Signaling through the neuropeptide GPCR PAC₁ induces neuritogenesis via a single linear cAMP- and ERK-dependent pathway using a novel cAMP sensor

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ABSTRACT Both cAMP and ERK are necessary for neuroendocrine cell neuritogenesis, and pituitary adenylate cyclase-activating polypeptide (PACAP) activates each. It is important to know whether cAMP and ERK are arranged in a novel, linear pathway or in two parallel pathways using known signaling mechanisms. Native cellular responses [cAMP elevation, ERK phosphorylation, cAMP responsive element binding (CREB) phosphorylation, and neuritogenesis] and promoter-reporter gene activation after treatment with forskolin, cAMP analogs, and PACAP were measured in Neuroscreen-1 (NS-1) cells, a PC12 variant enabling simultaneous morphological, molecular biological, and biochemical analysis. Forskolin (25 μM) and cAMP analogs (8-bromo-cAMP, dibutyryl-cAMP, and 8-chlorophenylthio-cAMP) stimulated ERK phosphorylation and neuritogenesis in NS-1 cells. Both ERK phosphorylation and neuritogenesis were MEK dependent (blocked by 10 μM U0126) and PKA independent (insensitive to 30 μM H-89 or 100 nM myristoylated protein kinase A inhibitor). CREB phosphorylation induced by PACAP was blocked by H-89. The exchange protein activated by cAMP (Epac)-selective 8-(4-chlorophenylthio)-2′-O-Me-cAMP (100-500 μM) activated Rap1 without affecting the other cAMP-dependent processes. Thus, PACAP-38 potently stimulated two distinct and independent cAMP pathways leading to CREB or ERK activation in NS-1 cells. Drug concentrations for appropriate effect were derived from control data for all compounds. In summary, a novel PKA- and Epac-independent signaling pathway: PACAP → adenylate cyclase → cAMP → ERK → neuritogenesis has been identified.—Emery, A. C., Eiden, L. E. Signaling through the neuropeptide GPCR PAC₁ induces neuritogenesis via a single linear cAMP- and ERK-dependent pathway using a novel cAMP sensor. FASEB J. 26, 3199–3211 (2012). www.fasebj.org

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Until recently, the only known pathway for cAMP signaling in the nervous system was via activation of PKA, which has been shown to regulate synaptic plasticity, memory consolidation, appetitive learning, and, at the molecular level, multiple neurotrophin, cytoskeletal, and ion channel proteins and transcription of their cognate genes (1, 2). PKA-independent cAMP-regulated pathways, however, are slowly being reported, and their functional importance in neuronal signaling is being recognized (3). We previously reported that a neuropeptide-activated noncanonical cAMP pathway mediates morphological differentiation in neuroendocrine cells (4, 5) and enhanced gene transcription in neurons (6). In each case, ERK phosphorylation appears to be an obligate component of the overall neuropeptide-initiated signaling event (4–6). It remains unclear whether neuropeptide and GPCR signaling through cAMP and ERK are complementary pathways converging on a downstream target or whether cAMP activation of ERK is a feature of this type of signaling.

Numerous reports support the involvement of both cAMP and ERK in differentiation and neuritogenesis in neuroendocrine and neuronal cells in response to GPCR ligands, such as pituitary adenylate cyclase-activating polypeptide (PACAP) and other neurotrophic molecules (5, 7–9). However, there are competing mechanisms proposed for how this occurs (10). In PC12 cells, cAMP and ERK were found to be linked in a single signaling pathway, which requires phosphory-
lation of the tyrosine kinase Src by PKA, which leads to Rap1 activation and ERK phosphorylation (11). Although this pathway appears to mediate regulation of some neuronal-specific genes, such as transin (12), PC12 cell neuritogenesis is clearly PKA independent (5, 13–15). Another possible linear signaling pathway connecting cAMP and ERK involves PKA-independent Rap1 activation via a cAMP-guanine nucleotide exchange factor (GEF; refs. 9, 16). However, the laboratories of both Bos (17) and Stork (18) have shown that the best-characterized cAMP-GEF, exchange protein activated by cAMP (Epac), is incapable of activation of ERK in neuroendocrine cells because of its cytosolic intracellular localization. In contrast, known pathways for GPCR-mediated activation of ERK require β-arrestins rather than cAMP (19) and would therefore be candidates for a mechanism of neuritogenesis in which ERK activation in parallel with cAMP-signaling, perhaps via Epac or a related cAMP-GEF, results in neurite formation. The aim of the current study was to assess both the nature of the cAMP sensor required and whether cAMP and ERK are arranged as serial components in a single pathway, leading to neuritogenesis in neuroendocrine cells.

MATERIALS AND METHODS

Cell culture

Neuroscreen-1 (NS-1) cells, purchased from Cellomics (Pittsburgh, PA, USA), were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA), which was supplemented with 10% equine serum (HyClone, Logan, UT, USA), 5% heat-inactivated fetal bovine serum (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were grown at 37°C in 95% air and 5% CO₂. Cells were grown in plates (BD Falcon, Billerica, MA, USA) to a density of 6500 cells/cm². All plates were coated with collagen I from rat tail (Sigma-Aldrich, St. Louis, MO, USA), which was diluted in 0.02 M glacial acetic acid to 50 μg/ml, and 0.1 ml/cm² of this solution was applied to plates for 1 h. Coated plates were then washed twice with 0.4 ml/cm² sterile PBS and dried before use.

Drugs

PACAP38 was purchased from Phoenix Pharmaceuticals (Mountain View, CA, USA). Forskolin and nerve growth factor (NGF) were obtained from Sigma-Aldrich. Cholera toxin (CTx; NaN₃-free) and inhibitors H-89, KT 5729, myristoylated PKA inhibitor 14-22 amide (PKI), U0126, PD98059, and toxins of both Bos (17) and Stork (18) have shown that the best-characterized cAMP-GEF, exchange protein activated by cAMP (Epac), is incapable of activation of ERK in neuroendocrine cells because of its cytosolic intracellular localization. In contrast, known pathways for GPCR-mediated activation of ERK require β-arrestins rather than cAMP (19) and would therefore be candidates for a mechanism of neuritogenesis in which ERK activation in parallel with cAMP-signaling, perhaps via Epac or a related cAMP-GEF, results in neurite formation. The aim of the current study was to assess both the nature of the cAMP sensor required and whether cAMP and ERK are arranged as serial components in a single pathway, leading to neuritogenesis in neuroendocrine cells.

Neurite outgrowth assay

NS-1 cells were plated in 6-well plates and treated 24 h later. After 48 h of treatment, images of cells were randomly acquired on a computer-assisted microscope (NIS-Elements BR; Nikon, Tokyo, Japan) using a ×20 objective. Cells and neurites were counted, and the length of the neurites was measured in each field. Neurites were defined as cell processes > 6 μm to eliminate inadvertent inclusion of cell membrane ruffling or irregularities as neurites. Data from neuritogenesis assays are expressed as average neurite length per cell.

Measurement of cAMP

Accumulated intracellular cAMP was assayed using the cAMP Biotrak enzyme immunoassay kit (Amersham Biosciences, Piscataway, NJ, USA). The day after NS-1 cells were plated in 96-well plates, cells were pretreated for 20 min in medium containing 0.5 mM 3-isobutyl-1-methylxanthine to inhibit endogenous phosphodiesterases with and without 100 μM adenylate cyclase (AC) inhibitor ddAd. After pretreatment, cells were challenged with agonists for 20 min at 37°C. Cells were then lysed in the buffer provided, and cAMP levels were measured using the nonacetylation protocol provided by the manufacturer. Each plate included standards, and data were fit to standard curves using 3-parameter logistic regressions in SigmaPlot 11.0 (Systat Software, San Jose, CA, USA).

cAMP responsive element binding (CREB) activation assay

CREB activation was measured using the PathDetect CREB Trans-Reporting System luciferase assay (Agilent Technologies, Santa Clara, CA, USA). NS-1 cells, cultured in 48-well plates, were transiently transfected with the reporter plasmid pFR-Luc (200 ng) and fusion transactivator plasmid pFA2-CREB (10 ng) using Lipofectamine 2000 (Invitrogen). The next day, cells were pretreated with indicated PKA inhibitors for 30 min and stimulated with agonists for 6 h, after which cells were washed with PBS and lysed in the buffer included. In opaque 96-well plates, 20-μl samples of lysates were added to 100 μl of the luciferase assay reagent provided, and light emission was immediately measured using a Victor3 microtiter plate reader (PerkinElmer, Waltham, MA).

Western blots

NS-1 cells, grown in 6-well plates, were treated as indicated and lysed in a buffer containing 1% Triton X-100, 50 mM Tris-HCl, 10 mM EDTA, and Halt protease and phosphate inhibitor cocktails (Pierce Biotechnology, Rockford, IL, USA). The amount of protein for each sample was determined using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (20 μg) were added to a solution containing 1× NuPAGE LDS Sample Buffer and 1× NuPAGE Reducing Reagent (Invitrogen), which were then heated for 5 min at 95°C and microcentrifuged. Proteins were separated by SDS-PAGE (120 V for 90 min) on 4–12% Novex Bis-Tris gels (Invitrogen), which were electrophoretically transferred (30 V for 90 min) to nitrocellulose membranes with 0.45-μm pores (Invitrogen). Membranes were incubated in blocking buffer containing 2% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 3 h at room temperature. After blocking, blots were incubated overnight at 4°C with a 1:1000 dilution of rabbit polyclonal antibody specific for phosphorylated p44/42 MAP kinase (ERK), total p44/42 MAP kinase (ERK), phospho-CREB (Ser133), or total CREB (Cell Signaling Technology, Beverly, MA, USA). After
5 washes in TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 in blocking buffer). After 5 washes in TBST to remove unbound secondary antibody, membranes were incubated with SuperSignal West Pico Chemiluminescence Substrate (Pierce Biotechnology), and immunoreactive bands were visualized by a charge-coupled device camera. Bound phospho-p44/42 MAP kinase or phospho-CREB antibodies were removed with Restore Western Blot Stripping Buffer (Pierce) before incubation of the same blot with either total p44/42 MAP kinase or total CREB antibodies. Images were quantified using ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA; http://imagej.nih.gov/ij/).

Rap1 activation assay

Rap1 activation was measured using the active Rap1 pulldown and detection kit (Pierce Biotechnology) according to the manufacturer’s instructions. The day after plating, NS-1 cells were treated for 5 min and lysed using the buffer provided. Samples containing 500 μg of protein were subjected to affinity purification by incubation for 1 h at 4°C in a solution of 20 μg of GST-RalGDS-RBD in slurry containing 50% glutathione resin. In parallel, 500-μg samples were not subjected to affinity purification and served as an index of total Rap1 levels. Samples were then centrifuged through the spin cups provided, washed 3 times with lysis buffer, dissolved in reducing sample buffer, vortexed, and boiled for 5 min. Affinity-purified and control samples were analyzed by Western blots, which were probed with an antibody against Rap1A/B at a dilution of 1:1000 (Upstate Biotechnology, Lake Placid, NY, USA).

RT-PCR analysis of PACAP receptor expression

Total RNA was harvested from NS-1 cells cultured in 6-well plates using the RNAqueous Kit (Ambion, Foster City, CA, USA) following the manufacturer’s protocol. RNA samples were treated with RNase-free DNase I (Invitrogen), and 1-μg samples were reverse-transcribed the SuperScript II Reverse Transcriptase (Invitrogen). PACAP receptors were amplified from the resultant cDNA using AmpliTaq Gold DNA Polymerase (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s protocol and the following primers: PAC1, forward 5′-TCAAAGGCCCCGTGGTGGC-3′ and reverse 5′-GCCCGCTGTGGCTTGCAGTA-3′; VPAC1, forward 5′-GCCACATGCAGCACGCAGGTA-3′ and reverse 5′-TGTGGAGAGGGTGTGAAGGGGC-3′; VPAC2.

Figure 1. cAMP-elevating and mimicking agents are neuritogenic in NS-1 cells. A) Representative photomicrographs of cells treated with CTX (50 μg/ml), forskolin (25 μM), and db-cAMP (500 μM) in the absence or presence of 100 μM ddAd. Scale bars = 20 μm. CTX, forskolin, db-cAMP, and 8-Br-cAMP all caused significant increases in neurite length compared with that in vehicle-treated control cells (ANOVA, *P < 0.01). Bonferroni post hoc tests revealed that ddAd (100 μM) only significantly inhibited the effects of CTX and forskolin. Data are expressed as the average length of all neurites from each cell from 3 independent experiments; error bars = SEM. B) Inhibition of forskolin-induced CAMP by ddAd. Bars represent triplicate determinations from 3 independent experiments (average ± SEM). Forskolin caused a significant increase in CAMP levels (ANOVA), and ddAd significantly inhibited the effect of forskolin (Bonferroni). ***P < 0.001.
forward 5’-ACTGCTGGTTGCTGGTGCGG-3’ and reverse 5’-TCCCCAATGTCTGCAGGGCG-3’; and GAPDH, forward, 5’-GTACAGGGCTGGCCTCTC-3’ and reverse 5’-GGGTTTCCCCGGTATGACC-3’. Thermal cycling conditions were as follows: 5 min at 95°C, followed by 40 cycles at 95°C (30 s), 60°C (45 s), and 72°C (45 s), followed by a final extension at 72°C for 7 min. Amplicons were separated and imaged using 2% agarose/TAE gels containing 0.5 μg/ml ethidium bromide.

Calculations and statistics

All calculations and statistics were performed in SigmaPlot 11.0. For significance testing between multiple groups, 2-way ANOVA was used, followed by Bonferroni-corrected t tests. In dose-response experiments, all data were fit using 4-parameter logistic regressions. Statistical significance was deemed at P < 0.05.

RESULTS

cAMP-initiated neurite extension is noncanonical in NS-1 cells

We first established that NS-1 cells respond not only to NGF (20) but also to cAMP and compounds that mimic or elevate intracellular cAMP, by extending neurites characteristic of differentiation as previously reported in PC12 cells. Multiple neurites of ≥1 cell diameter in length were formed with 48 h of treatment after ADP-ribosylation of Gα by CTX (50 μg/ml), direct stimulation of AC with forskolin (25 μM), or exposure to the cAMP-generating compound db-cAMP or to the lipophilic cAMP analog 8-Br-cAMP (Fig. 1A). The neuritogenic effects of CTX and forskolin were blocked by the AC inhibitor ddAd (100 μM). Neurite extension elicited by either db-cAMP or 8-Br-cAMP was unaffected by ddAd, as expected from its reported specificity for inhibition of AC. The pharmacological efficacy of ddAd as an AC inhibitor is shown in Fig. 1B; at the dose at which ddAd blocked forskolin-induced neuritogenesis, it also caused an ~90% inhibition of forskolin-stimulated cAMP accumulation.

Pretreatment of NS-1 cells with the PKA inhibitors H-89, KT5720, or PKI failed to block forskolin-elicited neurite extension (Fig. 2A), suggesting that in NS-1 cells, as reported for PC12 cells, cAMP-dependent neuritogenesis does not require catalytic activation of PKA. Reports that cAMP induction of neuritogenesis is independent of PKA have not typically included con-

![Figure 2. Neuritogenesis is a PKA-independent process in NS-1 cells. A) Representative photomicrographs of cells treated with forskolin (25 μM) ± H-89 (30 μM). Scale bars = 20 μm. Quantification of 3 assays in which NS-1 cells were treated with forskolin (25 μM) ± H-89 (30 μM), KT5720 (30 μM), and PKI (100 nM). Forskolin caused a significant increase in neurite length in each condition (Bonferroni post hoc tests). Bars represent the average ± sem neurite length per cell from ≥3 independent experiments. B) CREB reporter gene assays. Treatment for 6 h with forskolin (25 μM) significantly increased luminescence generated by a reporter gene responsive to a transfected Gal4/CREB fusion protein (ANOVA, P < 0.001). Bonferroni post hoc tests indicated that H-89, KT5720, and PKI decreased the effect of forskolin in a concentration-dependent manner. Bars represent triplicate determinations from 3 independent experiments (average ± sem). **P < 0.01, ***P < 0.001.]
vincing demonstration that the inhibitors of PKA used were either effective or specific. We therefore titrated the concentrations of extracellularly applied PKA inhibitors H-89, KT5730, and PKI to provide doses affording >90% inhibition of endogenous PKA, as measured in NS-1 cells that were transiently transfected with a Gal4/CREB fusion protein and a reporter gene responsive to PKA-dependent Gal4/CREB fusion protein phosphorylation and transactivation (4), under culture conditions identical to those in which forskolin-induced neuritogenesis was measured. As seen in Fig. 2B, after 6 h of treatment with forskolin, CREB activation was partially inhibited by 10 μM H-89 and KT5720 and 30 nM PKI, whereas it was completely inhibited by 30 μM H-89 and KT5720 or 100 nM PKI, demonstrating that all three of these compounds effectively inhibit PKA in NS-1 cells at the concentrations used in the neuritogenesis assays in Fig. 2A.

Together, these data indicate that the signaling pathway for cAMP-induced neuritogenesis in NS-1 cells is noncanonical (PKA independent). To assess whether Epac may be the cAMP sensor responsible for neuritogenesis, we used 007, an Epac-selective cAMP analog (17) as a potential activator of neuritogenesis. As seen in Fig. 3A, 007 had no effect on neuritogenesis at either 100 or 500 μM, whereas the structurally related, yet nonselective cAMP analog 8-CPT-cAMP (100 μM) caused neurite extension to a similar extent as forskolin (25 μM). We performed positive control experiments for the pharmacological efficacy of 007 as an activator of Epac in NS-1 cells. Rap1 is a GTP-binding protein activated by Epac-dependent signaling but not by neurotrophin (i.e., NGF) signaling in PC12 cells (17). We therefore tested the efficacy of 007 to activate Rap1 to confirm that 007 crosses the plasma membrane and is pharmacologically active in NS-1 cells. Treatment with 007 (100 μM) but not NGF for 5 min caused a substantial increase in activated GTP-bound Rap1, indicating that although the pathway for Epac-dependent Rap1 activation exists in NS-1 cells, it is not required for cAMP-dependent neuritogenesis.

We next tested the hypothesis that cAMP-dependent neuritogenesis in NS-1 cells is ERK dependent. In Fig. 4A, treatment with the MEK1 inhibitor PD98059 (30 μM) and MEK1/2 inhibitor U0126 (10 μM) completely blocked forskolin-induced neuritogenesis. As seen in Fig. 4B, cAMP-elevating agents CTX (50 μg/ml) and forskolin (25 μM) caused a robust increase in ERK phosphorylation, and ERK phosphorylation due to forskolin treatment was sustained for ≥3 h. Treatment with forskolin (25 μM) caused an increase in ERK phosphorylation measured after 10 min of stimulation (Fig. 4C). As expected, forskolin-induced ERK phosphorylation was blocked by the MEK1/2 inhibitor U0126 (10 μM) and the MEK1 inhibitor PD98059 (30 μM). Forskolin-induced ERK phosphorylation was also inhibited by pretreatment with 100 μM ddAd (Fig. 4C), indicating that forskolin-induced ERK phosphorylation is dependent on AC, thus establishing that cAMP and ERK are arranged in the same signaling pathway. Consistent with our data showing that forskolin-induced neuritogenesis is PKA independent, forskolin-induced ERK phosphorylation was not inhibited by H-89 (30 μM), KT5720 (30 μM), or PKI (100 nM). Taken together, these results indicate that AC and ERK are connected in a single noncanonical signaling pathway in NS-1 cells, which leads to neuritogenesis, and that the known cAMP sensors PKA and Epac are not elements of the noncanonical pathway.

Figure 3. Treatment with the Epac agonist 007 activates Rap1 without inducing neurite outgrowth. A) Representative photomicrographs and quantification from neurite outgrowth assays (n=3). NS-1 cells were treated with 007, 8-CPT-cAMP (8-CPT), or forskolin. Scale bars = 20 μm. 8-CPT-cAMP and forskolin caused significant neurite extension relative to that in vehicle-treated controls (Bonferroni, P<0.001). At either concentration tested, 007 did not cause significant neuritogenesis relative to that in untreated controls. B) Measurements of Rap1-GTP. Duplicate samples were treated with NGF (100 ng/ml), 8-CPT-cAMP (8-CPT, 100 μM), or 007 (100 μM) for 5 min, and activated, GTP-bound Rap1 was affinity-purified from lysates. Basal refers to lysates from untreated control cells, and input refers to 20 μg of protein, which was not subjected to affinity purification. Top band corresponds to Rap1A, bottom band to Rap1B.

PACAP38 is a GPCR-activating first messenger for noncanonical cAMP signaling leading to ERK activation in NS-1 cells

An obvious first-messenger candidate for initiating cAMP-dependent signaling for neuritogenesis in NS-1 cells is the neuropeptide PACAP, which in PC12 cells stimulates neuritogenesis and differentiation distinct from that caused by NGF (14, 21, 22). As seen
in Fig. 5A, PACAP potently stimulated cAMP accumulation (EC₅₀ = 17.31±3.31 nM) in NS-1 cells. PACAP-induced cAMP elevation was inhibited by pretreatment with ddAd (100 µM), which significantly reduced the maximal effect of PACAP from 84.1 ± 4.7-fold over basal cAMP levels to 6.7 ± 4.1-fold over basal. Vasoactive intestinal peptide (VIP), highly homologous to PACAP, also stimulated cAMP accumulation, but was ~50 times less potent than PACAP38 (EC₅₀ = 863.89±53.03 nM), consistent with the rank order of potency of these two peptides at the PAC₁ receptor.

We used RT-PCR to confirm that NS-1 cells express only mRNA encoding PACAP-preferring PAC₁ recep-
tor and not mRNA encoding the VPAC₁ and VPAC₂ receptors that recognize PACAP and VIP with similar affinity. Because all three of these receptors are expressed in the brain, cDNA from rat brain was used as a positive control for all RT-PCR amplifications. As seen in Fig. 5B, PAC₁ receptor-encoding mRNA, but not VPAC₁ or VPAC₂ receptor-encoding mRNA, was readily detected by RT-PCR in NS-1 cells. In the same experiments, transcripts from all three receptors were amplified in cDNA from rat brain, indicating that unlike in brain, the PAC₁ receptor is the only receptor to which PACAP binds that is expressed at detectable levels in NS-1 cells. PACAP, like forskolin, caused a robust increase in ERK phosphorylation, which was sustained for ≥3 h (Fig. 5C), and its effects were, like those of forskolin, sensitive to inhibition by ddAd (Fig. 5D). These findings rule out the possibility that PACAP activation of ERK and elevation of cAMP are parallel and independent signaling events and demonstrate instead that ERK phosphorylation stimulated by PACAP is downstream of and is a direct consequence of elevation of cAMP.

We further confirmed that PACAP-induced ERK-dependent neuritogenesis occurs through the same PKA-independent pathway as that stimulated by forskolin. As seen in Fig. 6A, ERK phosphorylation due to treatment with PACAP (100 nM) was not significantly inhibited by pretreatment with PKA inhibitors H-89 (30 μM), KT5720 (30 μM), or PKI (100 nM), suggesting PACAP-induced ERK phosphorylation is also a PKA-independent process. In the same experiments, PD98059 (30 μM) and U0126 (10 μM) completely blocked PACAP-induced ERK phosphorylation (Fig. 6A). Finally, PACAP caused a dose-dependent neuritogenic effect (Fig. 6B) that was blocked by ddAd (100 μM), U0126 (10 μM), and PD98059 (30 μM) (Fig. 6C) and was insensitive to the PKA inhibitors H-89 (30 μM), KT5720 (30 μM), and PKI (100 nM) (Fig. 6C).

Mechanism for PACAP elevation of cAMP in NS-1 cells

PACAP-selective PAC₁ receptors couple not only to G₃, leading directly to activation of AC, but also to Gq, which generates PLC-dependent diacylglycerol release, phosphotidylinositol hydrolysis, calcium mobilization, and PKC phosphorylation. According to a pathFinder query for pathways linking G₃ and AC (23), activation of Gq can lead via PLC to elevation of cAMP through calcium-dependent stimulation of AC1, AC3, and AC8. Furthermore, activation of ERK by PKC can occur via Gq-coupled PLC activation. To determine whether either of these two Gq-coupled pathways influences PACAP signaling in neuritogenesis, two sets of experiments were performed. First, we measured neuritogenesis due to treatment with PACAP38 (100 nM) in the presence of inhibitors of either PLC or PKC activation. Blockade of PLC with U73122 (10 μM) or inhibition of PKC with chelerythrine (5 μM), Go6976 (10 μM), or Go6983 (10 μM) did not significantly decrease the neuritogenic ef-
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The effects of PACAP38 (Fig. 7A). The PKC activator PMA (100 nM) failed to significantly induce neuritogenesis (Fig. 7A). In control experiments to the test the efficacy of Go6976 (10 μM), Go6983 (10 μM), and chelerythrine (5 μM) to inhibit PKC in NS-1 cells, we measured phorbol ester PMA-induced ERK phosphorylation, which is a PKC-dependent process (24).

As seen in Fig. 7B, Go6976 (10 μM), Go6983 (10 μM), and chelerythrine (5 μM) all completely inhibited ERK phosphorylation due to treatment with PMA (100 nM), indicating that all of these compounds are effective inhibitors of PKC in NS-1 cells (Fig. 7B). Thus, PACAP signaling to ERK is probably mediated directly through Gs coupling to AC, and
this pathway is uniquely insulated from G_q-mediated signaling in NS-1 cells.

**PACAP activates both canonical and noncanonical cAMP signaling pathways**

The pleiotropic cAMP-dependent effects of PACAP in mediating neuroprotection, tumor suppression, gene expression, synaptic plasticity, and stress signaling throughout the nervous system begs the question of whether PACAP activation of PKA-dependent and PKA-independent signaling occurs within a single cell type. We therefore examined the characteristics of PKA-dependent cAMP signaling in NS-1 cells. Treatment with PACAP (100 nM) for 6 h caused an elevation of CREB activation to 49 ± 11% of basal, which was inhibited in a dose-dependent manner by PKA inhibitors H-89 (IC_{50} = 12.65 ± 0.44 μM), KT5720 (IC_{50} = 17.42 ± 0.38 μM), and PKI (30 nM) (Fig. 8A).

As seen in Fig. 8B, we obtained results similar to those for the CREB reporter gene assays by measuring endogenous phosphorylated CREB (Ser133) by Western blot. In triplicate experiments, we confirmed that PACAP-induced CREB phosphorylation was blocked by ddAd (100 μM), H-89 (30 μM), KT5720 (30 μM), and PKI (100 nM), demonstrating that PACAP-induced CREB phosphorylation is AC- and PKA-dependent. Furthermore, U0126 (10 μM) and PD98059 (30 μM) failed to inhibit PACAP-induced CREB phosphorylation (Fig. 8B), demonstrating that PACAP-induced CREB phosphorylation is a MEK-independent process.

Taken together, our data suggest that in NS-1 cells, PACAP stimulates cAMP elevation through G-protein-mediated activation of AC, stimulating both canonical PKA-dependent signaling, which results in CREB phosphorylation, and noncanonical PKA-independent cAMP signaling, which causes ERK phosphorylation and neuritogenesis (Fig. 9).

**DISCUSSION**

Our major finding is the existence of a cAMP sensor, termed here the neuritogenic cAMP pathway in NS-1 cells. Furthermore, our data indicate that cAMP elevation in neuroendocrine cells can potentially activate at least 3 separate cAMP sensors (PKA, Epac, and the neuritogenic cAMP sensor), which are dedicated to functionally insulated downstream targets. There is as yet no reason to suppose that Gs-coupled first-messenger activation of the pathway is unique to PACAP acting at the PAC1 receptor nor that the function of this sensor is restricted to mediation of morphological alterations of neuroendocrine cells. However, PACAP is a potent dendritic and axonal neuronal morphogen, whose actions are in many cases mimicked by cAMP (26–28). The neuritogenic cAMP
sensor characterized here may participate in some of these actions of PACAP on central and peripheral neurons and developing neuroblasts.

In addition to its roles in neuritogenesis and other morphological changes related to neuronal development and plasticity, PACAP-induced cAMP signaling has been shown to regulate the transcription of numerous target genes. We have recently reported that in cortical neurons, PACAP promotes signaling that triggers the expression of stanniocalcin 1, a putative endogenous neuroprotective protein in brain (6), through a pathway that is pharmacologically consistent with linear activation of ERK through cAMP, as described here for PACAP-stimulated neuritogenesis in NS-1 cells. In fact, the actions of secretin family neuropeptide ligands, of which PACAP is one, are important in an array of physiological contexts and are remarkably protean. PACAP and VIP are both important first messengers in control of medulloblastic cancer (29), immune encephalitides (30), acquired immunity (31), rheumatoid arthritis (32), and brain development (33). PACAP signaling specifically through the PAC1 receptor has been implicated in cerebellar and sympathetic neuronal development (34, 35), modulates cerebellar and hippocampal long-term potentiation (36, 37), and reduces tissue damage and motor deficits caused by ischemic stroke (38–40). In PC12 cells, PACAP has been shown to regulate 3 distinct sets of transcripts that underlie cell adhesion, viability, and differentiation (41). Other examples of regulatory functions of PACAP acting through the PAC1 receptor in vivo include anxiogenesis (42), activation of the hypothalamic-pituitary-adrenal axis by psychogenic stress (43), locomotor behavior (44), associative learning (36), and circadian function (45).

The existence of multiple cAMP-dependent pathways linked to activation of the PAC1 receptor may help to explain how PACAP signaling can be both highly specific and ubiquitous throughout mammalian organisms. For example, PACAP-dependent interdiction in sonic hedgehog signaling associated with medulloblastoma is mediated through PKA modulation of gli1 gene expression (46), as is PACAP signaling to fos, which promotes the activity-dependent survival of cerebellar granule cells (47). On the other hand, PKA is not likely to be involved in PACAP-mediated protection of cerebellar granule cells from neurotoxic insult by ceramide, which is mimicked by forskolin but not blocked by H-89 (48), or in potassium channel regulation in cerebellar granule cells, in which Epac involvement has been suggested (49). Likewise, in SH-SY5Y human neuroblastoma cells, PACAP induction of Bcl2 and GAP-43 exhibited differential resistance to inhibition by H-89, implying that multiple cAMP sensors might also exist in these cells (9).

The number and variety of PKA-dependent and PKA-independent events initiated by PACAP in both neuronal and non-neuronal cells in culture and in vivo referenced above are consistent with the existence of multiple cAMP-dependent functions for PACAP, possibly even within the same cell. Nevertheless, the present finding that multiple cAMP pathways initiated by PACAP are highly insulated from one another was a striking one, especially in light of the fact that cAMP has been shown to simultaneously stimulate PKA and Epac, generating either additive or opposing downstream effects depending on the cell type. A demonstration of PKA and Epac functional cooperation is seen in immortalized human smooth myocytes, where the maximal release of inflammatory IL-8 due to treatment with the cAMP-stimulating neuropeptide bradykinin required both PKA and Epac (50). In contrast, PKA and Epac exert opposing signaling roles in cortical neurons, where they act to inhibit (PKA) or enhance (Epac) phosphorylation of the prosurvival kinase Akt (51). In the current study, PACAP-initiated CREB phosphorylation and neuritogenesis are virtually exclusively PKA-dependent and ERK-independent or PKA-independent and ERK-dependent, respectively, in NS-1
cells. These observations are however quite consistent with the findings of other laboratories that activation of Rap1 by Epac and PKA occurs in noncommunicating cellular compartments, one near the nuclear envelope and one at the plasma membrane (17, 18). This result suggests that, like Epac, the neuritogenic cAMP sensor occupies a cellular location that is specific to its signaling tasks in neuroendocrine cells.

Regarding its biochemical characteristics, the neuritogenic cAMP sensor must be a signaling molecule that includes a cAMP-binding domain and at least one additional domain with a signaling function. The best candidates for the sensor, based on bioinformatic analysis of proteins bearing cAMP-binding domains, include members of cyclic nucleotide GEF family other than Epac1/2 and CNrasGEF, which is neither selectively responsive to cAMP nor insulated from the downstream ras signaling pathway (52). Another possibility is that rather than activating a single molecule, cAMP may activate a signaling pathway via MAPK phosphatase inhibition/ERK activation (53). However, in PC12 cells, cAMP in fact up-regulates MAPK phosphatase-1 expression (54), predicting inhibition rather than enhancement of ERK1/2 kinase activity. PKA inhibitors such as H-89 inhibit the kinase activity of PKA (55) without affecting the conformation change that occurs to the regulatory subunit on cAMP binding. Thus, an additional possibility is that the regulatory subunit of PKA, independently from the catalytic subunit, may act as a signaling molecule once bound to cAMP, and, in fact, some data indicate that regulatory subunits of PKA can influence cancer cell proliferation and viability (56). Whether the neuritogenic cAMP sensor is a GEF or whether it activates additional kinases or phosphatases, the demonstration of its existence and direct activation of ERK1/2 in NS-1 cells suggest that successful characterization will eventually afford new opportunities for therapeutic development, including serving as a novel target for rational drug design.

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