Separate cyclic AMP sensors for neuritogenesis, growth arrest and survival of neuroendocrine cells

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Running Title: Parcellation of cAMP signaling for differentiation

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Background: Three cAMP sensors (PKA, Epac1/2 and NCS/Rapgef2) co-exist in neuroendocrine cells: their roles in differentiation require elucidation.

Results: Epac2, PKA and NCS/Rapgef2 independently gate signaling for growth arrest, cell survival, and neuritogenesis after GPCR-Gs engagement in PC12 cells.

Conclusions: Parallel, insulated pathways effect cAMP-dependent neuroendocrine cell differentiation.

Significance: Assays for parcellated cAMP signaling in neuroendocrine cells have broad application for CNS drug discovery.

SUMMARY

Dividing neuroendocrine cells differentiate into a neuronal-like phenotype in response to ligands activating G protein-coupled receptors, leading to the elevation of the second messenger cyclic 3'-5'-adenosine monophosphate (cAMP). Growth factors that act at receptor tyrosine kinases, such as nerve growth factor, likewise cause differentiation. We report here that two aspects of cAMP-induced differentiation, neurite extension and growth arrest, are dissociable at the level of the sensor conveying the cAMP signal in PC12 and NS-1 cells. Following cAMP elevation, NCS/Rapgef2 is activated for signaling to ERK to mediate neuritogenesis, while Epac2 is activated for signaling to the MAP kinase p38 to mediate growth arrest. Neither action of cAMP requires transactivation of TrkA, the receptor for NGF. In fact, the differentiating effects of NGF do not require activation of any of the cAMP sensors protein kinase A, Epac, or NCS/Rapgef2, but rather depend on ERK and p38 activation via completely independent signaling pathways. Hence, cAMP- and NGF-dependent signaling for differentiation are also completely insulated from each other. Cyclic AMP and NGF also protect NS-1 cells from serum withdrawal-induced cell death, again by two wholly separate signaling mechanisms, PKA-dependent for cAMP, and PKA-independent for NGF.

An array of extracellular signaling molecules including hormones and neurotransmitters cause cellular changes by regulating levels of the intracellular second messenger cyclic 3'-5'-adenosine monophosphate (cAMP). In the central nervous system, regulation of intracellular cAMP has been shown to control intracellular processes underlying synaptic plasticity and memory formation (1), guide axonal elongation (2), and support neuronal survival in both developing and adult brain (3). PC12 cells isolated from rat adrenal pheochromocytoma have long been used as a model of neural development and differentiation. Both receptor tyrosine kinase-activating neurotrophins, such as nerve growth factor (NGF), and neuropeptides that elevate intracellular cAMP, such as pituitary adenylate cyclase-activating polypeptide (PACAP), cause PC12 cells to undergo the dissociable processes of morphological differentiation (neurite outgrowth) and growth arrest (4,5) which together comprise differentiation.
Elevation of cAMP by GPCR signaling leads to activation of multiple downstream cAMP-responsive proteins (cAMP sensors). Protein kinase A (PKA) is the best characterized cAMP sensor. However, additional proteins functioning as cAMP sensors have since been identified. These include the exchange proteins activated by cAMP (Epacs) (6,7) and the neuritogenic cAMP sensor (NTS) Rapgef2 (8-10). Each of these three cAMP sensors (PKA, Epac, and NCS/Rapgef2) have been implicated in some aspect of PC12 cell differentiation (8,11,12), however it has not been determined whether each cAMP sensor fulfills a particular set of cellular tasks in response to cAMP elevation. Furthermore, it remains unknown whether the three cAMP-induced signaling pathways cross-regulate each other or whether they act as functionally insulated signaling channels.

In contrast to neuropeptide ligands for Gs-coupled GPCRs, which lead to the elevation of intracellular cyclic AMP, neurotrophins such as NGF cause protein phosphorylation via engagement with receptor tyrosine kinases (RTKs). The presence of either type of extracellular ligand is sufficient to cause PC12 cells to differentiate into a neuronal-like phenotype via a process that includes growth arrest, neurite extension, and the expression of neuron-specific gene products (5,13). It is clear that neurotrophin/RTK and GPCR/cAMP-initiated signaling for differentiation are separate and distinct processes (14). However, the degree of functional insulation between the two respective signaling pathways remains equivocal. The NGF- and cAMP-dependent pathways for differentiation have been shown to be readily pharmacologically dissociable (15,16) and NGF-induced differentiation of PC12 cells does not appear to require PKA (14). On the other hand, crosstalk between the NGF and cAMP differentiation pathways has been reported (17); PKA has been noted to be necessary for long-term effects of NGF on MAP kinases (18); NGF has been reported to increase cAMP activation of soluble adenylate cyclase (19); and cyclic AMP has also been reported to cause differentiation of PC12 cells via transactivation of the receptor for NGF, tropomyosin-related kinase A (TrkA) receptor (20,21).

The discovery of NCS/Rapgef2 as the cAMP sensor mediating neuritogenesis in response to PACAP, and the use of NS-1 cells as a PC12 cell subclone in which differentiation events can be conveniently measured and precisely quantified, has provided new pharmacological and cell biological opportunities to test this working hypothesis definitively. Here, we have determined the downstream signaling pathways engaged by cyclic AMP to cause both differentiation (neurite extension and growth arrest), and cell survival upon serum withdrawal. We report that three separate cAMP sensors independently mediate these three effects of cAMP elevation, and further, that the actions of NGF, while using the same final cellular effectors for neuritogenesis and growth arrest as cAMP elevation, do so without the need for signaling through cAMP.

**EXPERIMENTAL PROCEDURES**

**Drugs and Reagents**

- 8- (4-Chlorophenylthio)adenosine-3',5'-cyclic monophosphate (8-CPT-cAMP), 8- (4-Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8-CPT-2'-O-Me-cAMP), 8- Bromoadenosine-3',5'-cyclic monophosphorothioate, Rp-isomer (Rp-8-Br-cAMPS), 8- Bromoadenosine-3',5'-cyclic monophosphorothioate, Sp-isomer (Sp-8-Br-cAMPS), and 3-[5-(tetr-butyl)isoxazol-3-yl]-2-[2-(3-chlorophenyl)hydrazono]-3-oxopropanenitrile (ESI-09) were purchased from Biolog Life Science Institute. The inhibitors 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22,536), 1,4-Diamino-2,3-dicyano-1,4-bis[2-aminophenyl]butadiene (U0126), N-[2-[3-(4-bromophenyl)-2-propenyl]-aminoethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89), and trans-4-[4-(4-Fluorophenyl)-5-(2-methoxy-4-pyrimidinyl)-1H-imidazol-1-yl]cyclohexanol (SB 239063) were purchased from Toecris Bioscience. Nerve growth factor (NGF) basic fibroblast growth factor (bFGF) were purchased from Sigma and (9S,10R,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindol-[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid methyl ester (K-252a) was from Calbiochem. Pituitary adenylate cyclase-activating polypeptide (PACAP-38) was from Phoenix Pharmaceuticals. Farnesylthiosalicylic acid (FTS) and...
farnesylthiosalicylic acid amide (FTS-A) are available from the Cayman Chemical Company. Fresh stocks of all drugs were prepared in culture media with the following exceptions – ESI-09, H-89, U0126, SB 239063 were prepared as 50 mM stocks in dimethyl sulfoxide (DMSO). FTS and FTS-A were usually prepared as 30 mM stocks in DMSO. K-252a was prepared as a 2 mM stock in anhydrous ethanol.

**Cell culture** – Neuroscreen-1 (NS-1) cells are a subclone of PC12 cells purchased from Cellomics. All solutions used for cell culture were purchased from Invitrogen unless otherwise noted. NS-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% horse serum (HyClone), 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were grown in flasks (Techno Plastic Products) coated with collagen type I from rat tail as described previously (9) at 37°C in a humidified incubator containing 5% CO₂. PC12 cells (PC12-G; see (22)) were cultured in DMEM supplemented with 7% horse serum, 7% heat-inactivated fetal bovine serum, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. PC12 cells were grown at 37°C in a humidified incubator containing 5% CO₂, and for experiments were plated on poly-D-lysine-coated 6-well plates as described previously (23). All cells routinely tested negative for mycoplasma and were used between passages 5 and 16 for the experiments reported here.

**Neurite outgrowth measurements** – NS-1 cells were dispensed into 6-well plates and the following day media were changed to media containing drugs. Cells were treated for 48 or 72 hours, as indicated. Using a 20× lens, photomicrographs were then acquired with a computer-assisted microscope. Images were randomized and a blinded observer counted the number of cells, the number of neurites, and the length of the neurites in each field using NIS-Elements BR (Nikon).

**Proliferation assays** – MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) was purchased from Sigma and was kept in the dark at all times. Stocks (1 mg/ml) were prepared in culture media and stored for two weeks or less at 4°C. NS-1 cells were plated in 96-well plates (1x10⁴ cells per well) and treated accordingly for varying lengths of time. Media were then aspirated and replaced with MTT (0.2 mg/ml) in culture media. Cells were exposed to MTT for 90 minutes in the dark at 37°C. MTT-containing media were then aspirated and formazan salts were extracted from each well with 70 µl of DMSO. The optical density of the extracted formazan product, proportional to the number of viable cells (24), was then measured using a microtiter plate reader (Perkin Elmer).

**Western blotting** – Cells were seeded in 12-well plates and grown overnight. Cells were pretreated with small molecule inhibitors for 30 minutes prior to the addition of agonists. For measurements of ERK and p38, cells were treated with agonists for 10 minutes, after which time media were aspirated and cells were collected in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-Cl, 1% NP-40, and 1 mM EDTA) containing Halt protease and phosphatase inhibitors (Pierce Biotechnology). Protein concentrations were normalized and samples for electrophoresis were diluted in LDS Sample Buffer (Invitrogen) to a final protein concentration of 1 µg/µl. Proteins (20 µg/plate) were then separated by SDS-PAGE on 4-12% polyacrylamide Bis-Tris gels (Invitrogen). Gels were blotted onto nitrocellulose membranes (Invitrogen) using a semi-dry transfer apparatus (Invitrogen) at 30 V for 2 hours at room temperature. Membranes were then blocked with 5% skim milk dissolved in Tris-buffered saline with 1% Tween-20 (TBST) for 2 hours. Membranes were incubated overnight at 4°C with the following primary phospho-specific antibodies, which were purchased from Cell Signaling Technology: phospho-ERK (recognizes dual-phosphorylated ERK1 and ERK2; CST Catalog #4370) or phospho-p38 (recognizes dual-phosphorylated p38alpha, beta, delta and gamma; CST Catalog #4511). Membranes were then washed five times in TBST and incubated with appropriate HRP-coupled secondary antibodies (Pierce) in blocking buffer for 1 hour. Membranes were again washed five times in TBST and incubated with appropriate HRP-coupled secondary antibodies (Pierce) in blocking buffer for 1 hour. Membranes were then stripped for 15 minutes using Restore Plus Western Blot Stripping Buffer (Pierce), washed five times in TBST, incubated in
blocking buffer for 1 hour and re-probed with primary antibodies raised against total ERK (CST Catalog #4695) or p38 (CST Catalog #8690).

G1/G0 biosensor measurements – An LNCX retroviral vector expressing HDHBc-tdimer2 was generated as described previously (23). PC12 cells transduced with LNCX-HDHBc-tdimer2 retroviral vector were cultured in selection media containing G418 (800 µg/ml). Cells were plated in poly-D-lysine-coated 6-well dishes, grown overnight, and treated as indicated. At the end of the experiment, cells were photographed with a Nikon microscope using a 20× objective with rhodamine filter. All cells were counted and those with nuclear-confined red fluorescence were counted as cells in G1/G0, while those with diffuse cytoplasmic red fluorescence were counted as being in S/G2.

Silencing of Epac1 and Epac2 – shRNA for rat Epac1 (target sequence: aggaaccgatatacggtaa) and Epac2 (target sequence: caagaaacacgaagcgtat) were expressed in psi-Lv-HIVH1 lentiviral vectors (GeneCopoeia). Lentiviral particles were generated by co-transfecting HEK293T cells, grown in 10 cm dishes, using the Profection calcium phosphate system (Promega) according to the manufacturer’s instructions with plasmids encoding shRNA expression vectors (10 µg), gag-pol-rev (6.5 µg), and VSV (3.5 µg). 48 hours post-transfection, supernatants were harvested, filtered through 0.45 micron filters, and applied to cultures of 60-70% confluent NS-1 cells growing in T25 flasks. 12 hours post-transduction, NS-1 cells were split and cultured in selection media containing 1 µg/ml puromycin. Following 2 passages in selection media, >98% of transduced NS-1 expressed visible GFP upon epifluorescence illumination. Knockdown of Epac1 and Epac2 protein abundance was confirmed by Western blotting as described above using antibodies from Cell Signaling Technology (Catalog #4155 and 4156).

Measurements of Rap1 activation – Rap1-GTP was measured using the Active Rap1 Pull-Down and Detection Kit (Pierce, Catalog # 16120) according to the manufacturer’s instructions. NS-1 cells were grown to near confluency in 6-well plates. Cells were treated for 10 min as indicated and then lysed in ice-cold lysis buffer provided by the manufacturer. An aliquot of protein from each sample (500 µg) was incubated in a solution of 20 µg of GST-RalGDS-RBD in glutathione resin slurry for 1 hour. Samples were then centrifuged through spin cups, washed 3 times with lysis buffer, dissolved in reducing sample buffer, vortexed, and heated to 95˚C for 5 min. Samples were then analyzed by Western blotting as described above and probed with an antibody raised against Rap1 at a dilution of 1:1000 (Pierce). To account for possible differences in Rap1 content between samples, unpurified protein samples (20 µg) that corresponded to affinity purified samples were also analyzed by Western blotting on separate gels using the same procedure.

Propidium iodide staining – Propidium iodide (PI) is a dye that fluoresces upon binding to DNA and is highly polar, thus it enters and stains only cells with damaged membranes. Stocks of PI (5 mg/ml) were prepared in PBS and stored at 4°C protected from light for up to 1 month. Following treatment, media were aspirated and cells were exposed 5 µM PI in PBS for 30 minutes in the dark at 37°C. Fluorescence was then viewed using an inverted fluorescence microscope using a rhodamine filter. PI positive and negative cells were then manually counted by a blinded observer using NIS Elements (Nikon).

Calculations and statistics – All statistical analyses were performed using Sigma Plot (Systat). For datasets with a Gaussian distribution, statistical comparisons of multiple groups within an experiment were made using ANOVA followed by Bonferroni-corrected t-tests comparing each condition to controls. In experiments where data were not normally distributed, data were analyzed by Kruskal-Wallis non-parametric ANOVA followed by Dunnet’s or Dunn’s post-hoc tests comparing treated groups to controls. For dose-response experiments, curves were fit to dose-response data using 4-parameter logistic regression where appropriate.

RESULTS – We previously reported that intracellular cAMP, acting at NCS/Rapgef2, causes neurite extension (neuritogenesis) in NS-1 cells. NCS/Rapgef2 enhances GTP loading on the small G protein Rap1, allowing its association with B-Raf, thus activating MEK and ERK (8). This pathway is activated by the neuropeptide PACAP through interaction with the GPCR PAC1, and subsequent Gs-dependent stimulation of adenylate
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cyclase and elevation of cAMP (8,9). NGF also stimulates both neurite elongation and growth arrest. ERK is necessary for neuritogenesis due to either cAMP or NGF, and we therefore wished to see whether cAMP and NGF share a common pathway for inducing either neuritogenesis, or growth arrest.

NGF and cAMP stimulate neuritogenesis via separate signaling pathways. Neuroscreen-1 (NS-1) cells were differentiated by treatment for 48 hours with the lipophilic cAMP analog 8-CPT-cAMP (100 µM) or nerve growth factor (NGF; 100 ng/ml). As seen in Figs. 1A-C, treatment with either 8-CPT-cAMP or NGF resulted in both neurite extension and growth arrest. NGF signaling for differentiation has been reported to involve formation of a TrkA multiprotein complex containing NCS/Rapgef2 (also called PDZ-GEF1) and requiring activation of Rap1 (25). To see whether cAMP interaction with NCS/Rapgef2 is necessary for NGF-induced neuritogenesis, we treated NS-1 cells with the inhibitor of adenylate cyclase and NCS/Rapgef2 SQ22,536 (26), and then challenged cells with either 8-CPT-cAMP or NGF. As seen in Figs. 1A-C, SQ22,536 blocked cAMP-dependent neurite extension, while not affecting NGF-dependent signaling or cAMP-induced growth arrest.

It has also been reported that cAMP or one of its downstream effectors signals via transactivation of TrkA receptors (20). We wished to determine whether cAMP-induced ERK activation and neurite extension may involve transactivation of TrkA receptors. NS-1 cells were treated with the either 8-CPT-cAMP (100 µM) or NGF (100 ng/ml) in the absence or presence of 200 nM of the TrkA inhibitor K-252a (16). K-252a significantly blocked NGF-induced ERK activation while not affecting cAMP-induced activation of ERK (Figs. 1D and E). In the same experiments SQ22,536 blocked cAMP-dependent, but not NGF-dependent, activation of ERK (Figs. 1D and E). Following the same pharmacological profile as observed for ERK activation, NGF-induced neuritogenesis was blocked by K-252a, while cAMP-dependent neurite outgrowth was unaffected by the addition of K-252a (Figs. 1F and G). Together, these data indicate that the signaling pathways for neuritogenesis activated by NGF and cAMP are functionally insulated insofar as cAMP-initiated neuritogenesis does not require TrkA receptor activation, and NGF-initiated neuritogenesis does not require NCS/Rapgef2.

Epac mediates cAMP-dependent growth arrest. NS-1 cells also undergo growth arrest when treated with NGF or cAMP. NCS/Rapgef2, while necessary for neuritogenesis, is apparently not required for cAMP-dependent growth arrest (Figs. 1A and C). To determine the mechanism through which cAMP mediates growth arrest in NS-1 cells, the proliferation rate of NS-1 cells grown in media containing 8-CPT-cAMP (100 µM) was monitored, by estimation of cell number at the indicated intervals with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. After three days of treatment, the effects of 8-CPT-cAMP (100 µM) on growth arrest were evident (Fig. 2A). To determine which cAMP sensor mediates growth arrest, NS-1 cells were treated with 8-CPT-cAMP (100 µM) for 3 days with varying concentrations of H-89 (PKA inhibitor), SQ22,536 (NCS/Rapgef2 inhibitor), or ESI-09 (Epac inhibitor). As seen in Fig. 2B, ESI-09 inhibited growth arrest due to 8-CPT-cAMP while neither H-89 nor SQ22,536 significantly inhibited growth arrest, suggesting that Epac is necessary for cAMP-dependent growth arrest. Consistent with the notion that Epac underlies cAMP-dependent growth arrest, the Epac-selective cAMP analog 8-CPT-2′-O-Me-cAMP (27) caused growth arrest to a similar extent as 8-CPT-cAMP, which activates all three cAMP sensors (Fig. 2C).

Signaling through Epac causes growth arrest in a Rap1-independent manner. Cyclic AMP-induced neuritogenesis in NS-1 cells requires NCS/Rapgef2-mediated stimulation of Rap1 (8). Rap is also the best characterized effector of Epac signaling. We therefore wished to determine whether Epac-induced growth arrest is Rap-dependent. As seen in Fig. 3A, 8-CPT-2′-O-Me-cAMP caused a robust increase in Rap1 activation. Farnesyl thiosalicylic acid amide (FTS-A; 30 µM), a compound previously shown to inhibit Rap1 activation (8,28), significantly inhibited Epac-induced GTP loading of Rap1 (Fig. 3A). To determine whether the Rap1 GEF activity of Epac is necessary for growth arrest, NS-1 cells were treated with 8-CPT-2′-O-Me-cAMP (100 µM) for 3 days in the absence or presence of varying concentrations of FTS-A. As seen in Fig. 3B, FTS-
A failed to inhibit growth arrest following selective stimulation of Epac with 8-CPT-2'-O-Me-cAMP. In control experiments, NS-1 cells were grown in parallel cultures and were treated for 48 hours with 8-CPT-cAMP (100 μM) in the absence or presence of FTS-A (30 μM). As seen in Figs. 3D and E, FTS-A inhibited cAMP-dependent neuritogenesis, indicating that it remains biochemically active during the extended treatment. Furthermore, while FTS-A blocked neurite extension in these experiments, it failed to inhibit cAMP-dependent growth arrest (Figs. 3C and E). Together these data suggest that cyclic AMP and Epac induce growth arrest via a Rap1-independent signaling pathway. We therefore examined other possible downstream effectors of Epac-mediated signaling.

The MAP kinase p38 is necessary for Epac-dependent growth arrest. ERK is necessary for PC12 cell neuritogenesis (9,29,30) and ERK has also been shown to directly mediate growth arrest in certain cell types such as transformed fibroblasts (31). To investigate a possible role for MEK/ERK in growth arrest, we treated NS-1 cells with 8-CPT-cAMP (100 μM) in the absence or presence of varying concentrations of the MEK inhibitor U0126. As seen in Fig. 4A, U0126 failed to inhibit the effect of 8-CPT-cAMP on growth arrest, suggesting that ERK is not a component of the cAMP growth arrest pathway. Activation of the MAP kinase p38 has been shown to contribute to growth arrest through multiple mechanisms (32). Moreover, Epac has been shown to activate p38 in cerebellar and hippocampal neurons (33,34). We accordingly measured cAMP-induced p38 activation using antibodies that specifically recognize p38 phosphorylated at Thr180 and Tyr182. As seen in Fig. 4B, treatment with either the pan-specific cAMP analog 8-CPT-cAMP or the Epac-selective 8-CPT-2'-O-Me-cAMP increased the abundance of phosphorylated p38. Similar results were obtained using PC12 cells treated with either 100 μM 8-CPT-cAMP or 100 μM 8-CPT-2'-O-Me-cAMP (Fig. 4C).

We next established that the p38 inhibitor SB 239063 (10 μM) effectively blocked Epac-dependent p38 activation, while U0126 (10 μM) had no effect in NS-1 cells (Fig. 4D). Similarly in PC12 cells, pretreatment with SB 239063 effectively inhibited p38 phosphorylation occurring in response to treatment with 100 μM 8-CPT-2'-O-Me-cAMP (Fig. 4E). To determine whether p38 is required for Epac-dependent growth arrest, NS-1 cells were treated with either 8-CPT-cAMP or 8-CPT-2'-O-Me-cAMP for 3 days in the absence or presence of varying concentrations of SB 239063. As seen in Fig. 4F, SB 239063 potently inhibited 8-CPT-2'-O-Me-cAMP-induced growth arrest with an IC_{50} value of 460 ± 10 nM.

The neuropeptide PACAP causes growth arrest via Epac/p38 activation. Since cyclic nucleotide analogs cause NS-1 cells to undergo growth arrest via a signaling pathway including Epac and p38, we wished to see whether a GPCR ligand could also activate this signaling pathway. The neuropeptide PACAP plays protean roles in the development of the nervous system (35) and is well known to cause differentiation in PC12 cells (36). As seen in Fig. 5A, treatment with PACAP (100 nM) caused growth arrest to a comparable extent as cAMP analogs. PACAP-induced growth arrest was also potently inhibited by the p38 inhibitor SB 239063 (IC_{50} = 710 ± 40 nM), suggesting that PACAP-induced growth arrest is likewise p38 dependent.

We next wished to see whether PACAP activates p38, and if so, whether it also occurs in an Epac-dependent manner. NS-1 cells were treated with PACAP (100 nM) for 10 min with or without ESI-09 (10 μM). As seen in Figs. 5B and C, PACAP caused a significant increase in phosphorylated p38. The effect of PACAP was significantly inhibited by the addition of ESI-09, indicating that Epac is required for PACAP-dependent p38 activation. PACAP-dependent p38 phosphorylation was also effectively blocked by ESI-09 (10 μM) in PC12 cells (Fig. 5D).

To confirm that the effects of PACAP on growth arrest are mediated by Epac, NS-1 cells were treated with either PACAP (100 nM) or 8-CPT-2'-O-Me-cAMP (100 μM) for three days in the absence or presence of ESI-09 (10 μM). As seen in Fig. 5E, both PACAP and 8-CPT-2'-O-Me-cAMP caused growth arrest. The effects of both were significantly inhibited by ESI-09, suggesting that PACAP indeed causes growth arrest of NS-1 cells via engagement of the Epac pathway.

In order to determine which Epac is required for cAMP-dependent growth arrest, Epac1 and
Epac2 were each stably silenced in NS-1 cells using shRNA. Cell lines wherein the abundance of the target protein product was reduced by ≥ 90% relative to that in untransduced controls were selected for further experiments (Fig. 5F). Parental NS-1 cells as well as lines deficient in Epac1 and Epac2 were treated for three days in the absence or presence of cyclic AMP-elevating/mimicking agents PACAP-38 (100 nM), forskolin (25 µM), 8-CPT-cAMP (100 µM), or the RTK-activating agents NGF (100 ng/ml) or basic fibroblast growth factor (bFGF; 100 ng/ml). As seen in Fig. 5G, exposure to PACAP, forskolin, or 8-CPT-cAMP caused growth arrest in NS-1 cells or Epac1-deficient NS-1 cells, while Epac2-deficient cells proliferated at a similar rate to untreated cells under these conditions. NGF and bFGF caused growth arrest in all three cell lines tested. Together, these data indicate that while Epac2 is necessary for cAMP-dependent growth arrest in NS-1 cells, Epac1 is dispensable for this process.

Epac mediates cAMP-dependent growth arrest in PC12 cells. PC12 cells undergo a similar pattern of differentiation as NS-1 cells in response to cAMP elevation. As seen in Fig. 6A, both 8-CPT-cAMP (100 µM) and 8-CPT-2′-O-Me-cAMP (100 µM) elicited growth arrest while only 8-CPT-cAMP promoted neuritogenesis. In order to quantitatively monitor cell cycle arrest, we employed PC12 cells transduced with LNCX-HDHBc-tdimer2, a retroviral vector encoding a G1/G0 biosensor fused to red fluorescent protein (RFP) that distinguishes proliferating cells from growth arrested cells by confining RFP expression to the nucleus in growth arrested cells while it is diffusely expressed in the cytoplasm in cells in S/G2 phase (23,37). Treatment with either 8-CPT-cAMP (100 µM) or 8-CPT-2′-O-Me-cAMP (100 µM) for 48 hours caused growth arrest in the majority of PC12 cells (Fig. 6B). Furthermore, as seen in Fig. 6C, treatment with 8-CPT-2′-O-Me-cAMP (100 µM) or PACAP-38 (100 nM) caused growth arrest as determined by nuclear restriction of RFP. ESI-09 (10 µM) blocked PACAP-dependent growth arrest while not affecting neuritogenesis. Growth arrest without neuritogenesis was observed in cells treated with 8-CPT-cAMP (100 µM) and SQ22,536 (1 mM), most likely due to the ability of cAMP to activate Epac, but not NCS/Rapgef2, under these conditions. Together these data indicate that Epac is responsible for cAMP-induced growth arrest in PC12 cells while not involved in neuritogenesis.

NGF- and cAMP-dependent signaling pathways both require p38 to induce growth arrest in NS-1 cells. It has long been appreciated that trophic factors such as NGF cause growth arrest in PC12 cells (5). However, it has not been determined whether cAMP and NGF cause growth arrest by parallel activation of a common target or by cross-activation of their respective signaling pathways. In NS-1 cells, treatment with NGF for 3 days also induced growth arrest which was sensitive to SB 239063 (Fig. 7A). We next measured NGF-induced p38 activation, and as seen in Figs. 7B and C, both NGF and 8-CPT-2′-O-Me-cAMP caused significant increases in p38 phosphorylation. Since Ras activation is one of the best characterized signaling effectors of NGF for differentiation (38), we co-treated NS-1 cells with NGF and farnesylthiosalicylic acid (FTS; 10 µM), a specific Ras inhibitor (39,40). FTS significantly inhibited NGF-dependent p38 activation while Epac-induced p38 activation stimulated by treatment with 8-CPT-2′-O-Me-cAMP was insensitive to FTS (Figs. 7B and C).

Both NGF (100 ng/ml) and 8-CPT-2′-O-Me-cAMP (100 µM) caused growth arrest (Fig. 7D). The effects of NGF were selectively blocked by the addition of 10 µM FTS or 200 nM K252-a (Fig. 7D), neither of which affected growth arrest induced by 8-CPT-2′-O-Me-cAMP. In the same experiments ESI-09 (10 µM) significantly inhibited growth arrest elicited by 8-CPT-2′-O-Me-cAMP, while not affecting NGF-dependent growth arrest (Fig. 7D). These data indicate that p38 activation is necessary for growth arrest caused by either NGF or cAMP; however NGF activates p38 through a Ras-dependent pathway, while cAMP signals through an Epac-dependent, Ras-independent pathway.

Protection of NS-1 cells from serum withdrawal-induced cell death by cAMP requires PKA activation. Along with causing neuritogenesis and growth arrest, cAMP has been shown to be cytoprotective in multiple cell types, including PC12 cells (41). We wished to determine whether cAMP-dependent cytoprotection is similarly parcellated to a single cAMP sensor, as growth arrest and neuritogenesis
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are, or if cytoprotection mediated by cAMP requires signaling through multiple cAMP sensors. NS-1 cells were cultured for 3 days in serum-free media to induce apoptosis (42), after which time cells were stained with propidium iodide (PI), a highly polar compound that fluoresces upon binding to DNA and thus only stains cells with damaged plasma membranes, i.e. dead or dying cells. Serum withdrawal caused the death of most of the cells (Fig. 8), while only 1-4% of control cells grown in serum incorporated PI. As seen in Figs. 8A and B, treatment with 8-CPT-cAMP (100 µM) significantly decreased the ratio of PI-positive cells after serum withdrawal, thus protecting NS-1 cells from apoptosis. Cyclic AMP-dependent survival in serum-free media was blocked by H-89 (30 µM) while unaffected by ESI-09 (10 µM) or SQ22,536 (1 mM), suggesting that survival may be PKA-dependent. Consistent with this notion, we found that PACAP-38 (100 nM) also supported cell survival in serum-free media (Figs. 8C and D), and PACAP-dependent survival was blocked by the addition of a specific agonist of cAMP binding to the regulatory subunit of PKA, Rp-8-Br-cAMPS (43), applied at 750 µM (Figs. 8C and D). Furthermore, a specific agonist of the regulatory subunit of PKA, Sp-8-Br-cAMPS (500 µM), protected NS-1 cells from serum withdrawal (Figs. 8C and D). Together these data suggest that cyclic AMP-dependent survival is mediated exclusively by the activation of PKA and does not require Epac or NCS/Rapgef2.

As expected, NGF also protected NS-1 cells from serum withdrawal-induced cell death (Figs. 8E and F). However, NGF-mediated rescue from serum withdrawal-induced cell death was not blocked by Rp-8-Br-cAMPS, indicating that NGF does not protect cells by activating PKA, and suggesting that cAMP- and NGF-dependent signaling may share a final common target for pro-survival signaling, but do not share upstream signaling components for such signaling.

Taken together, these data (summarized in Fig. 9) suggest that cAMP and NGF trigger distinct but coordinated differential processes by activation of multiple, parallel signaling pathways. Furthermore, we show that cAMP-dependent neurite extension, growth arrest, and cytoprotection are mediated by distinct pathways each gated by a separate cAMP sensor: NCS/Rapgef2, Epac, and PKA, respectively.

DISCUSSION

In NS-1 cells, neuropeptide-induced cyclic AMP elevation, and neurotrophin-induced TrkA activation, are both sufficient to cause differentiation, as defined by the parallel cellular processes of neuritogenesis and cell growth arrest. Our data are consistent with the widely-held model that NGF signaling for differentiation is Ras-dependent, and show here that neuritogenesis requires ERK, but not p38, while growth arrest requires p38, but not ERK activation. When initiated by NGF, neither of these processes requires cAMP. Neuropeptide-induced differentiation likewise requires activation of ERK for neuritogenesis, and p38 for growth arrest, but (at least for PACAP) these pathways were differentially activated by the cAMP sensor NCS/Rapgef2, and Rapgef4 (Epac2), respectively.

The Epacs, identified as Rap1 GEFs (6,7), are members of the Rap GEF gene family (44) and enhance Rap1 activation in NS-1 cells. However our results suggest that Rap1 activation is not a requisite step for Epac-dependent p38 activation. In fact, other Rap-independent functions of Epac have been noted. For example, Epac has been shown to activate the MAP kinase JNK via a Rap-independent mechanism in HEK 293T cells (45). Epac has also been shown to be an indirect activator of the small G protein Rit (11), that unlike Ras or Rap, does not require prenylation for its biological activity (46). Interestingly, Epac1-Rit-p38 signaling has been reported to be a component in PACAP-induced differentiation of PC6 pheochromocytoma cells (11). Rit or a related small G protein may be involved in the Epac2-dependent p38 activation reported here in NS-1 cells, especially in light of the fact that an inhibitor such as FTS failed to interfere with Epac-dependent p38 activation while completely blocking NGF-dependent p38 phosphorylation.

Our finding that Epac2, but not Epac1, mediates cAMP-dependent growth arrest may shed important light on the distinct roles of these two Rap GEFs on cell cycle regulation in other systems besides neuroendocrine cells. For example, it is not known if p38 mediates effects of either Epac1 (Rapgef3) or Epac2 (Rapgef4) on cell proliferation or growth arrest in non-
neuroendocrine cells. In fact, the Epacs have been reported to both positively and negatively affect cell cycle entry and exit, depending on the cell type and relative expression levels of Epac1 and Epac2 (47). P38 activation has been shown to exert anti-proliferative effects in many cell and tissue types, including tumors in skin, lung, and liver (48), and p38 hypoactivity has been noted in human tumors (32). In the current study, Epac-dependent activation of the p38 pathway was shown to be necessary for growth arrest of NS-1 pheochromocytoma cells, and it is therefore possible that Epac/p38 signaling may play an inhibitory role in the development of neuroendocrine tumors. In general, at least based on the current limited examples, it would appear that Epac exerts growth arrest through one of several MAP kinases in cells in which differentiation and growth arrest occur in parallel, and that Epac exerts a proliferative effect, through other MAP kinases, in cells in which differentiation is linked to proliferation, or at least not to obligate removal from the cell cycle.

The neuritogenic and growth arrest pathways activated by cAMP in NS-1 cells require the cAMP sensors NCS/Rapgef2 and Epac, respectively, and exclusively (vida supra). Remarkably, there is no apparent role for the classical cAMP sensor/effector PKA in either growth arrest or neuritogenesis in neuroendocrine cell differentiation. However, the pro-survival effect of cyclic AMP after serum withdrawal in NS-1 cells was blocked by a PKA inhibitor, but not by Epac or NCS/Rapgef2 inhibitors. Furthermore, the pro-survival effects of PACAP are sensitive to a specific antagonist of the regulatory subunit of PKA, and are fully mimicked with the PKA-specific cAMP analog Sp-cAMPS. These data imply that PKA is the sole sensor mediating these effects of PACAP, with no apparent contribution from the other two cAMP sensors, NCS/Rapgef2 and Epac. Thus, PKA plays a major role in cAMP-mediated survival following serum withdrawal from NS-1 cells. Ligands of Gs-coupled GPCRs, notably PACAP, have also been shown to exert PKA-dependent cytoprotective effects in numerous cell types, including neurons of the central nervous system and retina, cardiomyocytes, kidney, and hepatic cells (49-52). NGF also protected cells from death due to serum withdrawal. However, in contrast, NGF-mediated cytoprotection was PKA-dependent, which is consistent with reports that NGF-mediated survival effects require the activation of phosphatidylinositol-3 (PI-3) kinase and Akt (53,54).

In addition to cAMP signaling specificity conferred by differential expression of cAMP sensors in different tissues, it is evident that cAMP sensors are activated to perform distinct and specific tasks within the same cell, following cAMP elevation after stimulation of Gs-coupled GPCRs. Furthermore, while stimulation of any Gs-coupled GPCR is presumed to lead to the activation of PKA, a subset of Gs-coupled GPCRs including PAC1 (8) and β1-adrenoceptors (55) efficiently activate NCS/Rapgef2, while others such as β2-adrenoceptors do not (8). It is an intriguing possibility that these differential signaling properties could be leveraged for therapeutic potential to specifically regulate an aspect of cAMP-dependent signaling, such as morphological plasticity, proliferation, or cell survival, in vivo.

A further question of interest is whether biased GPCR ligands that lead to preferential activation of particular cAMP sensors can be identified. In fact, different structural analogs of NGF have been shown to differentially induce signaling leading to either differentiation or trophism/cytoprotection through TrkA receptors (56,57). GPCR ligands that specifically modulate these cellular outcomes in a biased fashion (58) could therefore be of great therapeutic potential.

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**FOOTNOTES**

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2 The abbreviations used are: bFGF, basic fibroblast growth factor; cAMP, 3'-5'-cyclic adenosine monophosphate; ERK, extracellular signal-regulated kinase; GEF, guanine nucleotide exchange factor; GPCR, G protein-coupled receptor; MAP, mitogen-activated protein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; NCS, neuritogenic cyclic AMP sensor; NGF, nerve growth factor; NS-1, Neuroscreen-1; p38, P38 mitogen-activated protein kinase; PACAP, pituitary adenylate cyclase-activating polypeptide; PI, propidium iodide; PKA, protein kinase A; RTK, receptor tyrosine kinase; SEM, standard error of the mean; shRNA, short hairpin RNA; TrkA, tropomyosin-related kinase A

**FIGURE LEGENDS**

**FIGURE 1.** NGF and cAMP stimulate neuritogenesis via separate signaling pathways. (A) NS-1 cells were cultured for 48 hours in the absence or presence of 8-CPT-cAMP (100 µM), NGF (100 ng/ml), and SQ22,536 (1 mM). Both 8-CPT-cAMP and NGF caused significant decreases in cell number (A) and significant increases in neuritogenesis (B). SQ22,536 blocked the effect of 8-CPT-cAMP on neuritogenesis, but not growth arrest. Bars represent means from 4 images from each condition, N=3, and error bars correspond to the SEM, *P<0.05 relative to untreated controls using Dunnett’s post hoc test. (C) Representative photomicrographs corresponding to experiments summarized in panels A and B, scale bar = 50 µm. (D) Western blot of ERK phosphorylation following treatment with 8-CPT-cAMP (100 µM) or NGF (100 ng/ml) in the absence or presence of K-252a (200 nM) or SQ22,536 (1 mM). (E) Densitometric analysis of blot shown in panel D combined with data from 3 replicate experiments. Note that data are presented in different order than shown on blot to show appropriate statistical comparisons,
which were performed using Bonferroni-corrected post hoc t-tests, **P<0.01, ***P<0.001 relative to untreated controls and ###P<0.001 comparing samples within a treatment group (either 8-CPT-cAMP or NGF) with those co-treated with inhibitors. (F) Quantification of 3 separate neurite outgrowth assays wherein NS-1 cells were treated with either 8-CPT-cAMP (100 µM) or NGF (100 ng/ml) along with either K-252a (200 nM) or vehicle (0.01% ethanol). Both 8-CPT-cAMP and NGF significantly promoted neurite outgrowth. K-252a blocked the effect of NGF. *P<0.05 as compared to untreated controls using Dunnet’s test. (G) Representative photomicrographs from experiment depicted in panel E. Scale bar = 50 µm.

FIGURE 2. Epac mediates cAMP-dependent growth arrest. (A) NS-1 cells were seeded at low density in 96-well plates in the absence or presence of 8-CPT-cAMP (100 µM). At the indicated time points, proliferation was measured by MTT assays. The relative number of cells per well is expressed as “Absorbance/well, as % of control” and represents total MTT absorbance per well as a percentage of MTT absorbance per well in the wells containing untreated cells grown for 72 hours in complete medium. Data points represent means from 3 experiments and error bars correspond to the SEM. (B) NS-1 cells were grown under differentiating conditions by adding 8-CPT-cAMP (100 µM) to the media for 3 days with and without inhibitors of the cAMP sensors expressed in NS-1 cells – ESI-09, H-89, or SQ22,536. Data were normalized to values obtained from untreated control cells grown in complete media, N=3. (C) NS-1 cells were grown for 3 days in media supplemented with varying concentrations of either 8-CPT-cAMP (100 µM) or 8-CPT-2'-O-Me-cAMP (100 µM), MTT absorbance data were expressed as a percent of absorbance values obtained from untreated control cells grown in complete media, N=3.

FIGURE 3. Signaling through Epac causes growth arrest in a Rap1-independent manner. (A) Measurements of Rap1 activation in NS-1 cells treated with 100 µM 8-CPT-2'-O-Me-cAMP in the absence or presence of 30 µM FTS-A. 8-CPT-2'-O-Me-cAMP significantly enhanced the abundance of active Rap1 and the effect of 8-CPT-2'-O-Me-cAMP was blocked by FTS-A, **P<0.01, Bonferroni, N=4. (B) NS-1 cells were treated for 3 days with 100 µM 8-CPT-2'-O-Me-cAMP in varying concentrations of FTS-A. MTT absorbance values are shown as a percent of values obtained by untreated control cells grown in adjacent wells. The addition of FTS-A did not affect Epac-dependent growth arrest, N=3. (C) Growth arrest and (D) neurite outgrowth measurements in NS-1 cells treated with 100 µM 8-CPT-cAMP in the absence or presence of 30 µM FTS-A. Bars represent averages from 4 determinations over 3 experiments and error bars correspond to the SEM, *P<0.05 compared to untreated control by Dunn’s post-hoc test. (E) Representative photomicrographs from data summarized in panels C and D. Scale bar = 50 µm.

FIGURE 4. The MAP kinase p38 is necessary for Epac-dependent growth arrest. (A) NS-1 cells were seeded in 96-well plates and treated with 100 µM 8-CPT-cAMP to cause growth arrest. U0126, applied at varying concentrations, failed to inhibit 8-CPT-cAMP-dependent growth arrest. Data represent MTT absorbance values as the percent of absorbance values obtained from control cells cultured in complete growth media in adjacent wells. Data points represent means from 3 experiments and error bars correspond to the SEM. (B) Analysis of p38 activation in NS-1 cells treated with cAMP analogs as indicated for 10 minutes. (C) Representative measurements (N=3) of p38 phosphorylation in PC12 cells treated for 10 minutes with 8-CPT-cAMP or 8-CPT-2'-O-Me-cAMP (100 µM each). (D) NS-1 cells were pretreated with inhibitors SB 239063 (10 µM), U0126 (10 µM), or vehicle (0.1% DMSO) for 30 minutes.
Cells were then stimulated with cAMP analogs (100 µM) for 10 minutes. (E) Measurement of p38 in PC12 cells pretreated with either SB 239063 (10 µM) or 0.1% DMSO and challenged with 8-CPT-2'-O-Me-cAMP (100 µM) for 10 minutes. (F) NS-1 cells were treated with either 8-CPT-cAMP (100 µM) or 8-CPT-2'-O-Me-cAMP (100 µM) for 3 days in the presence of various concentrations of SB 239063, which inhibited growth arrest due to either analog. Data are expressed as a percent of absorbance values from control cells cultured in complete growth media in adjacent wells. Data points represent means from 3 experiments and error bars correspond to the SEM. Note: all four isoforms of (phospho)p38, alpha, beta, gamma and delta, are recognized by the pp38 antibody employed here. The signals detected here are from pp38alpha, or beta, or delta, or a combination thereof (apparent molecular weights of these isoforms ca. 40-43 kDa), but not pp38gamma (apparent molecular weight ca. 46 kDa).

**FIGURE 5.** The neuropeptide PACAP causes growth arrest via Epac/p38 activation. NS-1 cells were treated with PACAP-38 (100 nM) in the presence of varying concentrations of SB 239063 for 3 days. Data are MTT absorbance values as a percent of absorbance values from control cells cultured in complete growth media in adjacent wells. Data points represent means from 3 experiments and error bars correspond to the SEM. (B) Measurements of p38 activation in NS-1 cells pretreated with ESI-09 (10 µM) for 30 min followed by stimulation with PACAP (100 nM), blot depicted is representative of three experiments. (C) Quantification of data depicted in panel B along with 2 additional replications. Data are a ratio of phosphorylated p38 to total p38 immunoreactivity and were normalized to the basal response from untreated controls, ***P<0.001 compared to controls treated only with vehicle, ### P<0.001 comparing the effect of ESI-09 within one treatment group, N=3. (D) Representative Western blot from replication of experiment depicted in Panel B using PC12 cells, N=3. (E) NS-1 cells were grown for 3 days and treated with either PACAP (100 nM) or 8-CPT-2'-O-Me-cAMP (100 µM), each of which caused growth arrest, ***P<0.001 as compared to untreated controls, Bonferroni. ESI-09 (10 µM) significantly inhibited the effects of both PACAP and 8-CPT-2'-O-Me-cAMP, ###P<0.001, Bonferroni, N=3. (F) Western blots probing for Epac1 (upper gel, Lanes 1-3) and Epac2 (lower gel, Lanes 4-6). The abundance of Epac1 and Epac2 protein measured in untransduced NS-1 cells (Lanes 1 and 4) was compared to NS-1 cells stably expressing shRNA against Epac1 (shEPAC1; Lanes 2 and 5) or Epac2 (shEPAC2; Lanes 3 and 6). (G) Untransduced NS-1 cells (Parent), NS-1 cells stably expressing Epac1 shRNA (shEPAC1), and NS-1 cells stably expressing Epac2 shRNA (shEPAC2) were plated and grown for 3 days in the absence or presence of PACAP-38 (100 nM), forskolin (25 µM), 8-CPT-cAMP (100 µM), NGF (100 ng/ml), and basic FGF (100 ng/ml). Bars correspond to the means from three experiments each with triplicate determinations and error bars correspond to the SEM. Data were analyzed by two-way ANOVA followed by Bonferroni-corrected t-tests, ***P<0.001 compared to values obtained from untreated controls in each cell line, ###P<0.01, ###P<0.001 comparing the effect of each drug across the three cell lines.

**FIGURE 6.** Epac mediates cAMP-dependent cell cycle exit in PC12 cells. (A) Phase-contrast photomicrographs of PC12 cells treated for 48 hours with either 8-CPT-cAMP or 8-CPT-2'-O-Me-cAMP (100 µM each). Images are representative from three experiments with four determinations per condition. (B) Quantification of PC12 cells in G1/G0 phase following treatment with 8-CPT-cAMP or 8-CPT-2'-O-Me-cAMP (100 µM each) for 48 hours. G1/G0 phase was determined by nuclear expression of the HDHBc-timer2 G1/G0 biosensor following treatment. Bars represent the means of 4 determinations from three experiments and are plotted as the percentage of cells with RFP expression confined to the...
nucleus and bars correspond to the SEM. Data analyzed by one-way ANOVA followed by Bonferroni-corrected t-tests, ***P<0.001. (C) Representative photomicrographs of LNCX-HDHBc-tdimer2-expressing PC12 after treatment with 8-CPT-2'-O-Me-cAMP (100 µM), PACAP-38 (100 nM), PACAP-38 + ESI-09 (10 µM), or 8-CPT-cAMP (100 µM) + SQ22,536 (1 mM).

FIGURE 7. NGF- and cAMP-dependent signaling pathways converge on p38 to induce growth arrest in NS-1 cells. (A) NS-1 cells were treated with NGF (100 ng/ml) with varying concentrations of SB 239063 for 3 days. SB 239063 inhibited growth arrest caused by NGF. MTT absorbance values are expressed as a percent of absorbance values from control cells grown on the same plates in complete growth media. Data points represent means from 3 experiments and error bars correspond to the SEM. (B) Representative Western blot of p38 activation in NS-1 cells pretreated with Ras inhibitor FTS (10 µM) or vehicle (0.03% DMSO) for 30 min followed by stimulation with NGF (100 ng/ml) or 8-CPT-2'-O-Me-cAMP (100 µM). (C) Quantification of 4 experiments performed as shown in panel B. Data are expressed as a ratio of phosphorylated p38 to total p38 and normalized to untreated controls (basal), ***P<0.001 as compared to untreated controls, ###P<0.001 comparing cells treated with FTS and those treated with vehicle, Bonferroni. (D) NS-1 cells were grown for 3 days and treated with either NGF (100 ng/ml) or 8-CPT-2'-O-Me-cAMP (100 µM) to induce growth arrest. Inhibitors FTS (10 µM) and K-252a (200 nM) blocked the effect of NGF while ESI-09 (10 µM) blocked the effect of 8-CPT-2'-O-Me-cAMP. ***P<0.001 as compared to values from control cells grown in media containing only the indicated inhibitor, Bonferroni.

FIGURE 8. Cyclic AMP promotes the survival of NS-1 cells via PKA activation. NS-1 cells were grown in serum-free media for 3 days, after which time cell death was determined by propidium iodide (PI) incorporation. Data are expressed as a percent of PI-positive (dead) cells. In each experiment cells were also grown in media containing serum as a control. 1-4% of cells grown in serum-containing media incorporated PI. (A) In serum-free media, NS-1 cells were treated with 100 µM 8-CPT-cAMP in the absence or presence of H-89 (30 µM), ESI-09 (10 µM), or SQ22,536 (1 mM). Bars represent means from 3 experiments with 4 determinations per experiment and error bars correspond to the SEM, *P<0.05 compared to untreated control cells using Dunn’s post-hoc test. (B) Representative photomicrographs from experiment depicted in panel A. (C) NS-1 cells were grown in serum-free medium supplemented with either Sp-8-Br-cAMPS (500 µM) or Rp-8-Br-cAMP (750 µM) with and without PACAP-38 (100 nM). Bars represent means from 3 experiments with 4 determinations per experiment and error bars correspond to the SEM, *P<0.05 compared to untreated controls using Dunn’s post-hoc test. (D) Representative photomicrographs from experiments depicted in panel C. (E) Summary of toxicity experiments where NS-1 cells were treated as indicated for 3 days. NGF (100 ng/ml) protected from serum-withdrawal induced cytotoxicity and its effect NGF was not abrogated by Rp-8-Br-cAMPS (750 µM). Bars represent means from 3 experiments with 4 determinations per experiment and error bars correspond to the SEM. *P<0.05 compared to untreated controls by Dunn’s post-hoc test. (F) Representative photomicrographs from experiments depicted in panel E.

FIGURE 9. Schematic diagram of signaling pathways leading to differentiation and cytoprotection. Cyclic AMP-dependent signaling connections are depicted by black arrows; NGF-dependent pathways are represented by dotted arrows. Pharmacological inhibitors are in red, and analogs/activators are in green.
Fig. 2

(A) Absorbance/well, as % of control

Treatment Duration (Hours)

(B) Absorbance/well, as % of control

Inhibitor, M

- ESI-09
- H-89
- SQ22,536

(C) Absorbance/well, as % of control

cAMP Analog, M

8-CPT-cAMP
8-CPT-2'-O-Me-cAMP
Fig. 3

A

B

C

D

E

No Agonist  
8-CPT-cAMP

-FTS-A  
+FTS-A
Fig. 8

A. % of cells stained with PI

| Condition       | Control | +8-CPT-cAMP | +H-89 | +8-CPT-cAMP +H-89 | +ESI-09 | +8-CPT-cAMP +ESI-09 | +SQ22,536 | +8-CPT-cAMP +SQ22,536 |
|-----------------|---------|-------------|-------|-------------------|---------|---------------------|-----------|-----------------------|
| 8-CPT-cAMP      | -       | +           | +     | +                 | +       | +                   | +         | +                     |
| H-89            | -       | -           | +     | +                 | +       | +                   | +         | +                     |
| ESI-09          | -       | -           | -     | +                 | +       | +                   | +         | +                     |
| SQ22,536        | -       | -           | -     | -                 | -       | +                   | +         | +                     |

B. Images of cell staining with PI

C. % of cells stained with PI

| Condition       | Control | +Sp-8-Br-cAMPS | +Rp-8-Br-cAMPS | +PACAP | +Rp-8-Br-cAMPS +PACAP |
|-----------------|---------|---------------|---------------|--------|-----------------------|
| Sp-8-Br-cAMPS   | -       | +             | -             | -      | -                     |
| Rp-8-Br-cAMPS   | -       | -             | +             | +      | +                     |
| PACAP           | -       | -             | -             | -      | +                     |

D. Images of cell staining with PI

E. % of cells stained with PI

| Condition       | Control | +NGF | +Rp-8-Br-cAMPS |
|-----------------|---------|------|---------------|
| NGF             | -       | +    | +             |
| Rp-8-Br-cAMPS   | -       | -    | +             |
Separate Cyclic AMP Sensors for Neuritogenesis, Growth Arrest and Survival of Neuroendocrine Cells
Andrew C. Emery, Maribeth V. Eiden and Lee E. Eiden

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