Cyclic Adenosine 3',5'-Monophosphate Elevation and Biological Signaling through a Secretin Family G_s -Coupled G Protein–Coupled Receptor Are Restricted to a Single Adenylate Cyclase Isoform

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Received January 21, 2015; accepted March 12, 2015

ABSTRACT

PC12 cells express five adenylate cyclase (AC) isoforms, most abundantly AC6 and AC7. These two ACs were individually silenced using lentiviral short hairpin RNAs, which lead to a decrease ($\geq 80\%$) of the protein product of each transcript. These stable PC12 sublines were then used to examine potential AC isoform preference for signaling through a family B G protein–coupled receptor (GPCR). Cells were challenged with the endogenous agonist of the pituitary adenylate cyclase–activating polypeptide type I receptor (PAC₁), pituitary adenylate cyclase–activating polypeptide (PACAP)-38, or the diterpene forskolin as an AC-proximal control. Intracellular cAMP levels were elevated by forskolin about equally in wild-type, AC6, and AC7 knockdown cells. The ability of PACAP-38 and forskolin to activate three cAMP sensors downstream of AC [protein kinase A (PKA), exchange protein activated by cAMP (Epac) 2/Rapgef4, and neuritogenic cAMP sensor (NCS)/Rapgef2] was examined by monitoring the

phosphorylation status of their respective targets, cAMP response element-binding protein, p38, and extracellular signal-regulated kinase. Forskolin stimulation of each downstream target of cAMP was unaffected by knockdown of either AC6 or AC7. PACAP-38 activation of all downstream targets of cAMP was unaffected by AC7 knockdown, but abolished following AC6 knockdown. Membrane cholesterol depletion with methyl- β -cyclodextrin mimicked the effects of AC6 silencing on PACAP signaling, without attenuating forskolin signaling. These data suggest that vicinal constraint of the GPCR PAC₁ and AC6 determines the exclusive requirement for this AC in PACAP signaling, but that the coupling of the cAMP sensors PKA, Epac2/Rapgef4, and NCS/Rapgef2, to their respective downstream signaling targets, determines how cAMP signaling is parcellated to physiologic responses, such as neuritogenesis, upon GPCR- G_s activation in neuroendocrine cells.

Introduction

The specificity of signaling through G protein–coupled receptors (GPCRs) is governed by coupling to three major classes of G proteins: G_{α_s} , G_{α_q} , and $G_{\alpha_{12}}$. Each of these subtypes also possesses $\beta\gamma$ subunits, so are thus heterotrimeric complexes (Oldham and Hamm, 2008). G_s -coupled GPCRs act as guanine nucleotide exchange factors for the α subunit of G_s (G_{α_s}), enabling it to release GDP, bind GTP, dissociate from its $\beta\gamma$ subunits, and acquire a conformation allowing activation of adenylate cyclase (AC). In contrast, $G_{\alpha_{12}}$ coupling facilitates GPCR-mediated inhibition of AC (Gilman, 1984). GPCR- G_{α_q} coupling enhances the catalytic activity of membrane phospholipase C β (Qin et al.,

2011). The mechanisms that impart signaling specificity downstream of GPCR- G_{α_s} have historically been little explored, possibly due to an assumption that activation of G_{α_s} creates a signaling entity spatially independent of the GPCR, and thus competent to activate any membrane AC. However, recent work clarifying the kinetics of GPCR- G_{α_s} -AC signaling has made it clear that GPCRs and ACs are not randomly distributed in the plasma membrane (Ostrom and Insel, 2004; Head et al., 2006). In fact, significant enrichment of specific GPCRs and specific AC isoforms, within specific membrane domains, has been well documented in several cell types (Insel et al., 2005). This plasma membrane compartmentation has been shown to affect (either enhance or diminish) the probability of G_{α_s} activation in the vicinity of one or another AC isoform. For example, studies in myocytes have revealed that AC5/6, as distinct from AC3, most likely occupies a caveolin-rich domain of the plasma membrane, and that this differential localization imparts

This work was supported by the National Institutes of Health National Institute of Mental Health Intramural Research Program [Projects 1ZIAMH002386 and 1ZIAMH002592]. A.C.E.'s project is supported in part by a 2014 NARSAD Young Investigator Grant from the Brain and Behavior Research Foundation [Grant 21356].

dx.doi.org/10.1124/mol.115.098087.

ABBREVIATIONS: AC, adenylate cyclase; CREB, cAMP response element-binding protein; DMEM, Dulbecco's modified Eagle medium; EIA, enzyme immunoassay; Epac, exchange protein activated by cAMP; ERK, extracellular signal-regulated kinase; GPCR, G protein–coupled receptor; IBMX, 3-isobutyl-1-methylxanthine; M β CD, methyl- β -cyclodextrin; NCS, neuritogenic cAMP sensor; PAC₁, pituitary adenylate cyclase–activating polypeptide type I receptor; PACAP, pituitary adenylate cyclase–activating polypeptide; PKA, protein kinase A; RT-PCR, reverse-transcription polymerase chain reaction; shRNA, short hairpin RNA.

preferential signaling for active β -adrenoceptors (Insel and Patel, 2009; Ostrom et al., 2012).

We have been exploring cAMP-coupling features of a secretin-family GPCR, pituitary adenylate cyclase-activating polypeptide type I receptor (PAC₁), which is selectively activated by its endogenous neuropeptide agonist pituitary adenylate cyclase-activating polypeptide (PACAP)-38. Previously, we have shown that PACAP signaling through PAC₁ to three separate cAMP sensors downstream of AC: protein kinase A (PKA), exchange protein activated by cAMP (Epac) 2/Rapgef4, and neurotogenic cAMP sensor (NCS)/Rapgef2, in neuroendocrine cells (Emery et al., 2014). To determine whether cAMP signaling initiated by the PAC₁ receptor involves AC isoform selectivity, we have generated PC12 cell lines with diminished expression of either AC6 or AC7, the two major AC isoforms expressed by these cells. By monitoring intracellular cAMP levels, as well as the activation of downstream targets of PKA, Epac2/Rapgef4, and NCS/Rapgef2, we report in this work that, in contrast to the effects seen following treatment with the diterpene forskolin, PACAP-PAC₁ signaling occurs specifically through AC6. The specificity of PACAP-PAC₁ signaling through AC6 is further supported by our results that, in wild-type PC12 cells, PACAP-initiated cAMP signaling, but not forskolin-initiated cAMP signaling, is sensitive to depletion of cholesterol.

Materials and Methods

Cell Culture. The PC12-G cell line (referred to in this work as PC12) was derived as previously described (Rausch et al., 1988). Unless otherwise specified, solutions for cell culture were obtained from Invitrogen (Carlsbad, CA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 7% horse serum (HyClone, Piscataway, NJ), 7% heat-inactivated fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Human embryonic kidney 293T cells, obtained from Cell Genesys (Foster City, CA), were cultured in DMEM containing GlutaMAX that was supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. All cell cultures were kept at 37°C in a humidified incubator containing 10% CO₂ for PC12 cells and 5% CO₂ for 293T cells. Cells were used between passages 5 and 23 for the experiments reported in this work and routinely tested negative for mycoplasma.

Drugs and Reagents. PACAP-38 was purchased from Phoenix Pharmaceuticals (Burlingame, CA) or AnaSpec (Fremont, CA) and was prepared as a 20 μ M stock in media. Forskolin was purchased from Tocris (Ellisville, MO) and was prepared as a 50 mM stock in dimethylsulfoxide. The phosphodiesterase inhibitor 3-isobutyl-1-

methylxanthine (IBMX) was purchased from Sigma-Aldrich (St. Louis, MO) and was prepared as a 125 mM stock in dimethylsulfoxide. The cholesterol-depleting reagent methyl- β -cyclodextrin (M β CD) was obtained from Sigma-Aldrich and was prepared as a 10 mM stock in serum-free media.

cAMP Measurements. cAMP was assayed by the colorimetric cAMP Direct Biotrak enzyme immunoassay (EIA) kit (Amersham/GE Healthcare, Pittsburgh, PA). Measurements were performed following the manufacturer's instructions for the nonacetylation EIA procedure. Briefly, PC12 cells were seeded in 96-well plates at a density of 5×10^5 cells/well and grown overnight. The next day, media were changed to DMEM containing IBMX (500 μ M) to inhibit phosphodiesterase activity. PACAP-38 or forskolin was then added at the indicated concentrations at 37°C. After 20 minutes of stimulation, media were removed and cells were lysed using the Novel Lysis Reagent provided by the manufacturer. For cholesterol depletion experiments, PC12 cells were plated, as described above. The following day, media were gently aspirated and cells were treated with M β CD dissolved in serum-free DMEM at the indicated final concentrations. Cholesterol depletion was accomplished by pretreatment with M β CD for 1 hour at 37°C. M β CD-containing media were then removed, followed by a gentle rinse in sterile PBS (200 μ l/well). IBMX, and PACAP or forskolin, dissolved in culture media, were then added, as described above. Detection of intracellular cAMP in lysates was performed by EIA, conducted according to the protocol provided by the manufacturer. Raw data were fit to cAMP standard curves and are expressed as fmol cAMP per well.

Reverse-Transcription Polymerase Chain Reaction. RNA was collected from PC12 cells in TRIzol and was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. cDNA was synthesized from 2 μ g RNA from each sample using SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Reverse-transcription polymerase chain reaction (RT-PCR) amplifications were carried out as 50 μ l reactions using Platinum Taq Polymerase (Invitrogen) using the manufacturer's components and suggested ratios. Transcript-specific primer sequences are listed in Table 1. Samples were heated to 94°C for 5 minutes, followed by denaturation at 94°C for 1 minute, annealing for 1 minute (temperatures for each pair listed in Table 1), and extension at 72°C for 1 minute. Following 40 cycles, there was a final extension (72°C for 10 minutes), and samples were kept at -20°C. Amplicons were visualized on gels made of 1% agarose dissolved in Tris-acetate EDTA buffer that were stained with ethidium bromide (100 ng/ml) and photographed under UV transillumination.

Short Hairpin RNA. The expression of AC6 and AC7 was silenced using short hairpin RNA (shRNA) expressed in feline immunodeficiency virus lentiviral vectors (Lenti-Pac FIV Expression Packaging Kit; GeneCopoeia, Rockville, MD). Lentiviral particles were harvested from cotransfected human embryonic kidney 293T cells following a method that we previously described (Emery et al., 2014). PC12 cells were transduced with four vectors for each target, followed by selection in puromycin (1 μ g/ml). Following selection of each cell line, $\geq 95\%$ of cells

TABLE 1
Primer sequences and conditions used for RT-PCR

Transcript	Forward (5'-3')	Reverse (5'-3')	Annealing Temperature (°C)
rAC1	CCTTTTGGTCACCTTCGTGT	GGTTTTCCGTGGCTAACTCA	55
rAC2	TTCAACGACTTCAAACCTGC	ACCTCTTCATCACTTCTTCC	55
rAC3	CTTCATAACGCTGCCACTCA	TCTGCAAACAGGATGCTGAC	55
rAC4	TGTGGAGAGAGCAGACATGG	GGAGAAGGAAGAGAAGGAAGG	65
rAC5	CGGAAAGAAGAGAAGGCCATG	CCAAATCCGTCATCCACCTGC	65
rAC6	AGTCACACCCACTGAGGAC	AGGGGTCATGTATTGAGCG	65
rAC7	AAGGCAGCTGAGGACTGGTA	CCCCAGGTATGCTGTTCACT	58
rAC8	AGCCCACTGGATTGCCTCA	TTATAGTCTTCTGCCGTGGT	55
rAC9	TAATGAAGCAGGGGGATGAG	CGATGGCTTTTATCATGCCT	65
rAC10	ACCACCTACATCACACTC	TCAATCACCTCTCCTTCC	55
rGAPDH	GTTACCAGGGCTGCCTTCTC	GGGTTTCCCGTTGATGACC	55

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

expressed visible green fluorescence protein. Knockdown efficiency of the target transcript and its protein product was evaluated by quantitative RT-PCR and Western blotting, respectively. The sequences that conferred the greatest knockdown were 5'-AGCGGTACTTCTTCCA-GAT-3' (rAC6) and 5'-GATCTCTTCATCTACACCG-3' (rAC7). PC12 cells stably expressing scrambled shRNA encoded in the same vector were used as controls. Knockdown of AC6 was confirmed by quantitative RT-PCR following a previously published protocol (Ravni et., 2008). AC6 transcript was detected using the following primers: 5'-GGCAC-CCGACACATCGCCTC-3', and 5'-CGCGGCGTGGAAGGTCAACA-3'.

Western Blotting. PC12 cells were grown overnight in six-well dishes coated with poly-D-lysine and treated as indicated. Cells were lysed in cold buffer (150 mM NaCl, 50 mM Tris-HCl, 1% IGEPAL CA-630, 1 mM EDTA), to which fresh protease and phosphatase inhibitors were added (Pierce Biotechnology/Life Technologies, Grand Island, NY). Lysates were snap-frozen, and protein concentrations were determined using a modified Lowry assay (Bio-Rad Detergent Compatible protein assay [Bio-Rad Laboratories, Hercules, CA]). Samples were diluted in loading and reducing buffers (Invitrogen) to a final protein concentration of 1 µg/µl. Samples were heated to 95°C for 5 minutes, cooled to room temperature, and loaded onto Bis-Tris-buffered polyacrylamide (4–12%) gels. Samples were electrophoretically separated in 4-morpholinepropanesulfonic acid buffer (Invitrogen) at 125 V for 90 minutes. Proteins were then electroblotted (25 V for 3 hours) onto nitrocellulose membranes. Membranes were rinsed in Tris-buffered saline with 0.1% Tween 20 (TBST) and blocked for 1 hour at room temperature in 5% nonfat milk dissolved in TBST. Incubations with primary antibodies were carried out overnight at 4°C with gentle agitation. Primary antibodies were diluted in TBST at the following dilutions: AC6 (Santa Cruz, Santa Cruz, CA; C-17), 1:200; AC7 (Santa Cruz; V-18), 1:500. All other primary antibodies were raised in rabbit and were purchased from Cell Signaling Technology (Danvers, MA). Each was used at a dilution of 1:1000: cAMP response element-binding protein (CREB) (48H2); extracellular signal-regulated kinase (ERK) (9102); glyceraldehyde-3-phosphate dehydrogenase (D16H11); p38 (D13E1); phospho-CREB Ser¹³³ (9191); phospho-ERK (9101); phospho-p38 (D3F9). Unbound primary antibodies were removed in five washes in TBST, followed by labeling with secondary antibodies dissolved in 5% milk-TBST for 1 hour at room temperature with gentle agitation. Horseradish peroxidase-coupled secondary antibodies used were goat anti-rabbit (CST; 1:2000) and mouse anti-goat (Pierce; 1:5000–1:10,000). Membranes were washed five times, and immunoreactive bands were visualized using a chemiluminescent substrate (West Pico; Pierce). Blots were photographed by a cooled charge-coupled device camera (Protein Simple, San Jose, CA). Bound antibodies were removed by 20-minute incubation in a commercially available stripping buffer (Restore; Pierce). Membranes were then washed extensively, blocked, and reprobed to confirm equal loading. Quantification of Western blots displaying both total and phosphorylated kinases (i.e., ERK1/2, CREB, and p38) was performed densitometrically using ImageJ Software (Schneider et al., 2012). Each image, from 30-second exposure, was

saved as TIFF file that was randomly assigned a file name for analysis. Following analysis, paired data from each membrane were combined and expressed as a ratio of intensity of phosphorylated to total protein.

Neurite Outgrowth Assays. PC12 cells were seeded into poly-D-lysine-coated 12-well plates. Media were changed the following day to initiate treatment with PACAP-38 (100 nM) or forskolin (25 µM). Following 48 hours of treatment, images of cells were randomly acquired using a computer-assisted inverted microscope with a 20× lens. Imagefile names were randomized, and a blinded observer counted the number of cells and neurites and traced the length of each neurite using NIS-Elements BR (Nikon, Tokyo, Japan).

Calculations and Statistics. Curves were fit to dose-response data using four-parameter logistic regressions using Sigma Plot (Systat, San Jose, CA). In experiments using only categorical variables, data were analyzed by two-way analysis of variance, followed by Bonferroni-corrected *t* tests to compare mean values observed in treated samples with those seen in untreated controls.

Results

Neuroendocrine cells are known to express multiple isoforms of adenylate cyclase. We wanted to see whether PACAP signaling through the PAC₁ receptor is mediated through stimulation of a particular repertoire of ACs or whether this receptor signals indiscriminately through AC isoforms (“collision coupling”). We first determined whether mRNA for each of the ten AC isoforms is expressed in PC12 cells by amplification in 40 cycles of RT-PCR. As seen in Fig. 1, PC12 cells express a subset of the AC isoforms present in the brain: mRNAs encoding AC3, AC4, AC6, AC7, and AC9 were detected in these cells.

To see whether PACAP/PAC₁ receptor signaling is mediated through a specific isoform, we used lentiviral shRNA to knock down the expression of the two most prominently expressed AC isoforms: AC6 and AC7. Lentiviral shRNA constructs that provided suitable knockdown of each enzyme were identified by Western blotting comparing the abundance of the respective protein product of the transcript in transduced cells with cells stably expressing scrambled shRNA using the same lentiviral vector backbone (Fig. 2). Given the presence of off-target immunoreactivity using an AC5/6 antibody, we first established that the immunoreactive band at the predicted molecular weight of the target (AC6) was sensitive to adsorption by the peptide antigen against which this polyclonal antibody was raised. Furthermore, we confirmed by quantitative RT-PCR that AC6 shRNA caused an approximate 75% reduction in AC6-encoding mRNA as compared either to the untransduced parental cell line, or to a PC12 subline generated

Fig. 1. Comparative expression of transcripts encoding each AC isoform in PC12 cells relative to rat brain. Images are photographs of gels resolving samples of cDNA from rat brain or PC12 cell mRNA following 40 cycles of RT-PCR amplification using transcript-specific primers (sequences and amplification conditions are listed in Table 1).

Fig. 2. AC6 and AC7 were individually silenced using shRNA. PC12 cells were transduced with shRNA targeting AC6 or AC7 expressed in retroviral vectors. Following transduction, cells were selected with puromycin. (A) AC6 knockdown: lane 1, untransduced cells; lane 2, cells expressing scrambled shRNA; lane 3, cells expressing AC6 shRNA. Note: band visible at ~38 kDa is most likely nonspecific, as it was seen following antibody adsorption with antigen peptide (data not shown). (B) AC7 knockdown: lane 1, PC12 cells expressing scrambled shRNA; lane 2, cells expressing AC7 shRNA. Similar results were obtained in three to four independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MW, molecular mass.

in parallel cultures to stably express scrambled shRNA that was introduced in the same vector.

AC6 Mediates PACAP-Dependent cAMP Elevation

PACAP-dependent cAMP elevation was measured in cell lines stably expressing AC6 or AC7 shRNA. As seen in Fig. 3A, AC6 shRNA caused an approximate 83% decrease in the maximal effect of PACAP on cAMP elevation. Forskolin-dependent cAMP elevation was not significantly affected by introduction of AC6 shRNA (Fig. 3B). In contrast, AC7 shRNA did not affect PACAP-dependent cAMP elevation (Fig. 3A). Forskolin-dependent cAMP elevation was attenuated in cells expressing AC7 shRNA by 16% (Fig. 3B). These data suggest that all PAC₁ receptor-induced cAMP elevation is mediated by AC6, whereas forskolin accomplishes this task by engagement of multiple AC isoforms, evidently including AC7, but not necessarily AC6.

AC6 Is Necessary for PACAP-Dependent ERK, p38, and CREB Phosphorylation

Because silencing AC6 caused a reduction in PACAP-dependent cAMP elevation, we hypothesized that PACAP should not cause differentiation in PC12 cells deficient in AC6, whereas agents acting downstream of the PAC₁ receptor to elevate or mimic cAMP should be capable of promoting differentiation in these cells. As seen in Fig. 4, PACAP-induced phosphorylation of ERK and p38 was obtunded in cells expressing AC6 shRNA relative to cells expressing scrambled shRNA. In contrast, the AC activator forskolin (25 μ M) promoted ERK and p38 phosphorylation to a similar extent in both cell sublines (Fig. 4). In contrast, AC7 shRNA did not have an apparent differential effect on PACAP- or forskolin-dependent phosphorylation of ERK or

Fig. 3. AC6 mediates PACAP-dependent cAMP elevation. (A) Measurements of cAMP elevation in PC12 cells treated as indicated for 20 minutes in the presence of phosphodiesterase inhibitor IBMX (500 μ M). Data points represent means from three determinations and error bars represent the S.E.M. Curves were fit to data by four-parameter logistic regressions. This experiment was repeated two to four times with similar results. (B) Forskolin (25 μ M)-dependent cAMP elevation in PC12 cells expressing scrambled shRNA or shRNA targeting AC6 or AC7 did not vary significantly. Bars represent means from three independent experiments performed with triplicate determinations. Data were analyzed by two-way analysis of variance followed by Bonferroni-corrected *t* tests comparing samples treated with IBMX and vehicle (0.02% dimethylsulfoxide) with those treated with IBMX and forskolin; ****P* < 0.001.

p38, but appears to have caused a minor attenuation in the effects of both (Fig. 4).

Likewise in cells expressing scrambled shRNA, both PACAP and forskolin caused an increase in the abundance of CREB phosphorylated at Ser¹³³ (Fig. 4). Consistent with the notion that AC6 is necessary for PACAP-dependent cAMP elevation, PACAP failed to promote CREB phosphorylation in cells expressing shRNA against AC6, whereas forskolin treatment caused CREB phosphorylation in these cells. In cells expressing AC7 shRNA, both PACAP and forskolin promoted CREB phosphorylation, but to a similar extent compared to cells expressing scrambled shRNA.

PACAP Requires AC6 to Cause PC12 Cell Differentiation. Previously, we and others have reported that PACAP causes differentiation (neurite extension and growth arrest) in

Fig. 4. AC6 mediates PACAP-dependent phosphorylation of ERK, CREB, and p38. (A) PC12 cell sublines stably expressing either AC7 shRNA (lanes 1–3), AC6 shRNA (lanes 4–6), or scrambled shRNA (lanes 7–9) were treated for 10 minutes with either PACAP-38 (100 nM) or forskolin (25 μ M). Following 10 minutes of treatment, cells were lysed and equal amounts of total protein from each sample were probed with antibodies targeting ERK1/2: dual phosphorylated (Thr²⁰²/Tyr²⁰⁴) and total; CREB, phosphorylated (Ser¹³³) and total; and p38 mitogen-activated protein kinase, dual phosphorylated (Thr¹⁸⁰/Tyr¹⁸²) and total. The images above are representative of results obtained from independent experiments performed three to five times. (B–D) For each phospho- and total protein-pair, data were quantified by densitometry and are expressed as the ratio of signal obtained measuring phosphorylated to total protein. Bars in each panel represent means with error bars corresponding to the S.E.M. Data were analyzed by two-way analyses of variance, followed by Bonferroni-corrected *t* tests comparing values obtained in untreated control cells with those treated with either PACAP or forskolin; ****P* < 0.001; NS, not significant.

PC12 cells via cAMP elevation (Ravni et al., 2008; Emery et al., 2014). As seen in Fig. 5, after 48 hours of treatment, PC12 cells expressing scrambled shRNA or AC7 shRNA extended neurites in response to either PACAP-38 (100 nM) or forskolin (25 μ M). In contrast, PACAP failed to induce neurite elongation, whereas forskolin promoted neurite extension normally in AC6-deficient cells. Taken together, these data suggest that AC6 is a necessary component for PACAP to signal via the PAC₁ receptor.

PACAP- but Not Forskolin-Induced cAMP Stimulation Requires Cholesterol. The requirement for AC6 expression for PACAP-, but not forskolin-induced stimulation of cAMP elevation, phosphorylation of ERK, p38, and CREB, as well as PC12 cell neurogenesis, suggests unique properties of AC6,

relative to the other AC isoforms present in PC12 cells. GPCR signaling in other cell systems has been associated with cholesterol-dependent membrane phenomena, including lipid raft formation, although this has not previously been associated with GPCR-AC proximity per se. To investigate the possibility that GPCR-AC signaling is dependent on an intact plasma membrane cholesterol-enriched compartment, we exposed PC12 cells to M β CD, a cholesterol-depleting agent, and examined its effect on cAMP elevation following treatment with either forskolin or PACAP. As seen in Fig. 6A, the maximum soluble concentration of M β CD (10 mM) had no effect on the potency of forskolin, nor did it have a significant inhibitory effect on the activity of forskolin, to stimulate cAMP production.

Fig. 5. PACAP requires AC6 to cause PC12 cell neuritogenesis. PC12 cells stably expressing scrambled shRNA, AC6 shRNA, or AC7 shRNA were treated for 48 hours with vehicle (0.02% dimethylsulfoxide), 100 nM PACAP-38, or 25 μM forskolin. (A) Representative phase-contrast images from experiment performed three times with four determinations per condition; scale bar = 50 μm . (B) Quantification of average length from all images acquired. Bars represent means with error bars corresponding to the S.E.M. Data were analyzed by two-way analysis of variance; Bonferroni-corrected t tests were used to compare values obtained from vehicle-treated controls with treated cells within each PC12 cell subline; *** P < 0.001; NS, not significant.

In contrast, although M β CD failed to influence the apparent potency of PACAP-dependent cAMP stimulation, it caused a $76 \pm 0.5\%$ reduction in the maximal effect of PACAP to stimulate cAMP elevation (Fig. 6A). Further examination of the differential effect of M β CD on cAMP generation revealed that PACAP-induced cAMP elevation was inhibited, in a dose-dependent manner, by pretreatment with M β CD, which had an IC₅₀ of 7.7 mM on PACAP-induced cAMP elevation (Fig. 6B). In contrast, M β CD caused an apparent dose-dependent enhancement in the efficacy of forskolin (Fig. 6B), which is an effect previously reported in similar experiments performed using cardiomyocytes (Rybin et al., 2000).

Taken together, these data indicate that, unlike activation of the other AC isoforms expressed in PC12 cells, PAC₁ receptor-mediated AC6 activation requires the presence of membrane cholesterol. This suggests that this AC isoform, and not the others expressed in PC12 cells, may reside in a plasma membrane compartment that is uniquely accessible to the family B G_s-coupled GPCR PAC₁.

Discussion

Identification of AC isoform-selective small molecule inhibitors is of translational interest for a number of disorders (Seifert et al., 2012), and indeed, compounds targeting several isoforms are currently undergoing rigorous characterization

(Brand et al., 2013; Conley et al., 2013). Our data show that PACAP-induced cAMP elevation is mediated mainly through a single isoform of adenylate cyclase, AC6. The approach we used to explore the subject of differential AC isoform utilization by the G_s-coupled GPCR responsible for PACAP signaling employed cell lines with stably suppressed AC isoform expression. This may serve as a complementary approach for characterization of AC isoform-selective inhibitors.

Downstream of PACAP/PAC₁ signaling, the phosphorylation status of the three kinases, CREB, p38, and ERK, is controlled by the cAMP sensors PKA, Epac2/Rapgef4, and NCS/Rapgef2, respectively, in PC12 cells (Emery et al., 2014). PACAP-dependent cAMP activation that persists after AC6 knockdown, whether due to residual AC6 or a small contribution from other AC isoforms, is clearly insufficient to promote an increase in phosphorylated CREB, p38, or ERK. In contrast, AC7 knockdown was without effect on PACAP-stimulated cAMP elevation, or CREB, p38, or ERK phosphorylation.

Whereas these results suggest that AC6 is necessary for PAC₁ signaling, knockdown of either AC6 or AC7 did not have a detectable effect on forskolin-induced stimulation of cAMP elevation or downstream signaling. This suggests that, in neuroendocrine cells, the PACAP/PAC₁ neuropeptide-GPCR dyad, and forskolin, a diterpene AC activator, may signal through completely separate AC isoforms, predominantly AC6 in the former case, and evidently via any available AC in the latter.

Fig. 6. PACAP- but not forskolin-induced cAMP accumulation requires cholesterol. PC12 cells, grown in 96-well plates, were pretreated with MβCD in culture conditions. After 1 hour, cells were washed and media were replaced with culture media containing IBMX (500 μM). After 20 minutes, PACAP-38, forskolin, or vehicle (0.02% dimethylsulfoxide) was added. After stimulation for 20 minutes, cells were lysed and the abundance of intracellular cAMP in each sample was determined by EIA. (A) Wild-type PC12 cells were pretreated with MβCD (10 mM), followed by IBMX pretreatment and stimulation with varying concentrations of PACAP-38 or forskolin. Data points are means from triplicate determinations, and error bars correspond to the S.E.M. (B) Wild-type PC12 cells were pretreated with MβCD for 1 hour at the indicated concentrations, followed by exposure to IBMX and the addition of either vehicle (0.02% dimethylsulfoxide), forskolin (25 μM), or PACAP-38 (100 nM). Data points represent means from three determinations, and error bars correspond to the S.E.M. Curves were fit to data by four-parameter logistic regressions. Each experiment was independently replicated two to three times with qualitatively similar results.

These overall findings are in agreement with previous reports indicating that the abundance of AC5 (not expressed in PC12 cells) and AC6 (expressed in PC12 cells) is augmented in cholesterol-rich plasma membrane compartments (Thangavel et al., 2009). In contrast, AC7, which we found to be fully

responsive to forskolin, has been reported to be excluded from cholesterol-rich compartments and expressed elsewhere in the plasma membrane (Smith et al., 2002; Crossthwaite et al., 2005). Finally, others have suggested that PAC₁ receptors, like AC5 and AC6, are found in cholesterol-rich membrane fragments upon cell disruption (Zhang et al., 2007). Although the existence of a cholesterol-rich compartment of the plasma membrane containing PAC₁ and AC6 may represent an interesting hypothesis to explain the cholesterol-dependence for PAC₁ signaling reported here, we are not aware of any evidence that provides a hypothetical structural/mechanistic basis to support the notion that movement to such a compartment could be regulated during PAC₁ activation by PACAP. In any event, following the resolution of the structure of the family A neurotensin receptor, it seems quite likely that GPCRs, as integral membrane proteins, must interact with both cholesterol and phospholipids to function as signaling molecules (White et al., 2012), regardless of their organization into a specific plasma membrane domain.

We have previously demonstrated that three cAMP sensors, PKA, Epac2/Rapgef4, and NCS/Rapgef2, parcellate cAMP signaling for cell survival, neuritogenesis, and growth arrest, respectively, in PC12 and NS-1 cells (Emery et al., 2014). Recent reports have suggested that trafficking of AC-activating GPCRs following agonist activation may help to control signaling to various cellular compartments (Zimmerman et al., 2012; Irannejad et al., 2013), leading to trafficking-dependent downstream effects on cellular function. In this work, we find that forskolin-induced CREB, p38, and ERK phosphorylation are equivalently activated in wild-type, AC6-deficient, and AC7-deficient PC12 cells. Furthermore, a cellular physiologic effect of ERK activation, neuritogenesis, is likewise equivalently supported by the collective activation of ACs in the absence of AC6, or in the absence of AC7. Our data support the notion that signaling parcellation is not determined by the association of the receptor to a particular trafficking pattern within the cell, but rather by relatively upstream plasma membrane events that enable GPCR-G_s proximity to AC isoforms differentially distributed in plasma membrane compartments.

Acknowledgments

The authors thank Chang-Mei Hsu and Jill Russ for expert assistance with cell culture and for cotransfections to generate lentiviral vectors encoding shRNAs for AC6 and AC7. We thank Dr. Reinhard Grisshammer for insightful and helpful discussions about this manuscript.

Authorship Contributions

Participated in research design: Emery, M. V. Eiden, L. E. Eiden.
Conducted experiments: Emery, Liu, Xu.
Contributed new reagents or analytic tools: Liu, Xu, M. V. Eiden.
Performed data analysis: Emery, L. E. Eiden.
Wrote or contributed to the writing of the manuscript: Emery, L. E. Eiden, M. V. Eiden.

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