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Differential Pharmacophore Definition of the cAMP Binding Sites of NCS-Rapgef2, PKA and Epac in Neuroendocrine Cells Using an Adenine-based Scaffold

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ABSTRACT

We recently reported that the adenylate cyclase (AC) inhibitor SQ22,536 (9-tetrahydrofuranyl-adenine) also has inhibitory activity against the neuroendocrine-specific neuritogenic cAMP sensor-Rapgef2 (NCS-Rapgef2), a guanine nucleotide exchanger and activator for the small effector GTPase Rap1. Cell-based assays that distinguish signaling through the three intracellular cAMP sensors NCS-Rapgef2, exchange protein activated by cAMP (Epac), and protein kinase A (PKA), as well as AC, were used. These, collectively, assess the activities of adenine (6-amino-purine) derivatives modified at several positions to enhance selectivity for NCS-Rapgef2 by decreasing affinity for adenylate cyclase (AC), without increasing affinity for PKA or Epac. Testing of each adenine derivative in whole-cell assays incorporates features of cell permeability, target selectivity, and intrinsic potency into a single EC_{50} or IC_{50}, making robust extrapolation to compound activity in vivo more likely. N6-MBC-cAMP is a selective PKA activator (EC_{50} = 265 μM) with low efficacy at NCS-Rapgef2. 8-CPT-2'-O-Me-cAMP and ESI-09 are confirmed as Epac-selective, for stimulation and inhibition, respectively, versus both PKA and NCS-Rapgef2. The compound N6-Phe-cAMP is a full agonist of NCS-Rapgef2 (EC_{50} = 256 μM). It has little or no activity against Epac or PKA. The compound N6-phenyl-9-tetrahydrofuranyladenine is a novel and potent NCS-Rapgef2 inhibitor without activity at PKA, Epac or ACs, as assayed in the neuroendocrine NS-1 cell line. This line has been engineered to allow high-content screening for activation and inhibition of AC, PKA, Epac and NCS-Rapgef2, and the cellular activities initiated by these signaling pathway protein components.

KEYWORDS: Cyclic AMP, Epac, Rapgef2, ERK, p38, CREB

INTRODUCTION

For several decades after the discovery of protein kinase A (PKA), it was assumed that cAMP binding to the regulatory subunit of PKA (PKAR), with activation of the catalytic subunit of PKA (PKAc) was the sole mode through which cAMP signal transduction occurs in mammalian cells [1]. PKAc is inactive in association with PKAR. PKAc dissociates from PKAR when homodimers of PKAR (RIα-RIα, RIβ-RIβ, RIIα-RIIα or RIIβ-RIIβ; products of the RIα, RIβ, RIIα and RIIβ genes), are each occupied by two molecules of cAMP at sites A and B, found on each R monomeric subunit. The A and B sites of each of the four monomeric subunits RIα, RIβ, RIIα and RIIβ differ in their affinity for cyclic AMP, with the A site having a faster, and the B site having a slower dissociation rate for cAMP [2-3]. In addition, the absolute affinity for cAMP differs somewhat at both A and B sites, when compared across the four isoforms RIα, RIβ, RIIα and RIIβ**.** Consequently, a large number of pharmacological agents directed to sites A and B have been developed, and used in combination, to differentially activate the four forms of PKAR (RIα-RIα, RIβ-RIβ, RIIα-RIIα and RIIβ-RIIβ in various tissue and cell types [4]. These compounds have given significant insight into the cAMP pharmacophore for each of the two sites A and B, but have not been particularly useful for pharmacological experiments in vivo. For this reason, pharmacological study of PKA-dependent cellular signaling has relied mainly on competitive *inhibition* of cAMP binding to PKAR (e.g. by the Rp isomer of cyclic adenosine-3′,5′ monophosphorothioate (Rp-cAMPS) or of the kinase activity of PKAc, with isoquinolines such as H89 and KT5720. Each of these approaches has its own limitations of specificity and potency [5-6].

In the late 1990s, a second set of sensors for cAMP in mammalian cells was discovered. These were not protein kinases, but rather guanine nucleotide exchange factors (GEFs): the Exchange Proteins Activated by cAMP (Epac 1 and 2, also called cAMP-GEF-I/II, or Rapgef3/4). These proteins were shown to catalyze the activation of the small GTPase effector molecule Rap, a member of the Ras superfamily, by releasing GDP from, and allowing GTP binding to, Rap [7-8]. The existence of a new set of cAMP sensors, besides PKAR, made it necessary to reexamine the specificity of existing pharmacological activators and inhibitors of PKA, for their actions at Epacs. Rp-cAMPS and Sp-cAMPS were found to have similar activities at both PKAR and Epac. Thus, these compounds could no longer be considered specific for PKA [9-11]. Two compounds were developed, however with a high degree of specificity for Epacs, compared to PKA. These were 8-chlorophenylthio-2'-O-methyl-cAMP, an Epac activator, and ESI-05/09, which are Epac inhibitors [10-13].

This operational definition of specificity seemed to be sufficient for the analysis of cAMP signal transduction in most cell types. A third potential cAMP sensor, CNrasGEF/PDZ-GEF1 was dismissed as a cAMP-regulated GEF, since it seemed not to function in a cAMP-dependent manner [14-15], except perhaps in melanoma cells [16-18]. In fact, its non-canonical cyclic nucleotide binding site (CNBD) was deemed unlikely to mediate cAMP-dependent steric alteration/activation of the GEF functionality of the protein in vivo [19]. Subsequently, however, we have identified a protein product of the Rapgef2 gene, termed NCS (for neuritogenic cAMP sensor)-Rapgef2, which, although apparently not expressed in non-neuronal tissues of adult mammals, appears to mediate cAMP-dependent activation of ERK, through Rap1, in neuronal and endocrine cells [20-24]. The discovery of this third type of intracellular cyclic AMP sensor necessitates the development of a new cAMP-related neuropharmacology which takes into account differential affinities and effects of cAMP at its binding site(s) at PKAR, Epac, and NCS-Rapgef2. Herein, we describe cell-based signaling assays that probe the specificity of existing PKA- and Epac-directed cAMP analogs, as extended to interaction with NCS-Rapgef2. Furthermore, we report the synthesis and pharmacological properties of some novel adenine derivatives with specific activity at NCS-Rapgef2 compared to PKA and Epac, based on a previous report of NCS-Rapgef2 inhibitory activity of the adenylyl cyclase inhibitor 9-tetrahydrofuranyl-adenine [21].

RESULTS AND DISCUSSION

Overview

Our strategy for development of potentially specific NCS-Rapgef2 inhibitor proceeds from the observation that 9-THF-adenine, an adenine derivative modified at the 9-p0sition relative to cyclic AMP, is both an AC inhibitor and an NCS-Rapgef2 inhibitor, with apparently little activity to block cAMP-dependent activation of Rap by Epac or PKA-dependent transcriptional activation of a CRE reporter gene, in NS-1 cells [21]; and systematic modification at the N6-position of adenine to further improve potency and selectivity for NCS-Rapgef2 inhibition.

In order to achieve this, we have engineered a battery of cell-based biochemical assays that quantitatively distinguish between PKA-, Epac- and NCS-Rapgef2-dependent signaling in the NS-1 neuroendocrine cell line, based on our previous demonstration using genetic and pharmacological manipulations, that CREB phosphorylation, p38 activation and growth arrest, and ERK phosphorylation/activation and neurite extension are uniquely and exclusively stimulated by PKA, Epac and NCS-Rapgef2, respectively, in NS-1 cells [22]. To validate the use of phosphospecific antibody cell-based ELISAs (PACE) to assess the phosphorylation (activation) status of ERK, CREB, and p38 to be used as biochemical surrogates for NCS-Rapgef2, PKAc, and Epac, NS-1 cells were treated for 30 minutes with the pan-specific cAMP analog 8-CPT-cAMP (100 μM) in the absence or presence of inhibitors of a component of one of the three signaling pathways. Compounds included H89 (PKAc inhibitor), U0126 (MEK inhibitor), ESI-09 (Epac inhibitor), and SB2239036 (p38 inhibitor). Inhibitors were applied to cells for 30 minutes prior to the addition of 8-CPT-cAMP. As seen in Figure 1A-D, 8-CPT-cAMP caused a robust

increase in ERK phosphorylation that was only inhibited by U0126 (Figure 1B). CREB phosphorylation (Figure 1 E-H) was blocked by inhibition of PKAc with H89 (Figure 1E), but unaffected by the other compounds. P38 phosphorylation (Figure 1 I-L) was sensitive to Epac inhibitions with ESI-09 (Figure 1K) and SB239036 (Figure 1L).

Fig. 1

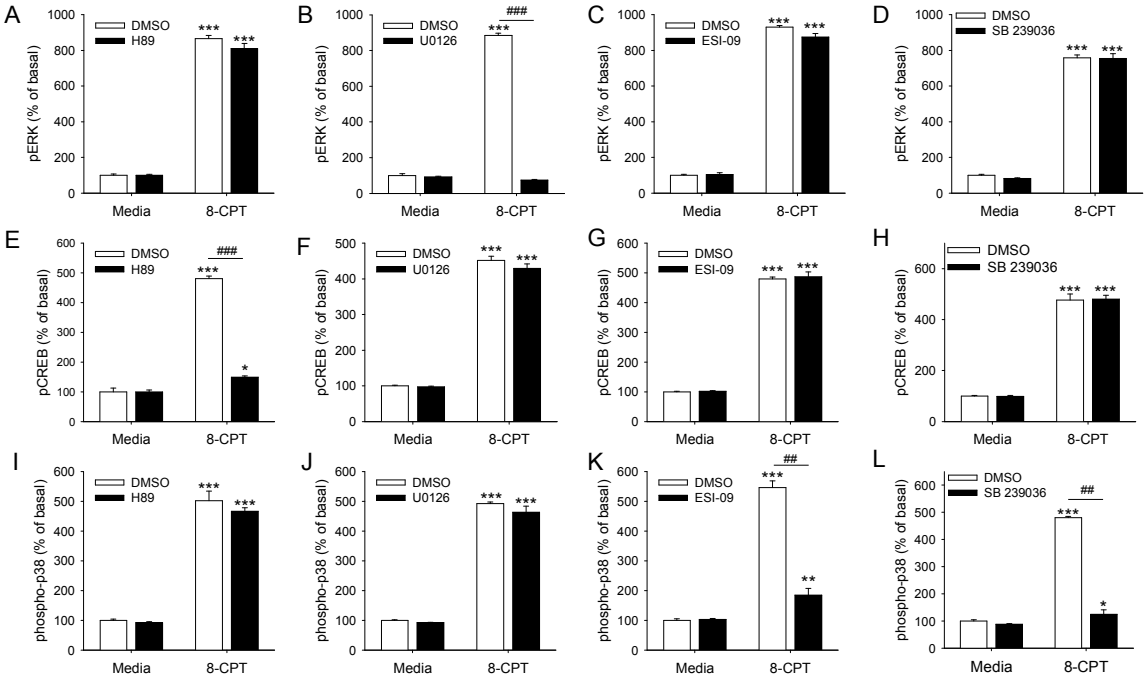


Figure 1 *Measurements of phosphorylated ERK, CREB, and p38 by PACE (phosphospecific antibody cell-based ELISA). NS-1 cells were grown overnight in 96-well plates at a density of 30,000 cells/well. The next day, inhibitors or DMSO was added as 10x stocks to the following final concentrations: 10 μM H89 or 0.02% DMSO; 10 μM U0126 or 0.02% DMSO; 10 μM ESI-09 or 0.02% DMSO; and 10 μM SB 239036 or 0.1% DMSO. Cells were pre-treated for 10 min followed by the addition of 100 μM 8-CPT-cAMP (8-CPT) or an equal volume of media. 30 minutes later, media were aspirated, cells were fixed and immunodetection of phospho-proteins was carried out as described in Methods. Bars are means from three experiments are errors correspond to standard errors of the mean. Data were analyzed by 2-way ANVOA and Bonferroni-corrected t-tests, *P < 0.05, **P < 0.01, *** P < 0.001 comparing the effect of 8-CPT-cAMP to media; ##P < 0.01, ###P < 0.001 comparing these effect of each inhibitor within a treatment condition (media or 8-CPT-cAMP).*

Identification of sensor-selective agonists

8-CPT-2'-O-Me-cAMP, while inactive at PKAR/CREB (as previously described [13]) or NCS-Rapgef2/ERK (Figure 2A-B), was a more potent Epac agonist than the parent compound 8-CPT-cAMP (Figure 2C). Together these data show that 8-CPT-2'-O-Me-cAMP is indeed a highly Epac-selective agonist. Since 8-CPT-cAMP does not discriminate among the three sensors and 8-CPT-2'-O-Me-cAMP is Epac-specific, we wished to see whether additional known cAMP analogs could be used as subtype-preferring agonists. A number of cyclic nucleotide analogs that preferentially bind to a single site of a regulatory subunit of PKA have been identified. The typical motif for these is that N6 modifications confer selectivity for the A site (also referred to as site I, or the rapid site), while halogenation of C2 and addition of some functional groups to C8 (methylamino or piperidino) confers selectivity for the B-site (also called site II, or the slow site). Highly selective ligands for either the A or B site would not be predicted to act as activators of the PKA holoenzyme unless used in combination. Accordingly, we compared the potencies and efficacies of five readily-available cAMP analogs on ERK phosphorylation in NS-1 cells to 8-CPT-cAMP (Figure 3A). 8-CPT-cAMP caused a maximal increase in ERK activation similar to that observed in cells treated with N6-Benzoyl-cAMP (N6-Bn-cAMP), N6-Benzyl-cAMP (N6-Bz-cAMP), N6-Phenyl-cAMP (N6-Phe-cAMP), and N6-Phenyl-8-CPT-cAMP (N6-Phe-8-CPT-cAMP). In contrast, 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole- 3', 5'- cyclic monophosphorothioate (cBIMPS) and N6-mono-tert.butylcarbamoyl-cAMP (N6-MBC-cAMP) had approximately 60% and 80% less intrinsic activity than other analogs examined, respectively. In rank order of potency for ERK activation: N6-Phe-8-CPT-cAMP > N6-Bn-cAMP = 8-CPT-cAMP > N6-Bz-cAMP = N6-Phe-cAMP. As seen in Figure 3B, 8-CPT-cAMP, Sp-DCl-5,6-cBIMPS, N6-Bn-cAMP, N6-Bz-cAMP, N6-MBC-cAMP are full PKA agonists in these cells. N6-Phe-cAMP is inactive at up to 1 mM at PKAR, i.e. to activate CREB phosphorylation. N6-Phe-8-CPT-cAMP, while a full agonist of PKA, was approximately 15 times more potent at NCS-Rapgef2. N6-MBC-cAMP and Sp-5,6-DCl-cBIMPS were the only agonists of PKA tested with limited intrinsic activity at NCS-Rapgef2. To see whether the N6-Phe-cAMP can be used as a selective agonist of NCS-Rapgef2 and N6-MBC-cAMP is a PKA-selective agonist, the ability these two compounds to stimulate Epac-dependent signaling (p38 phosphorylation) was evaluated in an assay with 8-CPT-2'-O-Me-cAMP as a point of reference. Both N6-Phe-cAMP and N6-MBC-cAMP were inactive at Epac at con-

centrations as high as 1 mM (Figure 3C). Using 8-CPT-cAMP as a point of reference, the relative potencies of all compounds tested are listed in Table 1.

Fig. 2

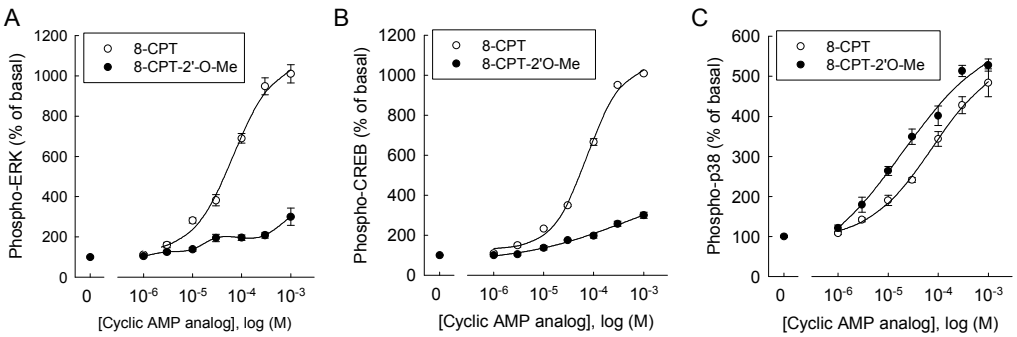


Figure 2 *Activity of 8-(4-Chlorophenylthio)-cAMP (8-CPT) and 8-(4-Chlorophenylthio)-2'-O-Me-cAMP (8-CPT-2'-O-Me) on the neuronal cyclic AMP sensors NCS-Rapgef2, PKAR, and Epac2. NS-1 cells were treated with varying concentrations of either compound for 30 min followed by fixation and measurement of (A) phosphorylated ERK, (B) phosphorylated CREB, and (C) phosphorylated p38 MAP kinase. Data points are means from 3-4 experiments performed in duplicate or triplicate with error bars corresponding to standard errors of the mean.*

Fig. 3

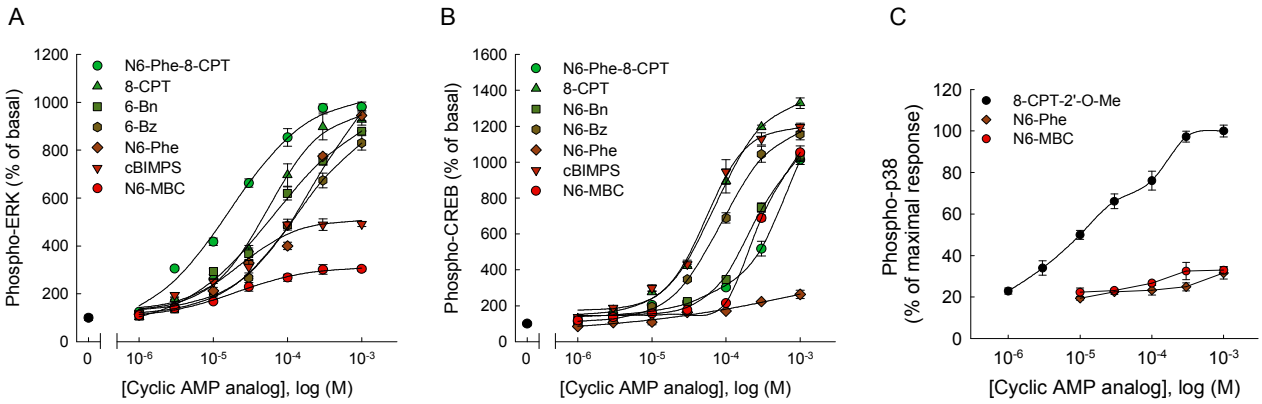


Figure 3 *Effects of N6-substituted cAMP analogs on (A) ERK and (B) CREB phosphorylation. 8-CPT-cAMP, N6-Benzyl (N6-Bn)-cAMP, N6-Benzoyl (N6-Bz)-cAMP are each full agonists at both ERK and CREB at approximately equivalent potencies. N6-mono-tert. butylcarbamoyl (N6-MBC)-cAMP and Sp-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3',5'-monophosphorothioate (cBIMPS) are low efficacy agonists for ERK and full agonists of PKAR/CREB. By contrast, N6-Phenyl (N6-Phe)-cAMP is active at ERK while inactive at CREB at concentrations as high as 1 mM. (C) Neither N6-Phe-cAMP nor N6-MBC-cAMP caused a significant increase in p38 phosphorylation relative to the effect of 8-CPT-2'-O-Me-cAMP.*

In order to confirm the NCS-Rapgef2 efficacy and specificity of the cAMP analogs that were either ERK or CREB selective in phosphorylation assays, we measured neurite elongation (an NCS-Rapgef2 and ERK-dependent phenotypic readout) in NS-1 cells treated for 48 hours with vehicle control (Figure 4A), 100 μM 8-CPT-cAMP (Figure 4B), which caused neurite elongation, 200 μM N6-Phe-cAMP (Figure 4C), which also caused neurite elongation, or 100 μM N6-MBC-cAMP (Figure 4D), which failed to elicit cause neurite elongation, suggesting that even though this compound had some efficacy to cause ERK phosphorylation, its intrinsic activity is likely insufficient to cause a sufficient magnitude of sustained ERK activation required for neurite elongation [25].

N6-Phe-cAMP has been reported to activate the PKA holoenzyme when applied in combination with a B-site-preferring agonist. As a control, we determined that additive effects on CREB phosphorylation were evident in cells treated with N6-Phe-cAMP (100 μM) and 1 μM of the B site ligand Sp-5,6-DCl-cBIMPS (Figure S1), supporting the notion that N6-Phe-cAMP may be quite specific for PKA's A sites over B sites, explaining why it is not an agonist of the PKA holoenzyme when applied alone.

Fig. 4

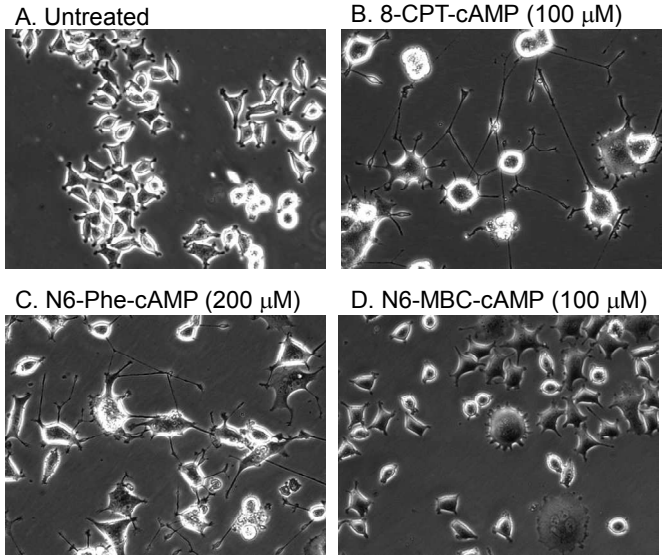
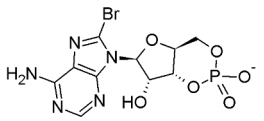
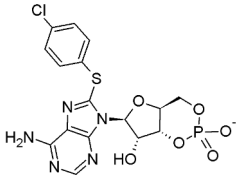
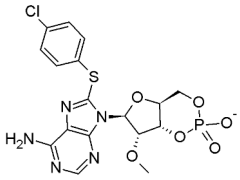
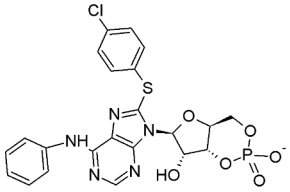
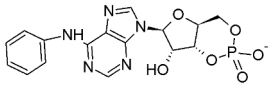
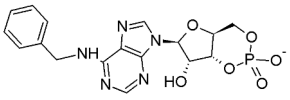
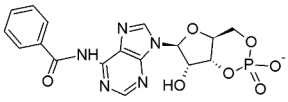
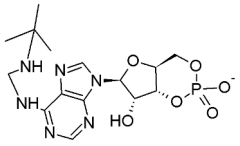
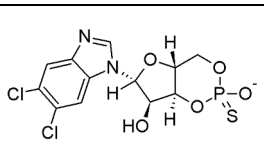


Figure 4 *Cyclic AMP analog-dependent neurite elongation. NS-1 cells were treated for 48 hours with (A) vehicle control, (B) 8-CPT-cAMP, 100 μM, slightly greater than the half-maximal concentration for the compounds effect on ERK phosphorylation, (C) N6-Phe-cAMP, 200 μM, an approximately half-maximal concentration for its effect on ERK phosphorylation, and (D) N6-MBC-cAMP, 100 μM, a supramaximal concentration for its effect on ERK phosphorylation. Photomicrographs were randomly acquired and are representative images from four determinations obtained in three independent experiments.*

Table 1. Potency of cAMP analogs to activate PKA-, NCS-Rapgef2-, and Epac2-dependent signaling processes relative to 8-CPT-cAMP. Results are means and standard errors from 2-4 independent experiments performed in triplicate. EC_{50} values were calculated by 4-parameter logistic regressions. Values >1000 μM indicates that compound did not reach the half-maximal effect of 8-CPT-cAMP when applied at up to 1 mM; n.d., not determined.

Compound	PKA/CREB EC_{50} (μM)	Rapgef2/ERK EC_{50} (μM)	Epac2/p38 EC_{50} (μM)	Structure
8-Br-cAMP	245 ± 61	158 ± 32	163 ± 31	
8-CPT-cAMP	74 ± 8	68 ± 14	79 ± 17	
8-CPT-2'-O-Me-cAMP	>1000	>1000	25 ± 3	
N6-Phe-8-CPT-cAMP	426 ± 91	25 ± 6	>1000	
N6-Phe-cAMP	>1000	256 ± 33	>1000	
N6-Bn-cAMP	231 ± 38	77 ± 29	n.d.	
N6-Bz-cAMP	88 ± 8	171 ± 46	n.d.	
N6-MBC-cAMP	265 ± 14	>1000	>1000	
Sp-5,6-DCl-cBIMPS	54 ± 7	>1000	>1000	

Activity of 9-tetrahydrofuranyl adenine (SQ22,536) to inhibit cAMP-dependent activation of PKA, Epac and NCS-Rapgef2

Given the specificity of known inhibitors of PKA and Epac shown in Figure 1, we were able to use cAMP-dependent ERK activation as a convenient assay for inhibition of NCS-Rapgef2 and AC, or combinations thereof, in the same cells. Previously, we have shown that cAMP-dependent ERK activation is completely abrogated by silencing NCS-Rapgef2 expression in NS-1 cells [23]. As seen in Figure 5A, 9-THF-adenine is a relatively potent AC inhibitor (IC_{50} = 8.9 ± 0.9 μM). Although its potency at NCS-Rapgef2 is diminished relative to its action at AC (the IC_{50} for inhibition of 8-Br-cAMP stimulation of NCS-Rapgef2 is 825 ± 12 μM), 9-THF-adenine is capable of causing significant inhibition of activation of NCS-Rapgef2 by cAMP to a much greater extent than its effects of cAMP-mediated actions on PKA (Figure 5B) or Epac (Figure 5C).

Fig. 5

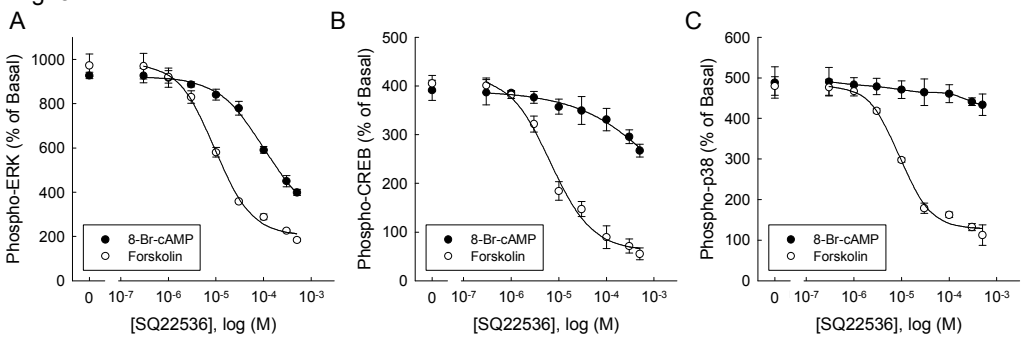


Figure 5 *SQ22,536 (9-THF-Ade) inhibits adenylate cyclase and NCS-Rapgef2. Measurements of (A) ERK phosphorylation, (B) CREB phosphorylation, and (C) p38 phosphorylation in NS-1 cells pre-treated for 30 min with varying concentrations of SQ22,536 or vehicle (0.3% DMSO) followed by treatment with EC_{80} concentrations of either 8-Br-cAMP (575 μM) or forskolin (3 μM) for 30 min. Data points correspond to means from three experiments performed in triplicate with error bars equal to the standard errors of the mean. As determined by ANOVA, 9-THF-Ade had significant inhibitory activity on the effects of both forskolin and 8-Br-cAMP on ERK activation (A), but only significantly blocked the effects of forskolin on (B) CREB and (C) p38 phosphorylation.*

Modification of 9-THF-Adenine to generate an NCS-Rapgef2-selective inhibitor

Previously we reported that the nucleoside 9-tetrahydro-2-furanyl-adenine (SQ 22,536, referred to here as 9-THF-Ade) inhibits both adenylate cyclase and NCS-Rapgef2. Since this compound has two mechanisms of action in the same signaling pathway, the only way to use it to examine NCS-Rapgef2 signaling is to examine the sensitivity to inhibition of a cAMP-mimicking agent that acts downstream of adenylate cyclase (i.e. a cAMP analog). For more experimental flexibility, and as a potential first step in NCS-Rapgef2-directed drug development, we wished to develop compounds with selectivity for NCS-Rapgef2 at the expense of AC, with a secondary goal of increasing overall potency/cell permeability. Since NCS-Rapgef2 was the only neuronal cAMP sensor that allowed the addition of an N6-phenyl group, and this group also increases the lipophilicity of nucleotides/nucleosides, we synthesized N6-phenyl-9-tetrahydro-2-furanyl-adenine (N6-Phe-9-THF-Ade).

As seen in Figure 6A, N6-Phe-9-THF-Ade inhibited forskolin and 8-Br-cAMP-induced ERK phosphorylation at similar potencies (IC_{50} values of 9 ± 1 μM). In contrast, as seen in Figure 6B, 9-THF-Ade was a more potent inhibitor of forskolin-dependent ERK phosphorylation (IC_{50} = 9 ± 2 μM) than of 8-Br-cAMP-dependent ERK phosphorylation (IC_{50} = 110 μM). Taken together, these data suggest that the observable mechanism of action of N6-Phe-9-THF-Ade is inhibition of NCS-Rapgef2 while 9-THF-Ade is about an order of magnitude more potent at adenylate cyclase than at NCS-Rapgef2.

To determine whether N6-Phe-9-THF-Ade is selective for NCS-Rapgef2 over PKA and Epac, NS-1 cells were pretreated with 100 μM of either 9-THF-Ade or N6-Phe-9-THF-Ade and exposed to EC_{80} doses of forskolin (3 μM) or 8-Br-cAMP (575 μM) followed by measurements of CREB (Figure 6D) and p38 phosphorylation (Figure 6E). Dose-response data of 9-THF-Ade and N6-Phe-9-THF-Ade on 8-Br-cAMP- and forskolin-induced CREB and p38 phosphorylation are shown in Figure S2. As seen in Figure 6D, N6-Phe-9-THF-Ade did not block the effect of forskolin or 8-Br-cAMP on CREB phosphorylation. On the other hand, 9-THF-Ade blocked the effect of forskolin, but not the effect of 8-Br-cAMP, consistent with its known profile as an AC inhibitor. Similar results were observed for p38 phosphorylation (Figure 6E), suggesting that neither 9-THF-Ade nor N6-Phe-9-THF-Ade block PKA or Epac and that 9-THF-Ade blocks adenylate cyclase, while N6-Phe-9-THF-Ade does not.

Fig. 6

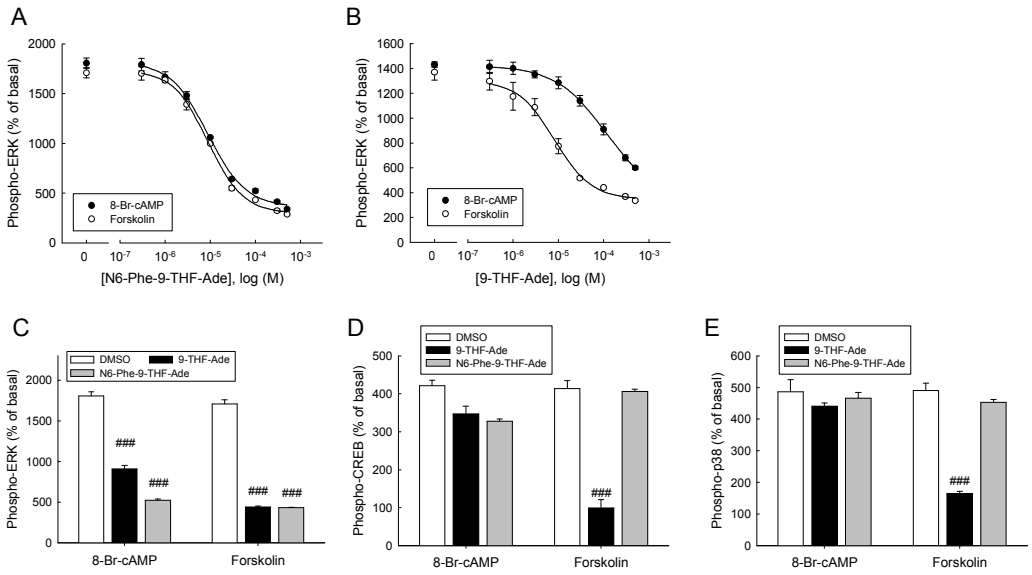


Figure 2 *N6-Phe-9-THF-Ade is a specific inhibitor of cAMP-dependent ERK phosphorylation and acts a single cAMP sensor, NCS-Rapgef2. (A) Measurements of ERK phosphorylation in NS-1 cells pre-treated for 30 min with varying concentrations of N6-Phe-9-THF-Ade or vehicle (0.3% DMSO) followed by treatment with either 8-Br-cAMP (575 μM) or forskolin (3 μM) for 30 min. (B) ERK phosphorylation in NS-1 cells after pre-treatment with varying concentrations of 9-THF-Ade followed by either 8-Br-cAMP (575 μM) or forskolin (3 μM). (C) ERK phosphorylation in NS-1 cells pre-treated with either N6-Phe-9-THF-Ade or 9-THF-Ade (100 μM each) followed by either 575 μM 8-Br-cAMP or 3 μM forskolin. Data are replotted from those shown in Panels A and B, ### $P < 0.001$ by Bonferroni-corrected t-test following 2-way ANOVAs comparing the effects observed in cells pretreated with N6-Phe-9-THF-Ade and 9-THF-Ade to those pre-treated with 0.3% DMSO (DMSO). Measurements of (D) CREB phosphorylation and (E) p38 phosphorylation after pretreatment with 100 μM N6-Phe-9-THF-Ade or 9-THF-Ade followed by 575 μM 8-Br-cAMP or 3 μM forskolin; N = 3-6 separate experiments.*

The neuropeptide PACAP-38 is a GPCR ligand that stimulates cAMP elevation in NS-1 cells and in vivo [20, 26]. It seemed appropriate to show that the results of stimulating the three cAMP pathways pharmacologically, and assessing the activities and potencies of inhibitors of the pathways, should be recapitulated with a physiological activator (i.e. GPCR ligand/first messenger) such as PACAP. To this end, we used a phenotypic assay (neurite elongation following 48 h of treatment with PACAP-38) to determine whether N6-Phe-9-THF-Ade can be used to effectively inhibit NCS-Rapgef2 for an extended time. To this end, NS-1 cells were treated PACAP (100 nM) and either 0.3% DMSO, or N6-Phe-9-THF-Ade, applied at either 3 μM or 10 μM, approximating the IC_{30} and IC_{50} of this compound determined by ERK phosphorylation measurements. After 48, cells were imaged, counted, and neurites were traced by a blinded observer. As seen in Figures 7A and 7B, both concentrations significantly reduced the effect of PACAP, to a similar magnitude seen when using the shorter-term (1 h) ERK phosphorylation assay. Inhibition of adenylate cyclase was measured in cells pretreated with either 9-THF-Ade or N6-Phe-9-THF-Ade followed by incubation with 3 nM PACAP-38 (Fig. 7C), which activates the Gs-coupled PAC1 receptor, or 3 μM forskolin (Fig. 7D), which directly activates adenylate cyclase. Cyclic AMP elevation caused by either agent was inhibited by 9-THF-Ade, but not significantly blocked by N6-Phe-9-THF-Ade at concentrations as high as 100 μM, thus demonstrating that N6-Phe-9-THF-Ade acts to inhibit PACAP-induced neurite elongation solely via direct inhibition of NCS-Rapgef2.

Fig 7

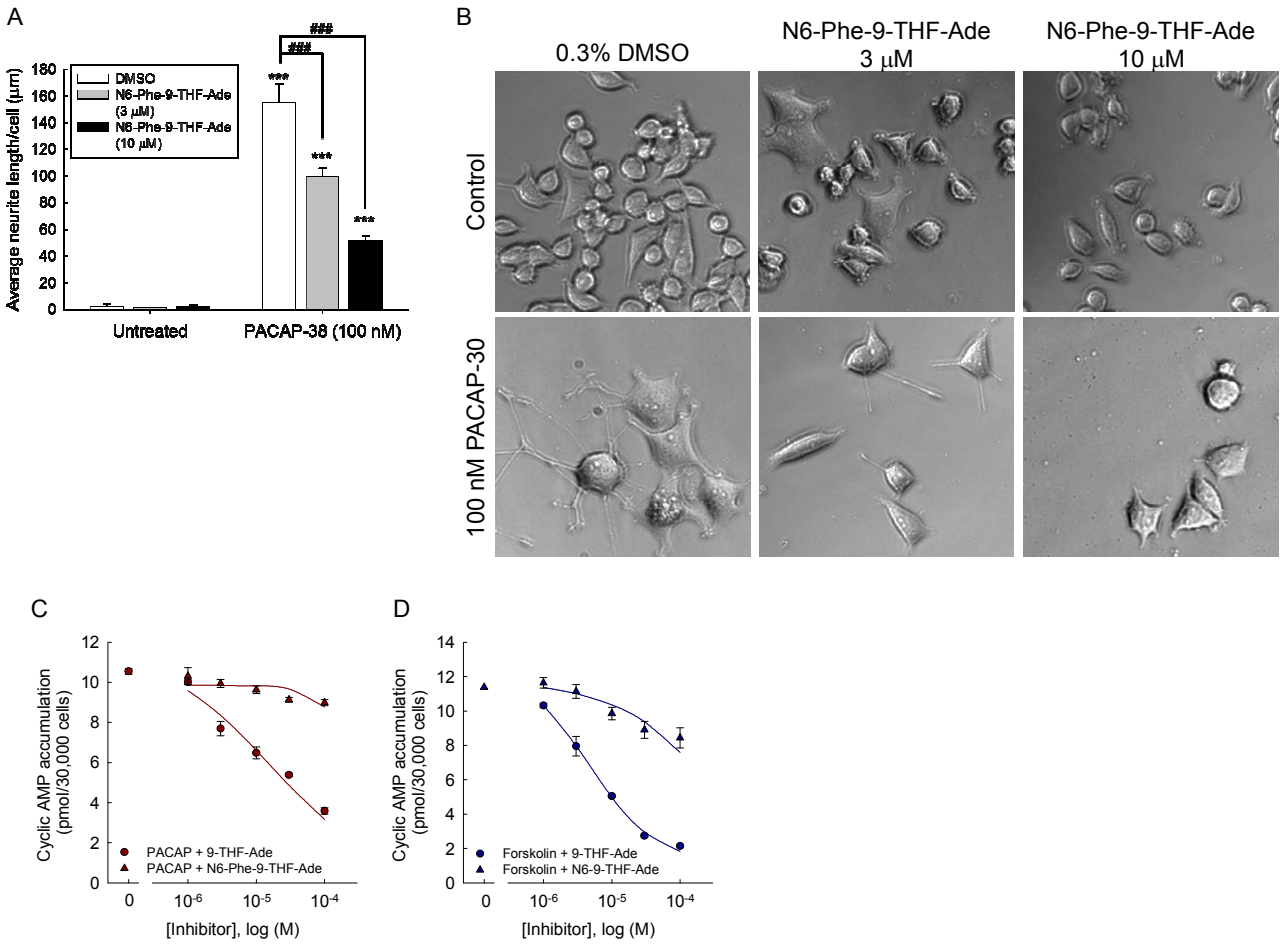


Figure 3 *N6-Phe-9-THF-Ade inhibits PACAP-dependent neurite elongation and cAMP elevation. (A) Neurite outgrowth was measured after cells were treated for 48 hours. Five images from each well were acquired, N=4. Bars correspond to means and errors represent standard errors of the mean. Data were analyzed by 2-way ANOVA followed by Bonferroni-corrected t-tests. Neurite extension promoted by PACAP was significant (***P<0.001) compared to untreated controls. 6-Phe-9-THF-Ade (either 3 or 10 µM) significantly blocked the effect of PACAP on neurite outgrowth (###P<0.001). (B) Representative phase-contrast micrographs from each condition plotted in A. D and E, measurements of intracellular cAMP in NS-1 cells pretreated for 30 min with 500 µM IBMX and either 9-THF-Adenine or N6-Phe-9-THF-Adenine or vehicle (0.3% DMSO) for 20 min. Cells were then treated with (C) PACAP-38 (3 nM) or (D) Forskolin (3 µM) for 20 min, N = 3. As determined by one-way ANOVAs, the inhibitory, 9-THF-Ade significantly inhibited the effects of both PACAP (C) and forskolin (D), while N6-Phe-9-THF-Ade did not.*

Summary and Conclusions

Our survey of the activities of some of the most commonly used cAMP analogs at each of three cAMP sensors expressed in NS-1 cells (relative potencies summarized in Table 1) has led to several conclusions. Modification to the N6 position is variably allowed by PKAR and NCS-Rapgef2 but is not permitted by Epac2. In fact, several analogs, typically used as PKA-specific ligands have equal apparent potencies at NCS-Rapgef2.

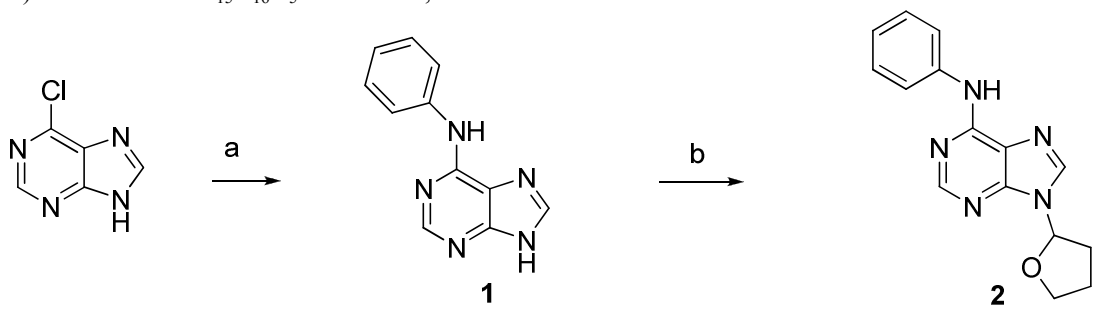
Where do we stand with specific intracellular cAMP sensor neuropharmacology that is potentially extendable to in vivo experimentation, and potentially therapeutics? As regards cAMP *agonists*, we show selectivity of N6-MBC-cAMP and Sp-5,6-DCI-cBIMPS for PKA (versus Epac and NCS-Rapgef2); selectivity of 8-CPT-2'-O-methyl-cAMP for Epac (versus NCS-Rapgef2 and PKA); and selectivity of N6-phenyl-cAMP and N6-phenyl-8-CPT-cAMP for NCS-Rapgef2 (versus PKA and Epac). As regards cAMP *antagonists*, we have established selectivity of ESI-09 for Epac (versus PKA and NCS-Rapgef2) and selectivity of N6-phenyl-9-THF-Ade for NCS-Rapgef (versus PKA and Epac). There is currently no candidate for development of a PKAR-specific inhibitor. Future development of agonists for NCS-Rapgef2 will need to focus on enhancing both the potency and the selectivity of the lead compounds N6-Phe-cAMP and N6-Phe-8-CPT-cAMP. Regarding further inhibitor development, the compound space defined by the novel adenine-based NCS-Rapgef2 inhibitor looks promising not only vis-a-vis other intracellular cAMP sensors (PKA and Epac) but also plasma membrane cAMP-gated ion channels, and the adenosine receptors, both of which have structure-activity relationships for adenine-based compounds considerably different than those defined here for the cAMP-binding domain of NCS-Rapgef2[27 28].

Development of potent and selective NCS-Rapgef2 inhibitors is likely to be of therapeutic relevance. Recent data indicates that cAMP-dependent activation of ERK in primary cortical, hippocampal and cortical rodent neurons in culture has pharmacological characteristics of NCS-Rapgef2 dependence [23, 29]. NCS-Rapgef2 mediates ERK-dependent aspects of psychomotor stimulant drug action in vivo [30]. Exploring the adenine-based structure space in which selectivity and efficacy for NCS-Rapgef2 appears greatest suggests that focusing at the eight position (likely to increase potency but without gains in selectivity) and the six position (likely to alter selectivity as well as potency) may yield compounds deployable in vivo, e.g. for inhibition of ERK in the context of psychomotor stimulant dependency, and stress-induced anxiety [31-32], to name two Gs-GPCR, ERK-dependent pathophysiological arenas of CNS neuropharmacology. In the meantime, we would propose as an urgent basic research priority deployment of N6-phenyl-9-THF-Ade in ERK-dependent Gs-GPCR signaling in vivo, in animal models.

METHODS

Drugs and reagents All reagents and solvents used for chemical synthesis were obtained from Sigma-Aldrich in the highest purity available (≥98%) and were used as purchased. Cyclic AMP analogs were made by the Biolog Life Sciences Institute (Breman, Germany) and purchased through their US distributor (Axxora). Small molecule inhibitors, forskolin, and IBMX were purchased from Tocris (Ellisville, MO). PACAP-38 was purchased from Anaspec (Fremont, CA). Solutions used for cell culture were purchased from Life Technologies (Grand Island, NY) unless otherwise specified.

Chemical synthesis. *N*-phenyl-9-(tetrahydrofuran-2-yl)-9*H*-purin-6-amine **2** was prepared in two steps based on established methods [33-34] (Figure 8). 6-Chloropurine was reacted with aniline under microwave irradiation to give the *N*-phenyl-9*H*-purin-6-amine **1** in 75%. Reaction of **1** with 2,3-dihydrofuran in the presence of *para*-toluenesulfonic acid (*p*-TSA) at reflux of acetonitrile gave the desired compound **2** in high yield (88%). Compound **2** was soluble to ≥ 100 mM in DMSO. For biological experiments, this compound was dissolved in DMSO (50 mM), from which stable aqueous working solutions were prepared. High-resolution mass spectra of **2** were acquired with electrospray ionization (Bio-Synthesis, Lewisville, TX). HRMS (ESI+): calculated for $C_{15}H_{16}H_5O$ 282.1349, measured 282.1351.



Reagents and conditions: a) Benzylamine, MW 130°C, MW 110 W, 5 min (75%); b) 2,3-Dihydrofuran. *p*-TSA, MeCN, 80 °C, 60 min (88%).

Figure 4 *Synthesis of N-phenyl-9-(tetrahydrofuran-2-yl)-9H-purin-6-amine (**2**) from 6-Chloropurine.*

Cell culture Neuroscreen-1 (NS-1) cells were cultured as described previously [20] and were used between passages four and 17 for the experiments reported here. Cells were grown in RPMI 1640 media supplemented with 10% horse serum (HyClone, Logan, UT), 5% heat-inactivated fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cultures were maintained in a humidified environment at 37 °C containing 95% air/5% CO_2. Cells grown in antibiotic-free media were routinely tested for mycoplasma.

Cell-based ELISA Phosphorylated ERK, p38, and CREB were measured using cell-based ELISA according to a protocol described previously [35]. As cAMP signaling, in NS-1 cells, is parcellated via activation of NCS-Rapgef2, Epac2, and PKA, to ERK, p38, and CREB, respectively, activation (phosphorylation) of the latter represent reliable biochemical surrogates for each of the cAMP sensors [22]. NS-1 cells were seeded at density of 3 x 10^5 per well in 96-well plates and grown overnight. Cells were treated for 30 minutes followed by removal of media and fixation in 4% formaldehyde in PBS for 20 min at room temperature. Fixed cells were permeabilized by three washes in 0.1% Triton X-100 in PBS (PBST) and endogenous peroxidase activity was quenched by a 20-minute incubation in PBS containing 0.6% H_2O_2. Following three washes in PBST, samples were blocked (10% FBS in PBST) for 1 h and incubated overnight with primary antibodies against phospho-ERK1/2 (Cell Signaling Technology #9101) at 1:500, phospho-p38 (CST, #4155) at 1:500, or phospho-CREB (CST #9198) at 1:250; at 4°C. Samples were washed three times in PBST and twice in PBS. An HRP-coupled anti-rabbit secondary antibody (CST #7074), diluted 1:500 in PBST containing 5% BSA, was added for one hour at room temperature. Following five washes in PBST, samples were exposed to the colorimetric substrate One-Step Ultra TMB-ELISA (Pierce, catalog number 34028).

After development in the dark for 10 minutes, the reaction was stopped by adding 4 M sulfuric acid, and absorbance was read at 450 nm.

Cyclic AMP measurements Cyclic AMP was measured using colorimetric cAMP Direct Biotrak EIA kits (GE, Catalog #RPN2255). Assays were performed following the protocol provided by the manufacturer without acetylating cAMP. NS-1 cells were dispensed into 96-well plates at a density of 3 x 10^5 cells per well and grown overnight. The next morning, media were aspirated and culture media containing IBMX (500 μM) and inhibitors or DMSO were added. After 30 minutes, agonists were added as 10x stocks at the indicated concentrations, followed by a 20-minute incubation at 37° C. Media were then aspirated and cells were lysed using the Novel Lysis Reagent provided by the manufacturer. Intracellular cAMP from lysates was measured by EIA conducted according the protocol provided by the manufacturer. Raw data were fit to cAMP standard curves and are expressed as pmol of cAMP per well.

High content analysis (HCA) An NS-1 cell line stably expressing enhanced GFP was generated for HCA. Cells were dispensed into collagen type I-coated 96-well plates in growth medium at a density 1 x 10^3 cells per well. Two hours after plating, cells were photographed using an inverted fluorescence microscope outfitted with motorized objectives, shutters, filters, stage, automated focusing and focus correction (TiE Eclipse, Nikon). Five pairs of phase-contrast and fluorescent images were captured per well during each acquisition. Following each acquisition, plates were returned to an incubator. Eighteen hours after plating, cells were treated as indicated. Images were then acquired every 24 hours for five days. Cell number in each field was determined by Nikon NIS-Elements High Content Software. Cell growth data were normalized to values observed in each well two hours post-plating and are expressed as a percent of cell growth over time. Neurite elongation was measured in NIS-Elements by manually tracing neurites in fields captured 48 hours following addition of agonists. Neurite outgrowth data were normalized as the ratio of neurite length per field to number of cell number per field.

Calculations and statistics SigmaPlot 11.0 (Systat, San Jose CA) was used for all calculations and statistical analysis. For dose-response experiments, curves were fit to data using 4-parameter logistic regressions. Simultaneously with curve fitting, whether or not each test compound had a statistically significant effect was determined by a one-way ANOVA. Unless otherwise specified, data from test compounds to which curves were fit had a statistically significant effect as determined by a one-tailed P value of 0.05 or less. For experiments using two or fewer concentrations of agonists and antagonists, data were analyzed by one- or two-way ANOVA followed by Bonferroni-corrected t-tests. Statistical tests were non-directional (two-tailed) and $P < 0.05$ was the criteria for statistical significance.

Supporting Information

Supplemental Materials and Methods and Supplemental figures S1-S3

Abbreviations

Ade, Adenine; AC, Adenylate cyclase; Bn, Benzoyl; Br, Bromo; Bz, Benzyl; cAMP, Cyclic adenosine 3′,5′-monophosphate; cAMPS, cyclic adenosine-3′,5′ monophosphorothioate; CREB, Cyclic AMP-responsive element-binding protein; CPT, 4-Chlorophenylthio; ERK, Extracellular signal–regulated kinase; Epac, Exchange protein activated by cyclic AMP; ESI-09, α-[(2-(3-Chlorophenyl)hydrazinylidene]-5-(1,1-dimethylethyl)-β-oxo-3-isoxazolepropanenitrile; GEF, Guanine nucleotide exchange factor; H89, N-[2-[[3-(4-Bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride; MAPK, Mitogen-activated protein kinase; MBC, mono-t.butylcarbamoyl; NCS-Rapgef2, Neuritogenic cyclic AMP sensor-protein product of the Rapgef2 gene; NS-1, Neuroscreen-1; p38, p38 Mitogen-activated protein kinases α, β, γ, and δ; PACAP, Pituitary adenylate cyclase-activating polypeptide; Phe, Phenyl; PKA, Protein kinase A; SB 239063, trans-4-[4-(4-Fluorophenyl)-5-(2-methoxy-4-pyrimidinyl)-1H-imidazol-1-yl]cyclohexanol; SQ22,536, 9-(Tetrahydro-2-furanyl)-adenine; THF, Tetrahydro-2-furanyl; U0126, 1,4-Diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene

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Author Contributions

A.C.E. and L.E.E. designed experiments and wrote the manuscript. W.X. and M.V.E. designed and created new molecular & cell biological reagents used in this work. A.C.E., R.A.A., and W.X. performed experiments. F.S. designed and synthesized novel compounds reported herein. L.E.E. oversaw the project.

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Conflict of Interest

The authors declare no competing financial interests.

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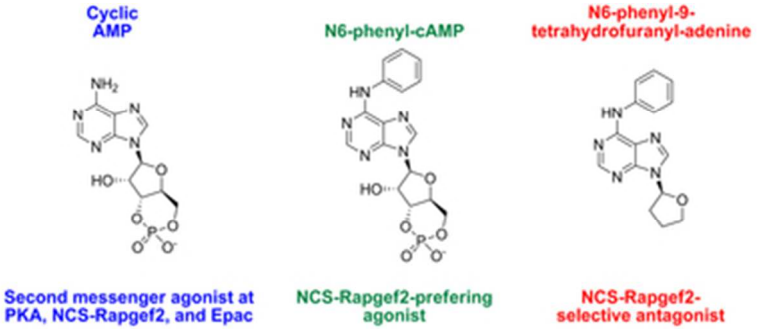

For Table of Contents Use Only: Graphical abstract

Differential Pharmacophore Definition of the cAMP Binding Sites of NCS-Rapgef2, PKA and Epac in Neuroendocrine Cells Using an Adenine-based Scaffold

Andrew C. Emery, Ryan A. Alvarez, Maribeth V. Eiden, Wenqin Xu, Fabrice G. Simeon, and Lee E. Eiden

34x13mm (300 x 300 DPI)