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Review

Epac-selective cAMP analogs: New tools with which to evaluate the signal transduction properties of cAMP-regulated guanine nucleotide exchange factors

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Abstract

The identification of 2′-*O*-methyl substituted adenosine-3′,5′-cyclic monophosphate (cAMP) analogs that activate the Epac family of cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs, also known as Epac1 and Epac2), has ushered in a new era of cyclic nucleotide research in which previously unrecognized signalling properties of the second messenger cAMP have been revealed. These *E*pac-*S*elective *C*yclic AMP *A*nalogs (ESCAs) incorporate a 2′-*O*-methyl substitution on the ribose ring of cAMP, a modification that impairs their ability to activate protein kinase A (PKA), while leaving intact their ability to activate Epac (the *E*xchange *P*rotein directly *A*ctivated by *C*yclic AMP). One such ESCA in wide-spread use is 8-pCPT-2′-*O*-Me-cAMP. It is a cell-permeant derivative of 2′-*O*-Me-cAMP, and it is a super activator of Epac. A wealth of newly published studies demonstrate that 8-pCPT-2′-*O*-Me-cAMP is a unique tool with which to asses atypical actions of cAMP that are PKA-independent. Particularly intriguing are recent reports demonstrating that ESCAs reproduce the PKA-independent actions of ligands known to stimulate Class I (Family A) and Class II (Family B) GTP-binding protein-coupled receptors (GPCRs). This topical review summarizes the current state of knowledge regarding the molecular pharmacology and signal transduction properties of Epac-selective cAMP analogs. Special attention is focused on the rational drug design of ESCAs in order to improve their Epac selectivity, membrane permeability, and stability. Also emphasized is the usefulness of ESCAs as new tools with which to assess the role of Epac as a determinant of intracellular Ca^{2+} signalling, ion channel function, neurotransmitter release, and hormone secretion.

Keywords: cAMP; Epac; PKA; Rational drug design; ESCA

Contents

Abbreviations: AKAP, A kinase anchoring protein; cAMP-GEF, cAMP-regulated guanine nucleotide exchange factor; CNBD, cyclic nucleotide-binding domain; CRF, corticotropin releasing factor; EGF, epidermal growth factor; Epac, exchange protein directly activated by cAMP; ESCA, Epac-selective cAMP analog; FRET, fluorescence resonance energy transfer; GLP-1, glucagon-like peptide-1-(7–36)-amide; GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; PACAP, pituitary adenylyl cyclase activating polypeptide; PDE, cyclic nucleotide phosphodiesterase; PKA, protein kinase A; PLD, phospholipase D; PLC-ε, phospholipase C-epsilon; PTH, parathyroid hormone; SUR1, sulfonylurea receptor-1.

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1. Introduction

Epac proteins are cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs) that mediate protein kinase A (PKA)-independent signal transduction properties of the second messenger adenosine-3′,5′-cyclic monophosphate (cAMP). Two variants of Epac exist (Epac1, Epac2), both of which are activated in living cells by physiologically relevant concentrations of cAMP [1–4]. The terms Epac1 and Epac2 (also known as cAMP-GEF-I and cAMP-GEF-II) refer to the products of the RAPGEF3 and RAPGEF4 genes, whereas the all-inclusive term Epac is an acronym for the *E*xchange *P*rotein directly *A*ctivated by *C*yclic AMP (2). This established terminology refers to the fact that upon binding of cAMP to Epac, the guanyl nucleotide exchange factor (GEF) activity of Epac is disinhibited, thereby allowing Epac to stimulate the exchange of GTP for GDP on small GTPases of the Rap family (Rap1, Rap2). Such nucleotide exchange activates the GTPases, thereby allowing Rap proteins to interact with and to stimulate effector signalling molecules (Fig. 1).

Both Epac1 and Epac2 contain a single high-affinity cAMP-binding domain (CNBD) (in vitro K_d of 1–4 μM) at which cAMP interacts to activate Epac (Fig. 2; note that Epac2 also contains a low-affinity CNBD of uncertain significance). Binding of cAMP to Epac results in a conformational change such that an autoinhibitory function present in the exchange factor's C-terminus is relieved [5–9]. This process of activation is initiated by agonist binding to a subset of G protein-coupled receptors (GPCRs) positively coupled to transmembrane adenylyl cyclases (TMACs) and cAMP production. An alternative mechanism by which Epac may be activated results from soluble adenylyl cyclase (SAC)-catalyzed production of cAMP [10], as has been suggested to occur in sperm [11,12] (Fig. 1).

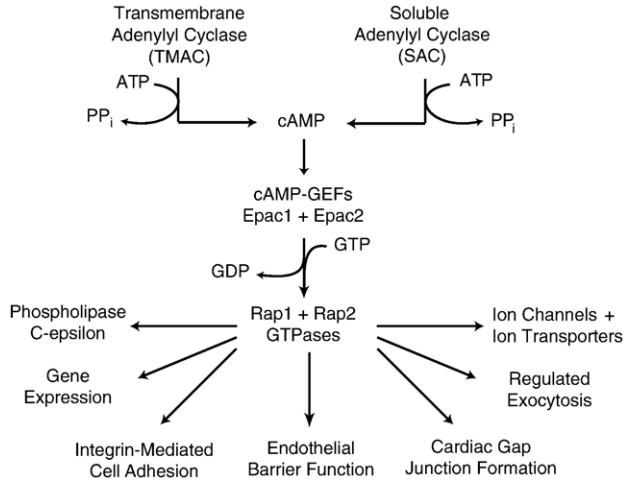

Fig. 1. Signal transduction properties of cAMP-GEFs Epac1 and Epac2. Transmembrane adenylyl cyclases (TMACs) and soluble adenylyl cyclases (SAC) catalyze the conversion of ATP to cAMP. The binding of cAMP to cAMP-GEFs activates the exchange factors, thereby allowing Epac1 and Epac2 to catalyze the exchange of GTP for GDP on Rap GTPases. The GTP-bound forms of Rap activate multiple cellular functions. Although not illustrated, the action of cAMP-GEFs at Rap is terminated by PDE-catalyzed hydrolysis of cAMP to generate 5′-AMP.

The Rap-dependent signalling pathways activated by Epac are known to be cell-type specific. For example, in human embryonic kidney (HEK) cells, Epac promotes Rap-dependent activation of phospholipase C-epsilon (PLC-ε), with concomitant hydrolysis of phosphatidylinositol bisphosphate (PIP_2), thereby generating inositol trisphosphate (IP_3) and diacylglycerol (DAG) [13]. There is also evidence for a Rap-mediated action of Epac to stimulate mitogen-activated protein kinases (MAPK; p38, ERK1/2) in neurons, endocrine cells, and T-cells [14–17]. In contrast, a Rap-mediated action of Epac to induce the expression of SOCS-3 (suppressor of cytokine signalling 3) and to inhibit interleukin 6 (IL-6) receptor-mediated signal transduction exists in vascular endothelial cells [18]. Through an as-yet-to-be defined mechanism, Rap is also reported to mediate the stimulatory action of Epac on neurotensin secretion from gut endocrine cells [19]. Finally, three particularly well-established roles for Epac and Rap exist in ovarian carcinoma cells, vascular endothelial cells, and cardiac myocytes, where Rap is demonstrated to mediate the stimulatory action of Epac on cell adhesion [20], endothelial barrier function [21], and gap junction formation [22], respectively.

It is important to note that not all actions of Epac are necessarily Rap-mediated. For example, Epac is reported to activate R-Ras, thereby stimulating phospholipase D (PLD) when HEK cells expressing M_3 muscarinic acetylcholine receptors are exposed to carbachol [23]. Furthermore, Epac might act independently of Rap to promote exocytosis of insulin from pancreatic beta cells. This secretagogue action of Epac is possibly a consequence of its direct interaction with secretory granule-associated proteins (Rim2, Piccolo) [24,25], the sulfonylurea receptor-1 (SUR1) subunit of ATP-sensitive K^+ channels [26–30], tubulin [31], or light chains 1 and 2 (LC1, LC2) of microtubule associated proteins 1A and 1B [32,33]. Since evidence exists for interactions of Epac with proteins other than

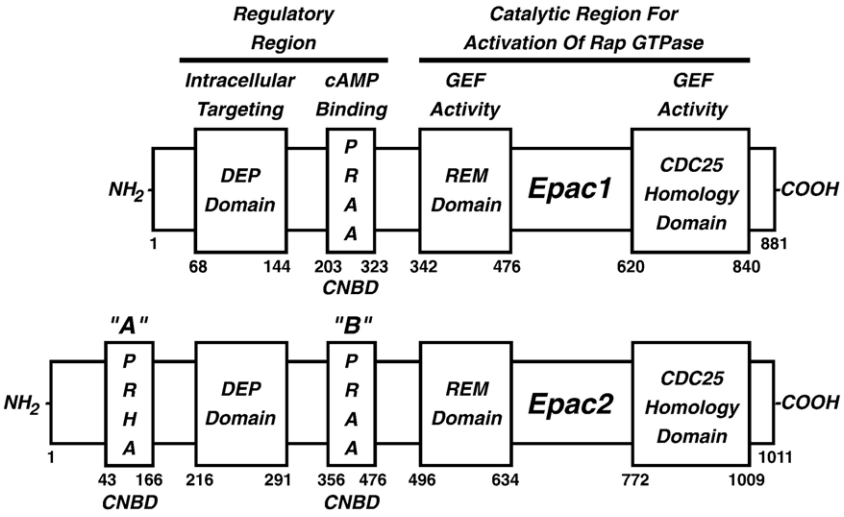

Fig. 2. Domain structures of Epac1 and Epac2 (amino acid residues numbered). A catalytic CDC25 homology domain located at the C-terminus of Epac1 and Epac2 promotes the exchange of GTP for GDP on Rap GTPases. The catalytic activity of the CDC25 homology domain is supported by a Ras-exchange motif (REM). A single cyclic nucleotide-binding domain (CNBD) in Epac1 contains a PRAA motif that binds cAMP with high affinity, whereas Epac2 contains two CNBDs, each designated as "A" or "B". The "A" CNBD of Epac2 contains a PRHA motif, and it binds cAMP with low affinity. The "B" CNBD of Epac2 contains the PRAA motif also found in Epac1, and it confers high affinity binding of cAMP.

Rap, it seems that the Rap-GEF activity of Epac might not be the sole determining factor by which this cAMP-binding protein exerts its effects.

Although cAMP activates both PKA and Epac, a process of rational drug design has culminated in the synthesis of *E*pac-*S*elective *C*yclic *A*MP analogs (ESCAs). These ESCAs activate Epac but not PKA when used at reasonable concentrations [34,35]. New studies demonstrate that in vitro administration of the first-in-class ESCA 8-pCPT-2′-*O*-Me-cAMP leads to significant alterations of ion channel activity [15,29,36,37], ion transporter activity [38,39], Ca^{2+} signalling [40–45], and exocytosis [10,19,26,40,46–48]. Of particular note are studies in which 8-pCPT-2′-*O*-Me-cAMP has been used to demonstrate

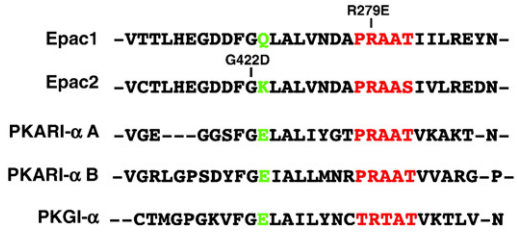

Fig. 3. Primary amino acid sequence alignments of CNBDs found in human Epac, PKA, and protein kinase G (PKG). In red is illustrated the PRAAT/S motif located within the CNBD of Epac1 and the "B" CNBD of Epac2. This same motif is found in the "A" and "B" CNBDs of PKA regulatory subunit Iα (PKARIα), but not in PKGIα which instead contains a TRTA motif. The TRTA motif of PKGIα confers selective binding of cGMP rather than cAMP. In green is illustrated the invariant glutamic acid residue (E) found in the CNBDs of PKARIα and PKGIα. It is missing in Epac1 and Epac2. The glutamic acid residue hydrogen bonds with the 2′-OH group of the ribose moiety of cAMP, but not the 2′-*O*-Me group of Epac-selective cAMP analogs. This explains the failure of ESCAs to activate PKA. Dominant-negative forms of Epac that fail to bind cAMP are derived by R279E or G422D substitutions introduced within the CNBDs of Epac1 and Epac2, respectively. Sequences illustrated correspond to Swiss-Prot accession numbers for human Epac1 (O95398), Epac2 (Q8WZA2), PKARIα (P10644) and PKGIα (Q13976). Figure modified from ref. [1].

a prominent role for Epac as a determinant of cardiac myocyte function [22,42,45,49,50] and pancreatic beta cell function [24–29,40,41,51–57]. Here we summarize the current state of knowledge regarding the molecular pharmacology and signal transduction properties of Epac, as assessed using ESCAs. For a more detailed discussion of the multiple biological actions and structural features of Epac proteins, the reader is referred to prior reviews of this subject matter [58–63].

2. Development of Epac-selective cAMP analogs

The molecular cloning of Epac cDNAs led to an appreciation that subtle differences in primary amino acid sequence exist when comparing the CNBDs present in Epac and PKA [1,2]. In particular, it was noted that an invariant glutamate residue (E) present in the CNBDs of the PKA regulatory subunits was not found in either Epac1 or Epac2 (Fig. 3). This glutamate residue plays a critical role in the hydrogen bonding of cAMP to the CNBDs of PKA regulatory subunits [64]. More specifically, the glutamate residue hydrogen bonds with the 2′-OH located on the ribose ring of cAMP (Fig. 4A). Deletion of the 2′–OH results in a cAMP analog (2′-deoxy-cAMP) that binds Epac selectively, mainly as a consequence of its poor affinity for PKA. Thus, in vitro binding assays demonstrated that 2′-deoxy-cAMP binds the "A" CNBD of PKA regulatory subunit I_α with an affinity that is approximately 15 000-fold less than that of cAMP. In marked contrast, 2′-deoxy-cAMP binds Epac1 with an affinity that is about 400-fold less than that of cAMP [35]. Despite these findings, 2′-deoxy-cAMP was judged to bind Epac1 with such low affinity as to not be a useful ESCA.

Attention then focused on the development of an ESCA that would exhibit higher affinity for Epac. A screen of cAMP analogs substituted at the critical 2′-position led to the appreciation that by introducing a 2′-*O*-alkyl group on the ribose moiety of cAMP, it would be possible to generate an

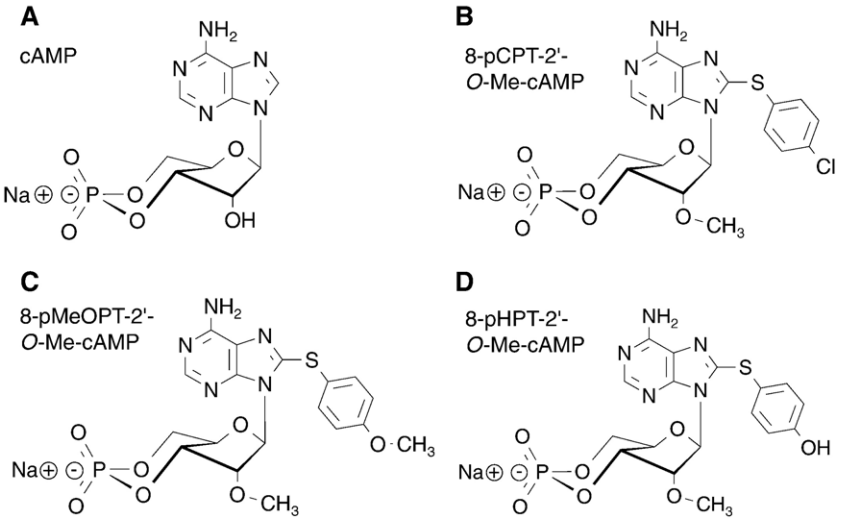

Fig. 4. Chemical structures of 2′-*O*-Me-cAMP analogs that activate Epac selectively. (A) Naturally-occurring cAMP, (B) 8-pCPT-2′-*O*-Me-cAMP incorporating the parachlorophenylthio substitution at position 8 on the adenine moiety of cAMP, (C) 8-pMeOPT-2′-*O*-Me-cAMP incorporating the paramethoxyphenylthio substitution at position 8, and (D) 8-pHPT-2′-*O*-Me-cAMP incorporating the parahydroxyphenylthio substitution at position 8. Hydrophobic substitutions at position 8 confer membrane permeability, thereby allowing the actions of ESCAs to be studied in living cells. See the BIOLOG Life Sciences Institute web site (http://www.biolog.de) for additional chemical structures of cAMP analogs.

analog that binds Epac selectively and with an affinity greater than 2′-deoxy-cAMP [34,35]. For example, a 2′-*O*-methyl substitution resulted in a cAMP analog (2′-*O*-Me-cAMP) exhibiting a 48-fold higher affinity for Epac1 when compared to 2′-deoxy-cAMP [35]. Unfortunately, 2′-*O*-Me-cAMP exhibited an affinity for Epac1 that was 8-fold less than that of cAMP.

A breakthrough occurred when it was realized that the affinity of an ESCA for Epac could be improved by the introduction of a parachlorophenylthio (pCPT) substitution at position 8 on the adenine moiety of 2′-*O*-Me-cAMP (Fig. 4B). This analog (8-pCPT-2′-*O*-Me-cAMP) bound Epac1 with an affinity 4.6-fold greater than cAMP. Moreover, when comparing the ability of 8-pCPT-2′-*O*-Me-cAMP to discriminate between Epac1 and PKA, the remarkable selectivity of the analog was revealed. The affinity of 8-pCPT-2′-*O*-Me-cAMP for Epac1 was 107-fold greater than the affinity of 8-pCPT-2′-*O*-Me-cAMP for the "A" CNBD of PKA regulatory subunit I_α [35]. Because in vitro assays demonstrated 8-pCPT-2′-*O*-Me-cAMP to activate Epac1 with an efficacy (maximal effect) greater than that of cAMP, 8-pCPT-2′-*O*-Me-cAMP was judged to be a "super activator" of Epac [65].

The development of 8-pCPT-2′-*O*-Me-cAMP was a fortuitous discovery because the presence of a hydrophobic pCPT substitution on 2′-*O*-Me-cAMP not only enhanced the affinity and selectivity with which the analog activated Epac, but it also conferred increased membrane permeability when tested using living cells [34]. This permeability property has been exploited in numerous studies examining the effects of extracellularly-applied 8-pCPT-2′-*O*-Me-cAMP (see below). Additional 2′-*O*-alkyl substituted ESCAs include 8-(4-methoxyphenylthio)-2′-*O*-methyl-cAMP (8-pMeOPT-2′-*O*-Me-cAMP) (Fig. 4C) and 8-(4-hydroxyphenylthio)-2′-*O*-methyl-cAMP (8-pHPT-2′-*O*-Me-cAMP) (Fig. 4D). As is the case for 8-pCPT-2′-*O*-Me-cAMP, these 2′-*O*-alkyl substituted ESCAs combine the desirable properties of membrane permeability, Epac/PKA selectivity, and high-affinity binding to Epac.

3. In vitro binding properties of 2′-*O*-alkyl substituted ESCAs at Epac and PKA

Døskeland and co-workers assessed the capacity of 2′-*O*-alkyl substituted cAMP analogs to displace the binding of [^{3}H]cAMP at Epac1 [35]. It was demonstrated that certain 2′-*O*-alkyl substituted cAMP analogs with S-phenyl ring substitutions displaced [^{3}H]cAMP more effectively than did cAMP itself. The rank order of potency was 7.1>6.9>4.6>1.0 for 8-pMeOPT-2′-*O*-Me-cAMP, 8-pHPT-2′-*O*-Me-cAMP, 8-pCPT-2′-*O*-Me-cAMP, and cAMP, respectively. These same ESCAs were dramatically less effective at displacing the binding of [^{3}H]cAMP at the "A" and "B" CNBDs of PKA regulatory subunits. For example, the rank order of potency for the displacement of [^{3}H]cAMP from the "A" CNBD of PKA regulatory subunit I_α was 0.025<0.043<1.0 for 8-pMeOPT-2′-*O*-Me-cAMP, 8-pCPT-2′-*O*-Me-cAMP, and cAMP, respectively [35]. Thus, unlike cAMP, 2′-*O*-alkyl substituted ESCAs interact preferentially with the CNBD of Epac.

As alluded to above, the 2′-*O*-alkyl substituted ESCAs with the highest affinity for Epac are those that incorporate on the adenine moiety at position 8 a *S*-phenyl ring in which the para-position is substituted with polar groups such as –Cl, –O–Me, or –OH (Fig. 4B–D). Thus, when comparing the effectiveness with which 2′-*O*-alkyl substituted or unsubstituted analogs displace the binding of [^{3}H]cAMP to Epac1, the rank order of potency was determined to be 7.1>6.9>4.6>1.0>0.12 for 8-pMeOPT-2′-*O*-Me-cAMP, 8-pHPT-2′-*O*-Me-cAMP, 8-pCPT-2′-*O*-Me-cAMP, cAMP, and 2′-*O*-Me-cAMP, respectively [35]. It is important to emphasize that *S*-phenyl ring substitutions of this sort, while conferring high-affinity binding, are not

necessary in order to generate a cAMP analog that interacts preferentially with Epac1. For example, when examining the potency with which 2′-*O*-Me-cAMP displaced the binding of [^{3}H]cAMP, it was demonstrated that this ESCA was 13-fold more effective at the CNBD of Epac1 as compared with the "A" CNBD of PKA regulatory subunit I_α [35]. Still, *S*-phenyl ring substitutions do provide an additional benefit in terms of Epac/PKA selectivity. When comparing the ability of an ESCA to discriminate between Epac1 and the "A" CNBD of PKA regulatory subunit I_α, the rank order of Epac/PKA selectivity was determined to be 284>107>13>1 for 8-pMeOPT-2′-*O*-Me-cAMP, 8-pCPT-2′-*O*-Me-cAMP, 2′-*O*-Me-cAMP, and cAMP, respectively. In summary, 8-pMeOPT-2′-*O*-Me-cAMP combines the highest affinity towards Epac with the highest Epac/PKA selectivity of all ESCAs described so far.

4. Activation of Epac by 8-pCPT-2′-*O*-Me-cAMP in vitro and in living cells

The in vitro activity of 8-pCPT-2′-*O*-Me-cAMP was characterized by Bos and co-workers using a test tube Rap1 activation assay [34]. It uses recombinant Epac1, Rap1, and a fluorescent GDP analog (MANT-GDP) that is released from Rap1 when the cAMP-bound form of Epac interacts with the GTPase. It was demonstrated that half-maximal activation of Rap1, as measured by a decrease of MANT-GDP fluorescence, was observed using 2.2 μM 8-pCPT-2′-*O*-Me-cAMP. In contrast, this same concentration of 8-pCPT-2′-*O*-Me-cAMP produced virtually no activation of PKA [34]. Interestingly, when compared with the action of cAMP, 8-pCPT-2′-*O*-Me-cAMP activated Rap1 with greater potency as well as greater efficacy [34]. However, the Epac selectivity of 8-pCPT-2′-*O*-Me-cAMP was not absolute since concentrations of the analog in excess of 100 μM activated PKA in vitro with either full efficacy at low concentrations of PKA [34], or partial efficacy at physiological concentrations of PKA [35]. Despite this fact, when 8-pCPT-2′-*O*-Me-cAMP was tested at a concentration of 100 μM in NIH3T3 cells stably transfected with Epac1, it failed to promote PKA-dependent phosphorylation of CREB, a known substrate for PKA. In contrast, under identical conditions, 8-pCPT-2′-*O*-Me-cAMP fully activated Rap1 [34]. Thus, in NIH3T3 cells, 1–100 μM 8-pCPT-2′-*O*-Me-cAMP appears to exert a highly selective action to activate Epac while having little or no stimulatory effect at PKA. It is important to emphasize that this selectivity is not necessarily representative of all cell lines or experimental conditions. Thus, when examining the action of 8-pCPT-2′-*O*-Me-cAMP in living cells, care must be taken to assure that the observed alterations of cellular function are mediated by Epac and not PKA (see below).

5. Target validation of Epac-selective cAMP analogs

Although numerous studies demonstrate that ESCAs alter cellular functions, most studies have not incorporated the necessary control experiments that would eliminate the possibility that an ESCA exerts its effects by activating PKA. This is an important consideration because in one study of pancreatic beta cells, it was reported that the potentiation of Ca^{2+}-dependent exocytosis by 100 μM 8-pCPT-2′-*O*-Me-cAMP was reduced by myristoylated PKA inhibitor 14–22 amide (PKI; a pharmacological inhibitor of PKA) [66]. The action of PKI measured in this assay might reflect its ability to block the activation of PKA by 8-pCPT-2′-*O*-Me-cAMP. Alternatively, it is possible that Ca^{2+}-dependent exocytosis, while being Epac-regulated, also requires some minimal level of PKA activity [67]. In this scenario, PKA would act in a permissive manner to support Epac-regulated exocytosis. Such a level of uncertainty illustrates a fundamental problem associated with the use of ESCAs. This problem arises because at the present time, no specific pharmacological antagonist of Epac exists. Although the cAMP analog Rp-cAMPS [68] was reported to inhibit cAMP-dependent activation of Epac in vitro [65], subsequent studies performed with living cells demonstrated that it fails to block actions of 8-pCPT-2′-*O*-Me-cAMP that are Epac-mediated [26,29,40,41,46].

To validate that an ESCA is acting via Epac, it is necessary to perform control experiments in which the confounding role of PKA is excluded. Ideally, it should be demonstrated that pretreatment of cells with an inhibitor of PKA (H-89, KT5720, PKI), or the cAMP antagonists Rp-cAMPS and Rp-8-Br-cAMPS, fails to block the action of 8-pCPT-2′-*O*-Me-cAMP. Conversely, it should be demonstrated that 8-pCPT-2′-*O*-Me-cAMP fails to act when Epac-mediated signal transduction is down-regulated by treatment of cells with Epac siRNA [69], Epac antisense deoxyoligonucleotides [24], or after transfection of cells with dominant-negative Epac proteins that fail to bind cAMP as a consequence of R279E or G422D substitutions (Fig. 3) [26,29,40,41,52,53]. It should also be demonstrated that the action of 8-pCPT-2′-*O*-Me-cAMP is mimicked by structurally-related ESCAs (8-pMeOPT-2′-*O*-Me-cAMP, 8-pHPT-2′-*O*-Me-cAMP), but not by a cGMP analog with identical substitutions (e.g., 8-pCPT-2′-*O*-Me-cGMP). Furthermore, the action of 8-pCPT-2′-*O*-Me-cAMP should not be mimicked by N^6-benzoyl-cAMP (N^6-Bnz-cAMP), a cAMP analog that activates PKA but not Epac [35]. If the experimental conditions allow the direct application of cAMP analogs to the inside of a cell by use of the patch clamp technique, it should be demonstrated that the action of 8-pCPT-2′-*O*-Me-cAMP parallels the effect that is measured upon application of 2′-*O*-Me-cAMP [29]. This control allows a determination to be made as to whether the action of an ESCA results from a non-specific effect conferred by the hydrophobic substitution at position 8 on the adenine moiety.

Although published studies demonstrate that the extracellular application of a cell-permeant ESCA produces major alterations of intracellular processes, care should be taken to validate that for the particular cell of interest, an ESCA such as 8-pCPT-2′-*O*-Me-cAMP does in fact enter the cell. This control experiment can be performed using cells transfected with fluorescent molecules that incorporate the CNBD of Epac1 or Epac2. These reporters exhibit a change of fluorescence resonance energy transfer (FRET) when cAMP binds to them [70–73]. By performing spectrofluorimetry, either in a multi-

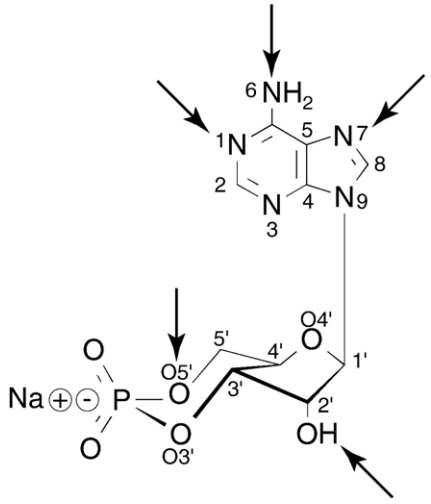

Fig. 5. Structural determinants of cAMP that confer its interaction with PDEs. Illustrated are the critical N^1, N^6, and N^7 positions on the adenine moiety that participate in hydrogen bond formation with the catalytic domains of all PDEs for which crystallographic data is available. For the special case of PDE10A2, additional structural determinants that are important to its interaction with cAMP are the 5′-O linkage of the phosphodiester bond and the 2′-OH on the ribose ring of cAMP (arrows). Since ESCAs contain a 2′-*O*-Me group on the ribose moiety, they are predicted to be able to interact with most PDEs other than PDE10A2.

well format, or by the imaging of individual cells, it is possible to confirm that an extracellularly-applied ESCA gains access to the cytoplasm, and that it reaches a concentration high enough to activate Epac. In general, the cell-permeability of an ESCA is expected to correlate with its lipophilicity. Data available at the BIOLOG Life Science Institute web site (http://www.biolog.de/technical-info/lipophilicity-data/) rank the lipophilicity of ESCAs and other commonly used cAMP analogs relative to that of cAMP. This web site also provides detailed technical information concerning the biochemical properties and chemical structures of ESCAs.

6. Potential confounding effects of ESCA degradation products

Using cultures of the protozoan *Trypanosoma brucei*, Beavo and co-workers reported that 8-pCPT-2′-*O*-Me-cAMP exerted an anti-proliferative effect, but that this action of the ESCA resulted from its degradation into bioactive metabolites, one of which was 8-pCPT-2′-*O*-Me-adenosine (8-pCPT-2′-*O*-Me-Ado) [74]. Importantly, no such anti-proliferative effect was measured in response to Sp-8-pCPT-2′-*O*-Me-cAMPS, a phosphorothioate derivative of cAMP that is resistant to enzymatic hydrolysis catalyzed by cyclic nucleotide phosphodiesterases (PDEs). Evidently, the PDEs expressed in Trypanosomes are capable of hydrolyzing 8-pCPT-2′-*O*-Me-cAMP to its 5′-AMP derivative, thereby generating metabolites that act independently of Epac to inhibit proliferation [74]. What remains uncertain is whether 8-pCPT-2′-*O*-Me-cAMP is also hydrolyzed by the PDEs expressed in mammalian cells. Numerous isoforms of PDEs are expressed in mammalian cells, and information exists concerning which functional groups on cAMP allow it to interact with PDEs [75–79]. Whereas the N^1, N^6, and N^7 positions on the adenine moiety are particularly important for the interaction of cAMP with most isoforms of PDEs (Fig. 5), the 2′-OH moiety on the ribose ring is not required for this interaction, except for the special case of PDE10A2 (Table 1). Thus, it may be predicted that most if not all ESCAs should interact with mammalian PDEs. For this reason, a cautious approach to the future use of ESCAs is to validate that the action of 8-pCPT-2′-*O*-Me-cAMP in living cells is reproduced by PDE-resistant Sp-8-pCPT-2′-*O*-Me-cAMPS, thereby minimizing the likelihood that the measured effect arises simply as a consequence of the generation of metabolites such as 8-pCPT-2′-*O*-Me-Ado.

The studies of Beavo and co-workers provide one additional surprising finding. Not only are ESCAs susceptible to hydrolysis by PDEs, but their interaction with PDEs may inhibit the hydrolysis of endogenous cAMP, thereby raising levels of the second messenger [74]. If this were to be the case in mammalian cells, one expected and confounding outcome might be the indirect activation of PKA by 8-pCPT-2′-*O*-Me-cAMP. Thus, when documenting Epac-mediated actions of 8-pCPT-2′-*O*-Me-cAMP, appropriate control experiments should be designed in which the action of the ESCA is demonstrated to be resistant to well-established inhibitors of PKA, and not secondary to increased cAMP production. Although Epac knockout mice are not available at the present time, it is anticipated that such a model system will eventually establish the selectivity with which ESCAs differentiate between Epac and PKA in vivo.

7. A likely role for Epac in GPCR signal transduction

G protein-coupled receptors that stimulate cAMP production include a subset of Class I (Family A) and Class II (Family B) seven transmembrane-spanning domain GPCRs [80]. These cAMP-elevating GPCRs are G_S-coupled because they interact with heterotrimeric G_s GTP-binding proteins that govern adenylyl cyclase activity [81]. Ligand-binding to G_S-coupled receptors produces an increase of $[cAMP]_i$ and the activation of

Table 1
Summary of structural determinants of cAMP that allow its interaction with PDEs

Nucleotide moiety	PDE isoform	Reference #
Adenine N^1	PDE1B	[75]
	PDE2A	[76]
	PDE4B/D	[77]
Adenine N^6	PDE1B	[75]
	PDE2A	[76]
	PDE3B	[78]
	PDE4B/D	[77]
	PDE10A2	[79]
Adenine N^7	PDE1B	[75]
	PDE2A	[76]
	PDE3B	[78]
	PDE4B/D	[77]
	PDE10A2	[79]
Ribose 5′-O	PDE10A2	[79]
Ribose 2′-OH	PDE10A2	[79]

PKA in numerous cell types [82]. However, GPCRs that interact with G_S are also known to exert effects independent of PKA, as demonstrated by the failure of PKA inhibitors such as H-89 or KT5720 to block cellular responses initiated by ligand binding to the receptors [61]. In some types of cells such effects are mediated by cyclic nucleotide-gated ion channels that bind cAMP directly [83]. In other cells the PKA-independent signalling properties of G_S-coupled receptors arise as a consequence of their interaction with G proteins other than G_S [84]. For example, the parathyroid hormone receptor (PTH-R), a member of Class II GPCRs, interacts not only with G_S, but also with G_q, thereby explaining its ability to mobilize an intracellular source of Ca^{2+} in COS cells [85]. It is also evident that G_S-coupled receptors have the capacity to activate signal transduction pathways that are "growth factor-like". For example, the glucagon-like peptide-1 (GLP-1) receptor is a Class II GPCR that acts through G_S but independent of PKA to transactivate the epidermal growth factor (EGF) receptor, thereby stimulating phosphatidylinositol-3-kinase (PI-3K) in pancreatic beta cells [86]. With these points in mind, it seems reasonable to speculate that additional PKA-independent signal transduction properties of G_S-coupled receptors remain to be discovered, and that such signalling properties might be explained by the cAMP-dependent activation of Epac.

What evidence is there for Epac-mediated actions of GPCRs? The main evidence is indirect and is based on the fact that for cells expressing Class I and Class II GPCRs, some PKA-independent effects of hormones or neurotransmitters are reproduced by the ESCA 8-pCPT-2′-*O*-Me-cAMP. This is the case for Class I GPCRs including: beta-1, beta-2, and beta-3 adrenergic receptors [20,22,45,87], the M_3 muscarinic cholinergic receptor [23,88], prostaglandin (PGE_2) receptor [21], the D_1 dopamine receptor [37], and the V_2 vasopressin receptor [43] (Table 2). For Class II GPCRs, 8-pCPT-2′-*O*-Me-cAMP reproduces the action of ligands active at CRF receptors [14], PACAP receptors [15,89], glucagon receptors [36,90], PTH receptors [38], and GLP-1 receptors [26,29,40,41,54,56] (Table 3).

Table 2
8-pCPT-2′-*O*-Me-cAMP replicates the effects of agonist stimulation at Class I GPCRs

GPCR	Cell type	Cellular response	Reference #
Beta-AR	Ovarian carcinoma	Integrin-mediated cell adhesion	[20]
	Cardiomyocyte	Gap junction formation	[22]
	Cardiomyocyte	Ca^{2+} mobilization via CICR	[45]
	Adipocyte	Inhibition of insulin-stimulated PKB phosphorylation	[87]
M_3-ACh-R	HEK cells	Activation of PLD	[23]
	HEK cells	Activation of PLC-ε Ca^{2+} mobilization	[88]
PGE_2-R	Vascular endothelium	Decreased endothelial permeability	[21]
D_1-Dopamine-R	Alveolar type II cell line	Na^+ channel activation	[37]
V_2-Vasopressin-R	Kidney inner medullary collecting duct cells	Ca^{2+} mobilization via CICR; Exocytosis	[43]

Abbreviations: AR, adrenergic receptor; CICR, Ca^{2+}-induced Ca^{2+} release; M_3-ACh-R, type-3 muscarinic acetylcholine receptor; PGE_2-R, prostaglandin-2 receptor; PKB, protein kinase B.

Table 3
8-pCPT-2′-*O*-Me-cAMP replicates the effects of agonist stimulation at Class II GPCRs

GPCR	Cell type	Cellular response	Reference #
CRF-R	LC neuron	Differentiation	[14]
PACAP-R	CG neuron	Big K^+ channel activation	[15]
	PC12 cells	Differentiation	[89]
Glucagon-R	Hepatocytes	Cl^- channel activation	[36]
	Islet α-cells	Exocytosis	[90]
PTH-R	Kidney proximal tubular cells	Na^+/H^+ exchanger 3 inhibition	[38]
GLP-1-R	Islet β-cells	Ca^{2+} mobilization via CICR	[40,41]
	Islet β-cells	K-ATP channel inhibition	[29]
	Islet β-cells	Exocytosis	[26,54,56]

Abbreviations: CG neuron, cerebellar granule cell neuron; CICR, Ca^{2+}-induced Ca^{2+} release; CRF-R, corticotropin releasing factor receptor; GLP-1-R, glucagon-like peptide-1-(7–36)-amide receptor; LC neuron, locus ceruleus neuron; PACAP-R, pitiuitary adenylyl cyclase-activating polypeptide receptor; PTH-R, parathyroid hormone receptor.

For many of these receptors it has yet to be demonstrated that the down-regulation of Epac expression ablates the actions of agonists active at GPCRs. However, a functional antagonism of GLP-1 action has been reported in INS-1 insulin-secreting cells co-expressing the GLP-1 receptor and a dominant negative Epac2 that fails to bind cAMP. In these cells, the action of GLP-1 receptor agonists to mobilize intracellular Ca^{2+} and to stimulate mitochondrial ATP production is inhibited [41,52,53]. Exactly how this inhibitory action of dominant negative Epac2 is achieved is not fully understood. Structural studies of crystallized Epac2 seem to indicate that an Epac2 incapable of binding cAMP would be unable to interact with Rap GTPase [7]. Thus, it may be speculated that dominant negative Epac2 competes with and inhibits the binding of endogenous Epac2 to an intracellular receptor, the function of which is to target Epac2 to its site of action.

8. Is the activation of Epac dependent on compartmentalized cAMP signalling?

Based on the differential capacity of isoproterenol and prostaglandin E_1 to stimulate an increase of cAMP levels in particulate vs. soluble fractions of purified cardiac myocytes, Buxton and Brunton first proposed that intracellular cAMP signalling is compartmentalized [91]. Modern views on how compartmentalization is achieved focus on the complementary roles of TMACs and PDEs, the catalytic activities of which generate heterogeneous concentration gradients of cAMP within the cell [92]. Critical to this phenomenon is the role of A-kinase anchoring proteins (AKAPs) that not only anchor

PKA, but which also target PDEs to the plasma membrane or intracellular membranes where cAMP hydrolysis occurs [82]. The prevailing view is that microdomains of elevated cAMP concentration are generated at the plasma membrane as a consequence of TMAC activity, and that under these conditions the concentration gradients of cAMP that extend into the cytoplasm are shaped by the activity of non-homogeneously distributed PDEs, of which the PDE4 family may play a particularly important role [93]. Gradients of cAMP formed in this manner are not static but are dynamic due to positive and negative feedback regulatory mechanisms that control TMAC and PDE activity. This allows for the spatiotemporal control of cAMP signalling which underlies the initiation, distribution, and termination of the second messenger's mode of action [94,95]. Although this dynamic form of signal transduction most likely plays an important role in determining how intracellular pools of PKA are activated, there is reason to believe that a similar process of cAMP compartmentalization might dictate how Epac is activated.

Evidence for a compartmentalization of cAMP signalling relevant to Epac signal transduction is provided by studies demonstrating that in cardiac myocytes there exists a macromolecular complex containing Epac1, PDE4D3, muscle-specific AKAP, PKA, ERK5 and ryanodine receptor (RYR) intracellular Ca^{2+} release channels [49]. Targeting of Epac1 to this complex is a consequence of the direct interaction of Epac1 with PDE4D3 [49]. Thus, it is expected that the activity of PDE4D3, which shapes the cytosolic cAMP concentration gradients, will strongly influence the activation state of Epac1. Given that ESCAs are reported to promote the release of Ca^{2+} from intracellular Ca^{2+} stores governed by ryanodine receptors [40,41,43], there may exist a process of compartmentalized cAMP signaling that serves to regulate cardiac myocyte Ca^{2+} handling.

Despite such findings concerning PDE4D3 and its interaction with Epac1, additional factors may determine where in a cell Epac1 is located. Bos and co-workers demonstrated that the presence of a Disheveled, Egl-10, Pleckstrin (DEP) domain in Epac1 is required in order for this exchange factor to be concentrated in particulate fractions of COS cells [4]. Confocal imaging studies using an Epac1-GFP fusion protein confirmed this finding and demonstrated that Epac1 was localized to the perinuclear region [96]. More recently, Yarwood and co-workers reported that the perinuclear targeting of Epac1 is also promoted by a portion of the catalytic GEF domain located at the exchange factor's C-terminus [33]. Thus, it may be speculated that the DEP and GEF domains act in concert to determine the subcellular distribution of Epac1.

The targeting of Epac2 may be somewhat more complicated than that of Epac 1 because Epac2 contains a functional Ras association (RA) domain that allows it to interact with the activated form of H-Ras GTPase [97]. Although Epac2 does not activate H-Ras, Epac2 is recruited to the plasma membrane by H-Ras under conditions of combined cAMP and growth factor stimulation [97]. This recruitment reflects a direct interaction of cAMP-bound Epac2 with the GTP-bound form of H-Ras [97]. Such findings prompt speculation that H-Ras-mediated recruitment of Epac2 to the plasma membrane might play a significant role in allowing Epac2 to interact with its effector proteins. If so, the Epac2-mediated actions of cAMP-elevating hormones or neurotransmitters might require simultaneous exposure of cells to growth factors with Ras signalling potential. Thus, Epac2 may be viewed as a potential locus of signal transduction crosstalk between cAMP and growth factor signalling pathways. In summary, to what extent these various targeting processes influence the responsiveness of Epac1 or Epac2 to the non-uniform concentration gradients of cAMP that exist within cells remains a question of interest.

9. Evidence for Epac-dependent activation of ERK mitogen-activated protein kinases

One controversy that has surrounded the interpretation of studies examining the actions of Epac-selective cAMP analogs concerns the potential role of Epac as a coupling factor linking cAMP production to the activation of the ERK MAPKs. For example, Bos and co-workers used 8-pCPT-2′-*O*-Me-cAMP to test for Epac1 and Rap1 dependent activation of ERK in multiple cell lines. Despite the fact that 8-pCPT-2′-*O*-Me-cAMP fully activated Rap1, no activation of ERK could be detected [34]. More recently, evidence has been presented that the Epac1-mediated activation of ERK does occur under defined experimental conditions. In studies of PC12 cells that express wild type Epac1 at the perinuclear membrane, Stork and co-workers found that 8-pCPT-2′-*O*-Me-cAMP failed to activate ERK [98]. However, activation of ERK by 8-pCPT-2′-*O*-Me-cAMP was observed after expression of an engineered Epac1 that incorporated a membrane-targeting (CAAX) motif at its C-terminus. Evidently, this recombinant Epac1 localized to the plasma membrane where a complex of Rap1 and its down stream effectors B-Raf and ERK were located [98]. Thus, it would appear that the subcellular localization of Epac1 might determine the efficacy of Epac-selective cAMP analogs as activators of ERK. To what extent Epac2 promotes the activation of ERK remains to be determined.

10. Epac-dependent regulation of ion channel function

An unanticipated role for Epac in the cAMP-dependent regulation of ion channel function is now known to exist. For example, Kang and co-workers reported an interaction of Epac with the SUR1 subunit of pancreatic beta-cell ATP-sensitive K^+ channels (K-ATP channels; a hetero-octamer comprised of SUR1 and the Kir6.2 pore-forming subunit) [29]. SUR1 is an ATP-binding cassette (ABC) protein, and it acts as a sensor of cytosolic adenine nucleotides, thereby coupling intracellular metabolism to the regulation of K-ATP channel function [99]. Co-immunoprecipitation studies demonstrated that Epac1 and Epac2 interact with full-length SUR1 [29], and for Epac2 this interaction appeared to be specific for nucleotide-binding fold-1 (NBF-1) of SUR1 [27,30]. It seems that the interaction of Epac2 with NBF-1 might serve to recruit Epac2 to the plasma membrane so that adenylyl cyclase-catalyzed cAMP production may modulate K-ATP channel activity [62]. This concept is

supported by the finding that 8-pCPT-2′-*O*-Me-cAMP inhibits K-ATP channel activity in INS-1 insulin-secreting cells [29].

An additional role for Epac in the regulation of ion channel function may exist in cardiac myocytes. Indeed, in these cells, 8-pCPT-2′-*O*-Me-cAMP stimulates an increase of $[Ca^{2+}]_i$ [42,45]. Could it be that as is the case for pancreatic beta cells [40,41], renal collecting duct cells [43], and cerebellar granule cells [15], the Epac located in cardiac myocytes plays some role in determining how cAMP influences ryanodine receptor (RYR) intracellular Ca^{2+} release channels? Such an effect seems plausible because in cardiac myocytes, Scott and co-workers report that Epac1 associates in a macromolecular complex with RYR, AKAP, PKA, PDE4D3, and MAPK [49]. Furthermore, 8-pCPT-2′-*O*-Me-cAMP is reported by Gomez and co-workers to increase the frequency of Ca^{2+} sparks originating from Ca^{2+} stores in cardiac myocytes [100]. This effect is associated with Ca^{2+}/calmodulin-dependent protein kinase-mediated phosphorylation of RYR [100]. Alternatively, the findings of Smrcka and co-workers seem to indicate that the Epac expressed in cardiac myocytes might mediate the cAMP-dependent activation of PLC-ε with concomitant mobilization of Ca^{2+} from IP_3 receptor-regulated Ca^{2+} stores [45].

11. Conclusion

Several unanswered questions immediately come to mind when evaluating the potential importance of Epac to cAMP signal transduction. First and foremost, what is the relative importance of Epac and PKA to cellular signalling? Is Epac a minor player, or does it play a major role, one that is only now becoming appreciated? Certainly, the multiplicity of cellular functions altered upon exposure of cells to 8-pCPT-2′-*O*-Me-cAMP provides preliminary evidence for the existence of physiological processes that are Epac-regulated. Yet, rather little is known concerning the ability of GPCRs to activate Epac. This dearth of knowledge is a consequence of the fact that there is no specific pharmacological antagonist of Epac with which to block GPCR signalling. Development of such an antagonist, perhaps based on the known structures of ESCAs, has been slowed by the lack of crystallographic data to provide detailed 3-dimensional structural information concerning the cAMP-bound form of either Epac1 or Epac2. So far, the only crystal structure available is that for Epac2 in its auto-inhibited state not bound to cAMP [7].

An additional question of interest concerns whether or not Epac1 and Epac2 serve similar or different functions. Both proteins are cAMP-GEFs, and both mediate cAMP-dependent activation of Rap, so it seems natural to suggest that Epac1 and Epac2 serve parallel if not identical functions. However, this conjecture is most likely a simplification since there may exist down stream effector proteins that interact with one form of Epac but not the other, possibly as a consequence of the differential targeting of Epac1 and Epac2 to non-identical subcellular compartments. Thus, as is the case for PKA [82], targeting or anchoring processes that govern where Epac is expressed in a cell, and just as importantly, which effector molecules it interacts with, may allow GPCRs to activate Epac1 or Epac2 in a selective manner. In this regard, it would be particularly interesting to assess whether in cells that express both Epac1 and Epac2, the targeted gene deletion of one but not the other isoform of Epac results in a selective loss of hormone or neurotransmitter responsiveness.

Finally, what role, if any, soluble adenylyl cyclase (SAC) plays in the regulation of Epac is a question not answerable at the present time [10]. Could it be that a pool of intracellular Epac is activated by SAC, and that this pool is not accessible to GPCRs as a consequence of the selective interaction of GPCRs with transmembrane adenylyl cyclases? Needless to say, such fundamental questions are likely to provide the impetus for future studies whereby the true physiological significance of Epac will be determined.

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