Minireview

cAMP effector mechanisms. Novel twists for an 'old' signaling system

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Abstract Cyclic AMP (cAMP) has traditionally been thought to act exclusively through cAMP-dependent protein kinase (cAPK, PKA), but a growing number of cAMP effects are not attributable to general activation of cAPK. At present, cAMP is known also to directly regulate ion channels and the ubiquitous Rap guanine exchange factors Epac 1 and 2. Adding to the sophistication of cAMP signaling is the fact that (1) the cAPK holoenzyme is incompletely dissociated even at saturating cAMP, the level of free R subunit of cAPK being able to regulate the maximal activity of cAPK, (2) cAPK activity can be modulated by oxidative glutathionylation, and (3) cAPK is anchored close to relevant substrates, other signaling enzymes, and local compartments of cAMP. Finally, we will demonstrate an example of fine-tuning of cAMP signaling through synergistic induction of neurite extensions by cAPK and Epac.
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Key words: Protein kinase A; cAMP-dependent protein kinase; Cyclic AMP; Epac; Extracellular signal-regulated kinase; Rap1; Rap2; Guanine exchange factor

1. Introduction

The second messenger concept of signaling was born with the discovery of cyclic AMP (cAMP) and its ability to influence metabolism, cell shape and gene transcription [1] via reversible protein phosphorylations. cAMP is produced from ATP adenylyl cyclase (AC) in response to a variety of extracellular signals such as hormones, growth factors and neurotransmitters. Elevated levels of cAMP in the cell lead to activation of different cAMP targets (Fig. 1). It was long thought that the only target of cAMP was the cAMP-dependent protein kinase (cAPK) [2], which has become a model of protein kinase structure and regulation [3–5]. In recent years it has become clear that not all effects of cAMP are mediated by a general activation of cAPK ([6]; see also article by Dremier et al. in this volume). Several cAMP binding proteins have been described: cAPK [2], the cAMP receptor of *Dictyostelium discoideum*, which participates in the regulation of development [7], cyclic nucleotide gated channels involved in transduction of olfactory and visual signals [8,9] and the cAMP-activated guanine exchange factors Epac 1,2, which specifically activate the monomeric G protein Rap [10,11]. This review will give a brief overview of some of the latest findings concerning cAPK and Epac.

2. Modulation of cAPK activity by R subunit expression level and localization

The cAPK exists as either an inactive holoenzyme composed of two regulatory (R) and two catalytic (C) subunits, or as dissociated subunits. According to current dogma the subunits are completely dissociated in cells with high cAMP levels, regardless of the concentration of the R subunit. This appears not to be correct, since inactive cAMP-saturated holoenzyme can form at the concentrations of subunit (0.2–2 μM) prevailing in cells (Fig. 2A) [12]. Note that cAPKI holoenzyme formation is less pronounced at high substrate concentration, suggesting that cAPK is more completely dissociated when in a compartment with high density of substrates. Another indication that holoenzyme can form at saturating cAMP levels is the decreased cAMP-responsive transcription in cells overexpressing R subunit (Fig. 2B). Furthermore, maximally cAMP-stimulated cells overexpressing RIα show less nuclear translocation of the microinjected C subunit (R. Kopperud, C. Krakstad, A. Christensen and S.O. Døskeland, unpublished observations). This suggests that the R subunit may limit the maximal dissociation of cAPK in the presence of excess cAMP, and that cAMP-induced upregulation of R subunit may decrease the response to a persistently high cAMP signal (Fig. 2C). In hepatocytes and neuroblastoma cells cAMP signaling is decreased both by induction of the RIα subunit and by degradation of the C subunit [13,14].

A striking example of regulation of R subunit level occurs during the memory-related long-term synaptic facilitation (LTF), which requires persistently elevated neuronal cAMP [15]. Increase in cAMP leads to dissociation of enough cAPK to allow the translocation of free catalytic subunit into the nucleus and phosphorylation of cAMP response element binding protein (CREB). This induces a ubiquitin carboxy-terminal hydrolase, which is essential for LTF and acts to degrade the RIα subunit of cAPK. This degradation of R subunit is a feed-forward mechanism of cAMP action. It allows more complete dissociation of cAPK (Fig. 2C), further

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Abbreviations: cAPK I and II, cAMP-dependent protein kinase I and II; Epac, exchange protein directly activated by cAMP; PKI, protein kinase inhibitor; AKAP, A-kinase anchor protein; CREB, cAMP response element binding protein; PKB, protein kinase B; ERK, extracellular signal-regulated kinase; NGF, nerve growth factor; CNBD, cyclic nucleotide binding domain

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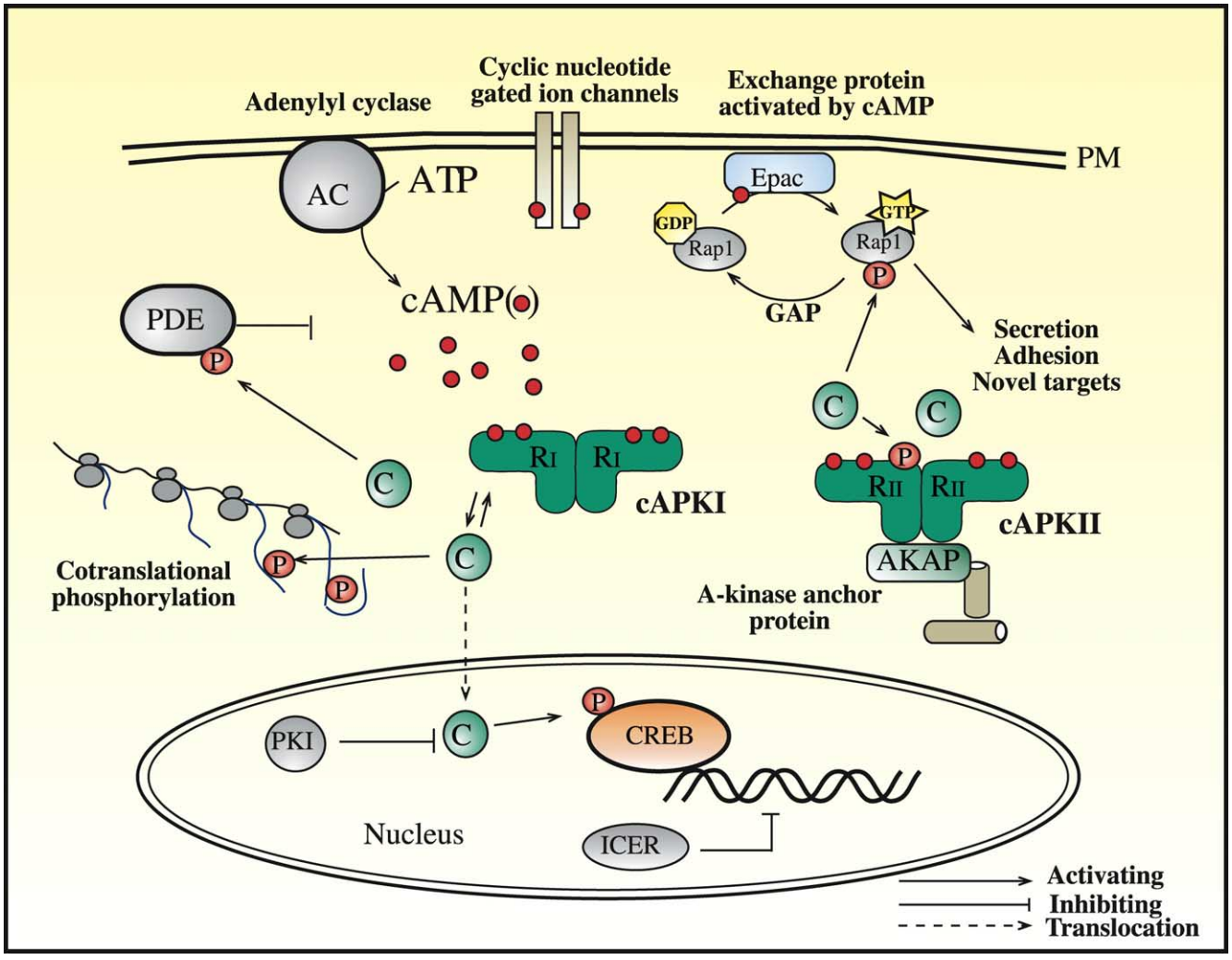

Fig. 1. Overview of cAMP signaling. Most cAMP effects are mediated by the cAMP-dependent protein kinase types I and II (cAPKI, II). Binding of cAMP to sites A and B of its R subunit lowers its affinity for the C subunit most of which dissociates from the holoenzyme. The C subunit can catalyze reversible protein phosphorylation and cotranslational, irreversible phosphorylation of newly synthesized peptides. It can also translocate to the nucleus to phosphorylate nuclear targets, like cAMP response element binding protein (CREB), or act locally close to where its R subunit is tethered by association with A-kinase anchor protein (AKAP). In the figure the RII subunit of cAPKII is bound to a centrosomal AKAP. Cyclic nucleotide gated ion channels are regulated by the binding of cAMP to their cytoplasmic tail. The generally expressed Epac are stimulated by cAMP to exchange GDP with GTP on Rap1,2. GTP activates downstream signaling pathways, such as secretion and cell adhesion. Rap may also be regulated by phosphorylation by the C subunit of cAPK. cAMP signaling is negatively regulated by members of the phosphodiesterase (PDE) enzyme family, which are themselves activated by cAPK. In the nucleus protein kinase inhibitor (PKI) may interact with and inhibit the C subunit of cAPK and enhance the nuclear export of free C subunit.

increasing the nuclear translocation of the C subunit. Another example of feed-forward signaling is the degradation of RIα observed in S49 cells treated with a lethal concentration of cAMP analog [16].

The R subunit of cAPK modulates kinase activity indirectly via binding to the A-kinase anchor protein (AKAP) family of scaffolding proteins [17,18]. AKAPs can tether the cAPK holoenzyme to a preferred substrate [19] at a particular subcellular localization. Several AKAPs serve to co-localize cAPK and other signaling enzymes, such as phosphatases and phosphodiesterases. Using fluorescent resonance energy transfer technique a close proximity (<5 nm) was demonstrated between the RII subunit of cAPKII and the phosphatase calcineurin [20]. The co-localization of the cAMP-degrading phosphodiesterase and cAPK [21] is one means to provide an intracellular microenvironment with a cAMP concentration different from that in the bulk of the cell cytoplasm [22].

Most members of the AKAP family bind the regulatory subunit (RII) of cAPK isozyme II [17,18]. So far only a few RI binding AKAPs have been identified [28–30]. Since several early observations demonstrated RI anchoring to membranes [23–26] and to a soluble multiprotein complex [27] more RI anchoring molecules probably remain to be characterized.

The AKAPs appear essential for cAMP effects such as modulation of ion channel activity [31]. The reason why cAPKII has been selected by AKAPs to microenvironments regulating ion channels is not known with certainty. One reason may be that cAPKII is particularly well suited to provide a single phosphorylation reaction or a short burst of activity in response to a pulse of cAMP. This is because catalytic subunit that just has completed phosphorylation of substrate still carries an ADP molecule in its active site. This C-ADP reassociates preferentially with RII subunit which is (auto)phosphorylated by the C subunit [3].

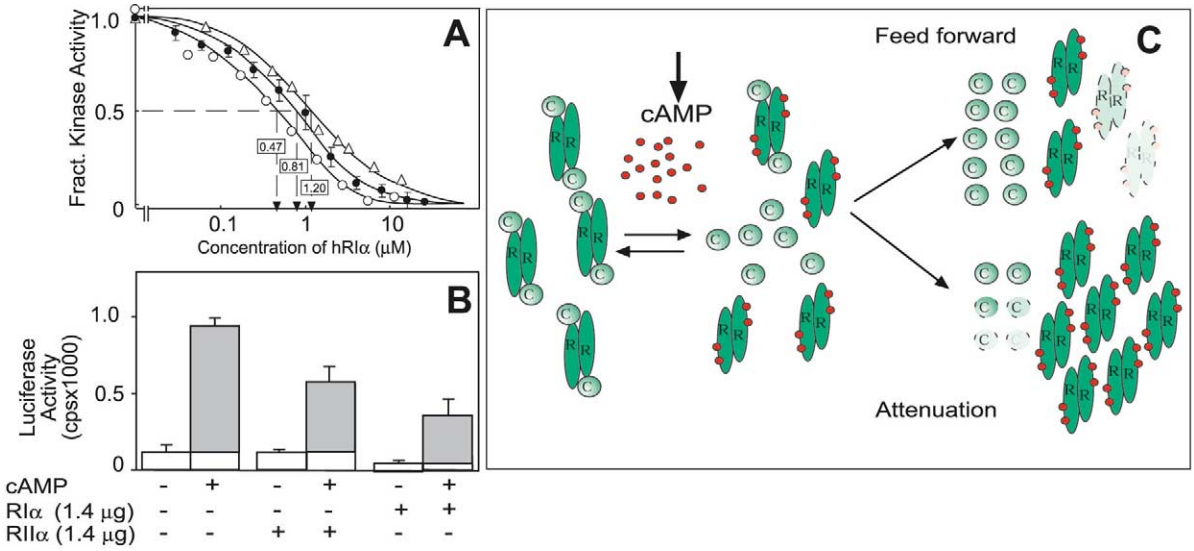

Fig. 2. Regulation of cAPK activity by R subunit at high (near saturating) cAMP levels. A: Inhibition of kinase activity by cAMP-RIα at various peptide substrate (kemptide) concentrations. Kemptide at 8 (○), 70 (●), and 140 (△) μM was phosphorylated by Cα at various concentrations of RIα subunit in the presence of saturating cAMP. Note that 1–1.5 μM RIα is sufficient to inhibit the kinase by about 50% even at saturating cAMP and near saturating substrate. Modified from [12]. B: Enforced expression of RIα or RIIα lowers CRE-dependent luciferase expression in cAMP-stimulated HEK 293 cells. Cells were transfected with RIα, RIIα, or control vector. The increments due to cAMP challenge are shown by the gray-colored bars. All the cells were transfected with 4CRE-Luc in addition to plasmids as indicated. Nineteen hours thereafter they were exposed to cAMP challenge (30 μM forskolin, 0.25 μM IBMX, 0.7 mM N^6-monobutyryl-cAMP, and 0.7 mM N^6-benzoyl-cAMP) or vehicle, and harvested 3 h thereafter for determination of luciferase activity. Modified from [12]. C: Scheme of positive feed-forward and negative regulation (attenuation) of cAPK activity in a maximally cAMP-stimulated cell. Degradation of RIα with bound cAMP (upper right) enhances further the dissociation of cAPK holoenzyme, thereby increasing the maximally obtainable kinase activity at saturating cAMP concentration. Degradation of C subunit and upregulation of RIα synthesis (lower right) decreases the maximal kinase activity.

Another feature of cAPKII is that phospho-RII can enhance the inhibition of protein phosphatase 1 by AKAP 220 [33]. This feature would serve to prolong the duration of the phosphorylated state of a phosphoprotein target. A third feature of cAPKII is that it offers reversible anchoring. The translocation of cAPKII from the centrosome to the chromatin during mitosis is due to phosphorylation of RII by cyclin-dependent protein kinase 1 [32].

As expected from the difference in anchoring a switch in cAPK isozyme distribution can influence the cellular response to cAMP. The profound consequence of altered isozyme expression is illustrated by the embryonically lethal phenotype of mice with a disrupted RIα gene [34]. Isozyme switching occurs physiologically during differentiation and in cells stimulated to grow [3].

An intriguing phenomenon, which must depend on the presence of cAPK near the site of protein synthesis, is cAPK-catalyzed co-translational phosphorylation. Actin [35,36] and at least four other proteins [36] are co-translationally phosphorylated in cells induced by cAMP to die by apoptosis. The phosphorylated site becomes buried when the peptide assumes its final conformation, and is therefore not available for dephosphorylation. This therefore represents an example of irreversible protein phosphorylation. The physiological significance of this phenomenon has still not been proven, but it can provide a prolonged (hysteretic) response to a cAMP stimulus.

3. Modulation of cAPK activity by mechanisms independent of the R subunit

A family of nuclear peptides (protein kinase inhibitors (PKI) α, β and γ) bind to and inhibit the C subunit without being regulated by cAMP. PKI is a pseudosubstrate for

Table 1
Predicted cAPK isozyme synergy between pairs of cAMP analogs

cA analog pairs	Predicted synergy cAPKI	Predicted synergy cAPKII
x+y	$\frac{\sqrt{(AI^x+AI^y)(BI^x+BI^y)}}{\sqrt{(AI^x)(BI^x)}+\sqrt{(AI^y)(BI^y)}}$	
8-AHA-cA+N^6-Bnz-cA	2.8	2.9
8-Pip-cA+8-MA-cA	3.2	1.0
N^6-BC-cA+Sp-5,6diCl-cBIMPS	1.3	8.3

Analogs preferring site *A* synergize with analogs preferring site *B* in activating cAPK. 8-AHA-cAMP selects site *B* of both cAPKI and II, and N^6-Bnz-cAMP selects site *A* of both cAPKI and II and will therefore synergize in activating both cAPKI and cAPKII. 8-Pip-cAMP selects site *AI* of cAPKI and site *BII* of cAPKII, and will therefore synergize only for activation of cAPKI when combined with the *BI*, *BII* preferring analog 8-MA-cAMP. Sp-5,6diCl-cBIMPS has a very strong selectivity for site *BII* and N^6-BC-cAMP for site *AII*. These compounds give therefore very strong synergism for cAPKII activation.

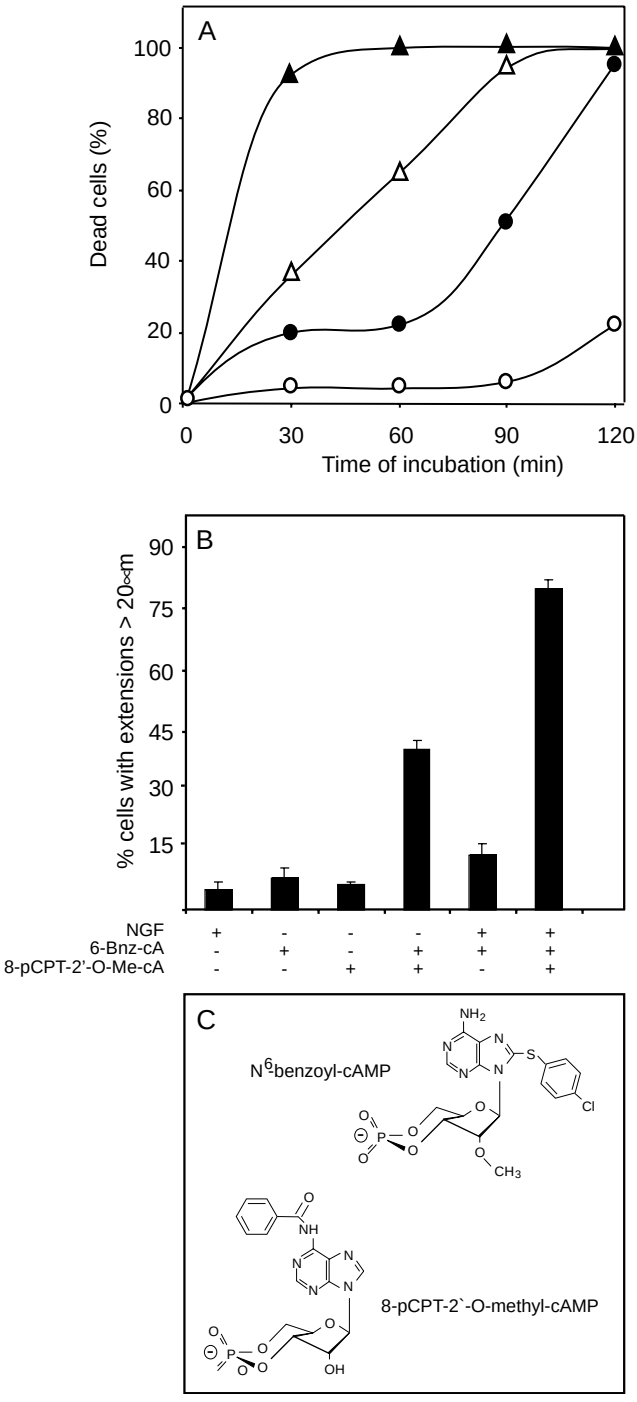

Fig. 3. cAPK-selective cAMP analog protects against NaN_3-induced cell death (A) and synergizes with Epac-activating analog and with nerve growth factor (NGF) in inducing neuronal differentiation (B). A: Hepatocytes were incubated with 5 (○, ●) or 10 (△, ▲) mM NaN_3 in the absence (solid symbols) or presence (open symbols) of N^6-benzoyl-cAMP. B: A sign of neuronal differentiation of PC12 cells is the extension of long neurites (>20 μm). The figure shows that the Epac activator 8-pCPT-2′-*O*-methyl-cAMP (0.6 mM) acts synergistically both with the cAPK activator N^6-benzoyl-cAMP (0.1 mM) and with NGF (50 ng/ml) to promote neuronal extensions. C: The structure of two cyclic nucleotide analogs 8-pCPT-2′-*O*-methyl-cAMP and N_6-benzoyl-cAMP, which are preferential activators of Epac and cAPK, respectively.

cAPK, and it blocks its active site. PKI also acts as a chaperone for nuclear export of the C subunit. PKI would therefore be expected to have two negative influences on cAPK activity in the nucleus [4,37]. The physiological significance of PKI inhibition of the C subunit is not known. Surprisingly, mice with targeted disruption of PKI had, if anything, decreased CREB-dependent transcription. This may be related to the compensatory increase of RIα in these mice [38].

The C subunit of cAPK can be inhibited directly through glutathionylation of Cys-199. This reaction occurs in intact cells exposed to oxidative stress [39]. Hepatocytes are protected from sodium azide and other agents inducing oxidative damage by agents elevating the endogenous cAMP or by cAMP analog-activating cAPK (Fig. 3A; unpublished observations). It is tempting to speculate that oxidative stress weakens the protection against cell death by inhibiting cAPK through glutathionylation, and that the transient nature of the protection (Fig. 3A) might be related to this process.

4. Use of cAMP analogs to dissect roles of cAMP receptors in intact cells

The two cAMP binding sites (A,B) of cAPK act in synergy to activate cAPK in intact cells [3,4]. Examples of combinations of cAMP analogs that preferentially activate cAPKI or cAPKII are given in Table 1. Analog combinations specific for cAPKI induce leukemia cell apoptosis [40] and T cell activation [25], whereas cAPKII activators induce fat cell lipolysis [4].

We have recently described cAMP analogs that selectively activate the newly discovered [10] Epac family of guanine exchange factors for Rap1 (Table 2) [41–43]. The two isoforms Epac1 and Epac2 (also known as cAMP-GEFII [11]) have a broad tissue distribution. Many cAMP effects previously ascribed only to activation of cAPK may turn out to be mediated fully or in part by Epac.

The analog 8-CPT-2′-*O*-Me-cAMP (Fig. 3C) is Epac-specific. It has more than 100-fold lower affinity than cAMP for cAPK, but five-fold higher affinity than cAMP for Epac1 (Table 2). Furthermore, it is a strong agonist for Epac, but only a partial agonist for cAPKI and II. In contrast, 6-modified cAMP analogs such as N_6-benzoyl-cAMP (Fig. 3C) are poor agonists for Epac and full cAPK activators [42]. Such discriminating cAMP analogs reveal that Epac synergizes with cAPK and nerve growth factor (NGF) to promote neurite extension in PC12 cells (Fig. 3B). Synergism between Epac and cAPK may turn out to be a common phenomenon in cAMP signaling, although it has been reported that Epac and cAPK regulate the protein kinase B (PKB) signaling cascade in opposite directions. Over-expressed Epac1 led to PKB activation, while stimulation of cAPK inhibited PKB [44]. The molecular basis for the synergy observed between Epac and cAPK (Fig. 3B) might be that cAPK activates extracellular signal-regulated kinase (ERK) [41] and Epac activates Rap, since both ERK and Rap have been strongly implicated in the control of neuronal differentiation [45,46]. It is noteworthy that the Epac activator also synergized with NGF (Fig. 3), even though NGF is a stronger activator of Rap1 in PC12 cells [42]. This may suggest that the Rap1 activated by Epac may reside in a cell compartment less efficiently activated by NGF. The Epac activator may also have hitherto unrecognized effects not mediated via activation of Rap.

Table 2
Analog affinity (relative to cAMP) for Epac-1, cAPKI and cAPKII

cA analog	Rel. aff. Epac	Rel. aff. RI: Av. (*AI*,*BI*)	Rel. aff. RII: Av. (*AII*,*BII*)	Epac/RI	Epac/RII
cA	1.0	1.0	1.0	1.0	1.0
8-pCPT-cA	65	2.6	1.01	25	64
2′-*O*-Me-cA	0.12	0.0048	0.0052	25	23
8-pCPT-2′-*O*-Me-cA	4.6	0.0089	0.0028	517	1626

The relative affinity of 8-pCPT-cAMP and 2′-*O*-Me-cAMP for Epac-1 is 20–70-fold higher than the average affinity for the binding sites (*AI*, *BI*) of the RI subunit of cAPKI or (*AII*, *BII*) of the RII subunit of cAPKII. The analog 8-pCPT-2′-*O*-Me-cAMP has an Epac preference of 500–1600-fold, i.e. can be considered completely Epac-specific for practical purposes.

5. How many cAMP receptors are there?

The cyclic nucleotide binding domain (CNBD) originated in bacteria. It serves in the CAP transcription factor to sense the binding of cAMP and induce a conformation change making the CAP protein transcriptionally active [47]. Later in phylogeny the CNBD is found in several types of ion channels directly modulated by cyclic nucleotides, in the cAMP- and cGMP-dependent protein kinases, and in the Epac family of guanine exchange factors. The role of proteins with sequence similarity to the CNBDs, but with expected or proven low affinity for cAMP or cGMP in vitro, is unclear. It is possible that the domain has useful functions not related to cyclic nucleotide binding, but it is also possible that the CNBD may have a conformation allowing physiologically significant binding of cAMP inside the intact cell, e.g. because of interaction with another protein. A possible example may be a Rap/Ras exchange factor (PDZ-GEF1, CNrasGEF, nRap-GEF), whose activity against Rap appears unaffected by cAMP, which it binds with very low affinity in vitro [48]. The protein has been proposed to be a cAMP-activated Ras exchange factor in some intact cells [49,50], although this has not been observed in other cell types [51].

There is also reason to believe that proteins may exist that can bind cAMP without having the canonical CNBD motif. One example is the extracellular cAMP receptor of *D. discoideum* [7], which is completely different from the CNBDs, and poses a particular challenge because the cAMP binding site may be formed by parts of surface loops of the protein distant in the primary sequence. Such cAMP receptors may be difficult to predict by database search for homology.

There may therefore still be new twists and surprises to be discovered for the cAMP second messenger signaling system.

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