outgrowth in the presence of NGF (50 ng/ml), 8-pCPT-2′-O-Me-cAMP (0.6 mM), 6-Bnz-cAMP (0.1 mM), or 8-pCPT-cAMP (0.2 mM). Panel D shows the stimulation of cell extensions by various concentrations of 8-pCPT-2′-O-Me-cAMP, 8-pMeOPT-2′-O-Me-cAMP, and 8-pCPT-2′-O-Me-cAMP, and panel E shows stimulation by various concentrations of 8-pCPT-2′-O-Me-cAMP (○) or the axial benzyl triester prodrug of 8-pCPT-2′-O-Me-cAMP (△).

DISCUSSION

The cAMP analog mapping revealed significant differences between the binding sites of Epac and the R subunits of cAPK. In general, Epac had less stringent requirements for binding of either adenine- or 2′-ribose-modified analogs than any of the cAPK binding sites (AI, B1, AII, BII). Molecular docking suggests that Epac could accommodate bulky adenine substituents because of little steric hindrance in the region facing the adenine. In addition, perfect stacking between the adenine of an analog and an aromatic residue is not required for binding to Epac, which has no aromatic residue in a position corresponding to Tyr-371 in bRI. Docking of 8-pCPT-2′-O-Me-cAMP into the CNBD of Epac1 (Fig. 10B) suggested that the high affinity of 8-thiophenyl substituted cAMP related to the ability of His-317 to provide stacking interactions with the phenyl ring. The further affinity enhancement from a polar group in the para-position of the thiophenyl substituent could be explained by the predicted solvent exposure of the apical part of the phenyl ring (Fig. 10B).

Modification of the 2′-position of the ribose was less detrimental for binding to Epac than to cAPK. Comparative docking of 8-pCPT-2′-O-Me-cAMP (Fig. 10B) and cAMP (Fig. 10A) into the CNBD of Epac1 suggested that the H-bonding of the oxygen of 2′-OH of cAMP with the side chain amino of Gln-270 was maintained by 2′-O-Me. On the other hand, 2′-O-Me loses the particularly strong (39) H-bond of the 2′-OH of cAMP to Glu-324 in site BI (Fig. 10C).

NGF and analyzed for the presence of extensions >20 μm (panels A, B, D, and E) or 15 μm (panel C). Panel A, cells were incubated with various concentrations of 6-Bnz-cAMP alone (○), with 0.5 mM Rp-cAMPS (□) or 0.5 mM Rp-8-Br-cAMPS (△), or with 0.6 mM 8-pCPT-2′-O-Me-cAMP (▲). Panel B, cells were treated with various concentrations of 6-Bnz-cAMP and either 50 ng/ml NGF (●) or NGF + 0.6 mM 8-pCPT-2′-O-Me-cAMP (△). Panel C shows the effect of the cAPK inhibitors H-89 (0.6 μM), Rp-cAMPS (0.5 mM), and Rp-8-Br-cAMPS (0.5 mM) on neurite outgrowth in the presence of NGF (50 ng/ml), 8-pCPT-2′-O-Me-cAMP (0.6 mM), 6-Bnz-cAMP (0.1 mM), or 8-pCPT-cAMP (0.2 mM). Panel D shows the stimulation of cell extensions by various concentrations of 8-pCPT-2′-O-Me-cAMP, 8-pMeOPT-2′-O-Me-cAMP, and 8-pCPT-2′-O-Me-cAMP, and panel E shows stimulation by various concentrations of 8-pCPT-2′-O-Me-cAMP (○) or the axial benzyl triester prodrug of 8-pCPT-2′-O-Me-cAMP (△).
8-pCPT-2'-O-Me-cAMP and other 2'-O-alkyl analogs of cAMP were unable to activate cAPK fully under physiologically relevant conditions. This is a novel finding and indicates that 2'-O-Me-cAMP analogs are partial rather than full cAPK agonists. Even in the unlikely event that they should reach millimolar concentration inside the cell, they would be unable to
activate cAPK above the normal resting level (20–30% of full activity). The CNBD of free Epac showed only partial discrimination (about 10-fold) between cAMP and cGMP (Table I). This would not be sufficient to prevent cGMP binding to Epac in cells where the cGMP level exceeds that of cAMP. We found that 6-modified cAMP analogs, including cGMP, were only weak partial agonists of Epac activation, indicating that Epac is only weakly activated by cGMP under physiological conditions. Basically, guanine nucleotide exchange factors like Epac act by stabilizing the free, relative to the GDP-complexed form, of G-proteins (40). An intact amino group in the 6-position appears therefore to be crucial for cAMP analogs to induce the active conformation of Epac with enhanced affinity for free Rap relative to Rap-GDP. The low efficiency of the 6-modified analogs was not because of introduction of bulk causing steric hindrance, because cPuMP and cIMP/cGMP have less bulk than cAMP at the 6-position but still were only weak agonists. The intact cell studies showed that 6-Bnz-cAMP and 6-MB-cAMP failed to activate Rap1. In contrast, 8-pCPT-2′-O-Me-cAMP, 8-pCPT-cAMP, 8-Br-cAMP, and the adenylate cyclase stimulator forskolin activated Rap1 in the same cells (fibroblasts, dog thyrocytes, PC-12 cells) under comparable conditions. This suggests that 6-modified analogs are indeed poor Epac activators in the intact cell. It also suggests that Rap1 activation by cAMP is more likely to be mediated by Epac than cAPK in the cells under the conditions of the present study.

Armed with discriminatory cAMP analogs it was possible to dissect the relative role of Epac and cAPK in cAMP-induced neurite extension in rat pheochromocytoma PC-12 cells. The Epac activator 8-pCPT-2′-O-Me-cAMP appeared able to induce neurite extensions on its own. Only analogs with a high affinity for Epac could substitute for 8-pCPT-2′-O-Me-cAMP, indicating that Epac and not another cAMP receptor was the target. This suggests Epac to have an important, hitherto unrecognized, role in cAMP-induced neurite extension. Rp-cAMPS analogs and H-89 counteracted the action of 6-Bnz-cAMP much more strongly than the action of 8-pCPT-2′-O-Me-cAMP, further underlining the Epac specificity of 8-pCPT-2′-O-Me-cAMP. Activation of Epac had a strongly enhancing effect on cAMP. One obvious consequence of the synergy between Epac and cAPK is to provide positive cooperativity of cAMP action, because activation of Epac enhanced the action of cAMP activator, which in turn enhanced the action of Epac activator (Fig. 9).

NGF sensitized the cells to both cAPK and Epac activators. A challenging observation was that the Epac stimulator 8-pCPT-2′-O-Me-cAMP acted more rapidly than NGF to induce neurite extensions, although NGF gave rise to a higher early activation of Rap1. This suggests that activation of Epac does not merely act to mimic and replace the Rap1 activation by NGF but probably has a distinct action, possibly by activating Rap1 in a different compartment than NGF. The Epac activator can also have hitherto unrecognized effects not mediated via activation of Rap (41).

In conclusion, we have mapped fine structural differences between the binding sites of Epac1 and cAPK and elucidated differences in the mechanism of activation of Epac and cAPK using cAMP analogs. Selective analogs have been demonstrated to discriminate activated Epac or cAPK and thereby point out a hitherto unknown synergism between Epac and cAPK in PC-12 cell differentiation.