Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

Cyclic AMP elevation and biological signaling through a secretin family G_s-coupled GPCR is restricted to a single adenylate cyclase isoform

Andrew C. Emery, Xiu-Huai Liu, Wenqin Xu, Maribeth V. Eiden, and Lee E. Eiden

Section on Molecular Neuroscience, Laboratory of Cellular and Molecular Regulation, National Institute of Mental Health, Bethesda, MD 20892 (A.C.E., X.H.L., L.E.E.)

Section on Directed Gene Transfer, Laboratory of Cellular and Molecular Regulation, National Institute of Mental Health, Bethesda, MD 20892 (W.X., M.V.E.)

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: PACAP/PAC_1 cyclic AMP signaling is mediated by AC6

Corresponding author:

Lee E. Eiden, Ph.D.

National Institute of Mental Health

49 Convent Drive

Building 49, Room 5A-38

Bethesda, MD 20892-4090

Telephone: +1-301-496-4110, E-mail: eidenl@mail.nih.gov

Number of text pages: 26

Number of figures: 6

Number of references: 23

Number of words in the abstract: 246

Number of words in the introduction: 438

Number of words in the discussion: 631

List of Abbreviations: AC, adenylate cyclase; cAMP, cyclic adenosine 3',5'-monophosphate; CREB, cyclic AMP response element-binding protein; EIA, enzyme immunoassay; Epac, exchange protein activated by cyclic AMP; ERK, extracellular signal-regulated kinases 1 and 2; GEF, guanine nucleotide exchange factor; GPCR, G protein-coupled receptor; IBMX, 3-isobutyl-1-methylxanthine; MAPK, mitogen-activated protein kinase; MβCD, methyl-β-

cyclodextrin; NCS, neuritogenic cAMP sensor; NS-1, Neuroscreen-1; p38, P38 mitogen-activated protein kinases; PAC_1, pituitary adenylate cyclase-activating polypeptide type I receptor; PACAP-38, pituitary adenylate cyclase-activating polypeptide; PDE, phosphodiesterase; PKA, protein kinase A; shRNA, short hairpin RNA

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

PC12 cells express five adenylate cyclase (AC) isoforms, most abundantly AC6 and AC7. These two ACs were individually silenced using lentiviral shRNAs, which lead to a decrease (≥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 GPCR. Cells were challenged with the endogenous agonist of the PAC_1 receptor, PACAP-38, or with the diterpene forskolin as an AC-proximal control. Intracellular cAMP levels were elevated by forskolin about equally in wild-type, and AC6 knockdown cells, with a slight diminution in cAMP elevation in AC7 knockdown cells. The ability of PACAP-38 and forskolin to activate three cAMP sensors downstream of AC (PKA, Epac2/Rapgef4, and NCS/Rapgef2), was examined by monitoring the phosphorylation status of their respective targets, CREB, p38, and ERK. 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 affecting forskolin signaling. These data suggest that vicinal constraint of the GPCR PAC_1, and AC6, determine the exclusive requirement for this AC in PACAP signaling, but that the downstream coupling of the cAMP sensors PKA, Epac2/Rapgef4, and NCS/Rapgef2, to their respective signaling targets, determines how cAMP signaling is parcellated to physiological responses, such as neuritogenesis, upon GPCR-G_s activation in neuroendocrine cells.

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

The specificity of signaling through G protein-coupled receptors (GPCRs) is governed by coupling to three major classes of G-proteins: $G\alpha_s$, $G\alpha_q$, and $G\alpha_{i/o}$. 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 (GEFs) for the alpha subunit of G_s ($G\alpha_s$), enabling it to release GDP, bind GTP, dissociate from its $\beta\gamma$ subunits, and acquire a conformation allowing activation of adenylate cyclase (AC). On the other hand, $G\alpha_{i/o}$ coupling facilitates GPCR-mediated inhibition of AC (Gilman, 1984). GPCR-$G\alpha_q$ coupling enhances the catalytic activity of membrane phospholipase Cβ (Qin et al., 2011). The mechanisms that impart signaling specificity downstream of GPCR-$G\alpha_s$ have historically been little explored, possibly due to an assumption that activation of $G\alpha_s$ creates a signaling entity spatially independent of the GPCR, and thus competent to activate any vicinal AC. However, recent work clarifying the kinetics of GPCR-$G\alpha_s$-AC signaling has made it clear that GPCRs and ACs are not randomly distributed in the plasma membrane (Head et al., 2006; Ostrom and Insel, 2004). 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 $G\alpha_s$ activation in the vicinity of one or another AC isoform. For example, studies in myocytes have revealed that AC5/6, as distinct from AC3, likely occupies a caveolin-rich domain of the plasma membrane, and that this differential localization imparts preferential signaling for active β-adrenoceptors (Insel and Patel, 2009; Ostrom et al., 2012).

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

We have been exploring cAMP coupling features of a secretin-family GPCR, pituitary adenylate cyclase-activating polypeptide type I receptor (PAC_1), which is selectively activated by its endogenous neuropeptide agonist PACAP-38. Previously, we have shown that PACAP-PAC_1 signals to three separate cAMP sensors downstream of AC: PKA, Epac2/Rapgef4, and NCS/Rapgef2, in neuroendocrine cells (Emery et al., 2014). In order to determine if cAMP signaling initiated by the PAC_1 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, NCS/Rapgef2, we report here that in contrast to the effects seen following treatment with the diterpene forskolin, PACAP-PAC_1 signaling occurs specifically through AC6. The specificity of PACAP-PAC_1 signaling through AC6 is further supported by our results that in wild-type PC12 cells, that PACAP-initiated cAMP signaling, but not forskolin-initiated cAMP signaling, is sensitive to depletion of cholesterol.

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

Cell culture. The PC12-G cell line (referred to here 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 μg/ml streptomycin. Human embryonic kidney (HEK) 293T cells, obtained from Cell Genesys Inc. (Foster City, CA), were cultured in DMEM containing GlutaMax™ that was supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell cultures were kept at 37° C in a humidified incubator containing 10% CO_2 for PC12 cells, and 5% CO_2 for 293T cells. Cells were used between passages five and 23 for the experiments reported here and routinely tested negative for mycoplasma.

Drugs and reagents. PACAP-38 was purchased from Phoenix Pharmaceutics (Burlingame, CA) or AnaSpec (Fremont, CA) and was prepared as a 20 μM stock in media. Forskolin was purchased from Tocris (Ellisville, MO) and was prepared as a 50 mM stock in DMSO. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was purchased from Sigma (St. Louis, MO) and was prepared as a 125 mM stock in DMSO. The cholesterol-depleting reagent methyl-β-cyclodextrin (MβCD) was obtained from Sigma and was prepared as a 10 mM stock in serum-free media.

Cyclic AMP measurements. Cyclic AMP was assayed by the colorimetric cAMP Direct Biotrak EIA kit (Amersham, GE Catalog #RPN2255). Measurements were performed following the manufacturer's instructions for the non-acetylation EIA procedure. Briefly, PC12 cells were

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

seeded in 96-well plates at a density of $5x10^5$ cells per well and grown overnight. The next day, media were changed to DMEM containing IBMX (500 μM) 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 MβCD dissolved in serum-free DMEM at the indicated final concentrations. Cholesterol depletion was accomplished by pretreatment with MβCD for one hour at 37° C. MβCD-containing media were then removed followed was gentle rinse in sterile PBS (200 μl/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 the protocol provided by the manufacturer. Raw data were fit to cAMP standard curves and are expressed as fmol of cAMP per well.

RT-PCR. 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 μg of RNA from each sample using SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. RT-PCR amplifications were carried out as 50 μl reactions using Platinum Taq Polymerase (Invitrogen) following the manufacturer's components and suggested ratios. Gene-specific primers 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

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

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 shRNA expressed in feline immunodeficiency virus lentiviral vectors (Lenti-Pac™ FIV Expression Packaging Kit, GeneCopoeia, Rockville, MD). Lentiviral particles were harvested from co-transfected HEK 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 μg/ml). Following selection of each cell line, $\geq$ 95% of cells expressed visible GFP. Knockdown efficiency of the target transcript and its protein product was evaluated by qRT-PCR and Western blotting, respectively. The sequences that conferred the greatest knockdown were AGCGGTACTTCTTCCAGAT (rAC6) and GATCTCTTCATCTACACCG (rAC7). PC12 cells stably expressing scrambled shRNA encoded in the same vector were used as controls.

Western blotting. PC12 cells were grown overnight in 6-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, Catalog #78440). Lysates were snap-frozen and protein concentrations were determined using a modified Lowry assay (Bio-Rad DC protein assay). 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 MOPS buffer (Invitrogen) at 125 V for 90 minutes. Proteins were then electroblotted (25 V for 3 hours) onto nitrocellulose membranes. Membranes were

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

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, 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 – CREB (48H2); ERK (9102); GAPDH (D16H11); p38 (D13E1); phospho-CREB Ser^{133} (9191); phospho-ERK (9101); phospho-p38 (D3F9). Unbound primary antibodies were removed in five washes in TBST, followed by labeled with secondary antibodies dissolved in 5% milk-TBST for 1 hour at room temperature with gentle agitation. Horse radish peroxidase-coupled secondary antibodies used were goat anti-rabbit (CST, 1:2000) and mouse anti-goat (Pierce, 1:5000-1:10000). Membranes were washed five times and immunoreactive bands were visualized using a chemiluminescent substrate (Pierce, West Pico). Blots were photographed by CCD 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 re-probed to confirm equal loading. Quantification of Western blots examining 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 analyzed and then saved as TIFF file that was randomly assigned file names for analysis. Following analysis, paired data from each membrane was 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

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

forskolin (25 μM). Following 48 hours of treatment, images of cells were randomly acquired using computer-assisted inverted microscope with a ×20 lens. Images file 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).

Calculations and statistics. Curves were fit to dose-response data using 4-parameter logistic regressions using Sigma Plot (Systat). In experiments using only categorical variables, data were analyzed by 2-way ANOVA followed by Bonferroni-corrected t-tests to compare mean values observed in treated samples to those seen in untreated controls.

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Neuroendocrine cells are known to express multiple isoforms of adenylate cyclase. We wanted to see whether PACAP signaling through the PAC_1 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/PAC_1 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 as compared to 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 IR 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 qRT-PCR that AC6 shRNA caused an approximate 75% reduction in AC6-encoding mRNA as compared either to either the untransduced parental cell line, or to a PC12 subline generated 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

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

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). On the other hand, 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 PAC_1 receptor-induced cAMP elevation is mediated by AC6, while 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. Since silencing AC6 caused a reduction in PACAP-dependent cAMP elevation, we hypothesized that PACAP should not cause differentiation in PC12 cells deficient in AC6, while agents act downstream of the PAC_1 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. On the other hand, the AC activator forskolin (25 μM) 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 p38, but appears to have caused a minor attenuation in the effects of both (Fig. 4).

In cells expressing scrambled shRNA, both PACAP and forskolin caused an increase in the abundance of CREB phosphorylated at Ser^{133} (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, while forskolin treatment caused CREB phosphorylation in these cells. In cells expressing AC7 shRNA, both PACAP and

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

forskolin promoted CREB phosphorylation, but to a lesser extent than in 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 PC12 cells via cyclic AMP elevation (Emery et al., 2014; Ravni et al., 2008). As seen in Fig. 5, after 48 hours of treatment, PC12 cells expressing scrambled shRNA or AC7 shRNA differentiated in response to either PACAP-38 (100 nM) or forskolin (25 μM). In contrast, PACAP failed to induce neurite elongation while forskolin promotes neuritogenesis in AC6-deficient cells. Taken together, these data suggest that AC6 is a necessary component for PACAP to signal via the PAC_1 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 neuritogenesis 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-AC6 signaling is dependent on an intact plasma membrane lipid raft-enriched compartment, we exposed PC12 cells to MβCD, 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 MβCD (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. On

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

the other hand, although MβCD failed to influence the apparent potency of PACAP-dependent cAMP stimulation, it caused a 76 ± 0.5% reduction in the maximal effect (E_{MAX}) of PACAP to stimulate cAMP elevation (Fig. 6A). Further examination of the differential effect of MβCD on cAMP generation revealed that PACAP-induced cAMP elevation was inhibited, in a dose-dependent manner, by pretreatment with MβCD, which had a half-maximal inhibitory effect (IC_{50}) of 7.7 mM on PACAP-induced cAMP elevation (Fig. 6B). In contrast, MβCD 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, that PAC_1 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 PAC_1.

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 cyclic AMP 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 was by the use of cell lines with stably suppressed AC isoform expression. This may serve as a complementary approach for characterization of AC isoform-selective inhibitors.

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

Downstream of PACAP/PAC_1 signaling, the phosphorylation status of the three kinases CREB, p38, and ERK is mediated by the cAMP sensors PKA, Epac2/Rapgef4, and NCS/Rapgef2, respectively, in PC12 cells (Emery et al., 2014). PACAP-dependent cyclic AMP activation that persists after AC6 knock-down, 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, to equivalent levels as those reached with AC6, was without effect on PACAP-stimulated cyclic AMP elevation, or CREB, p38, or ERK phosphorylation.

While these results suggest that AC6 is necessary for PAC_1 signaling, ablation of either AC6 or AC7 did not have a detectible effect on forskolin-induced stimulation of cAMP elevation or downstream signaling. This suggests that in neuroendocrine cells, the PACAP/PAC_1 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.

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-enriched 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 expressed excluded from cholesterol-enriched plasma membrane compartments and expressed elsewhere in the plasma membrane (Crossthwaite et al., 2005; Smith et al., 2002). Finally, others have suggested that PAC_1 receptors are also expressed at relatively high levels in caveolin/cholesterol-enriched plasma membrane compartments (Zhang et al., 2007). While the existence of a cholesterol-enriched compartment of the plasma membrane comprising PAC_1 and

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

AC6 may represent an interesting hypothesis, we are not aware of any evidence that provides a hypothetical structural/mechanistic basis to support the notion that such an interaction could be regulated during PAC_1 activation by PACAP. In fact, following the resolution of the structure of the Family A neurotensin receptor (NTSR1), it seems quite likely that GPCRs, as integral membrane proteins, must interact with both cholesterol and phospholipids in order to function as signaling molecules (White et al., 2012),

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 (Irannejad et al., 2013; Zimmerman et al., 2012), and leading to trafficking-dependent downstream effects on cellular function. Here, we find that cAMP-dependent CREB, p38, and ERK phosphorylation are equivalently activated in the absence of either AC6 or AC7 in PC12 cells. Furthermore, a cellular physiological 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.

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgments

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

Authorship Contributions

Participated in research design: Emery, M. V. Eiden, L. E. Eiden

Contributed new reagents or analytical tools: Liu, Xu, M. V. Eiden

Conducted experiments: Emery, Liu, Xu

Performed data analysis: Emery, L. E. Eiden

Contributed to the writing of the manuscript: Emery, L. E. Eiden, M. V. Eiden

REFERENCES

Brand CS, Hocker HJ, Gorfe AA, Cavasotto CN and Dessauer CW (2013) Isoform selectivity of adenylyl cyclase inhibitors: characterization of known and novel compounds. *J Pharmacol Exp Ther* **347**: 265-275.

Conley JM, Brand CS, Bogard AS, Pratt EP, Xu R, Hockerman GH, Ostrom RS, Dessauer CW and Watts VJ (2013) Development of a high-throughput screening paradigm for the discovery of small-molecule modulators of adenylyl cyclase: identification of an adenylyl cyclase 2 inhibitor. *J Pharmacol Exp Ther* **347**: 276-287.

Crossthwaite AJ, Seebacher T, Masada N, Ciruela A, Dufraux K, Schultz JE and Cooper DM (2005) The cytosolic domains of Ca2+-sensitive adenylyl cyclases dictate their targeting to plasma membrane lipid rafts. *J Biol Chem* **280**: 6380-6391.

Emery AC, Eiden MV and Eiden LE (2014) Separate cyclic AMP sensors for neuritogenesis, growth arrest, and survival of neuroendocrine cells. *J Biol Chem* **289**: 10126-10139.

Gilman AG (1984) G proteins and dual control of adenylate cyclase. *Cell* **36**: 577-579.

Head BP, Patel HH, Roth DM, Murray F, Swaney JS, Niesman IR, Farquhar MG and Insel PA (2006) Microtubules and actin microfilaments regulate lipid raft/caveolae localization of adenylyl cyclase signaling components. *J Biol Chem* **281**: 26391-26399.

Insel PA, Head BP, Patel HH, Roth DM, Bundey RA and Swaney JS (2005) Compartmentation of G-protein-coupled receptors and their signalling components in lipid rafts and caveolae. *Biochem Soc Trans* **33**: 1131-1134.

Insel PA and Patel HH (2009) Membrane rafts and caveolae in cardiovascular signaling. *Curr Opin Nephrol Hypertens* **18**: 50-56.

Irannejad R, Tomshine JC, Tomshine JR, Chevalier M, Mahoney JP, Steyaert J, Rasmussen SG, Sunahara RK, El-Samad H, Huang B and von Zastrow M (2013) Conformational biosensors reveal GPCR signalling from endosomes. *Nature* **495**: 534-538.

Oldham WM and Hamm HE (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol* **9**: 60-71.

Ostrom RS, Bogard AS, Gros R and Feldman RD (2012) Choreographing the adenylyl cyclase signalosome: sorting out the partners and the steps. *Naunyn Schmiedebergs Arch Pharmacol* **385**: 5-12.

Ostrom RS and Insel PA (2004) The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology. *Br J Pharmacol* **143**: 235-245.

Qin K, Dong C, Wu G and Lambert NA (2011) Inactive-state preassembly of G(q)-coupled receptors and G(q) heterotrimers. *Nat Chem Biol* **7**: 740-747.

Rausch DM, Iacangelo AL and Eiden LE (1988) Glucocorticoid- and nerve growth factor-induced changes in chromogranin A expression define two different neuronal phenotypes in PC12 cells. *Mol Endocrinol* **2**: 921-927.

Ravni A, Vaudry D, Gerdin MJ, Eiden MV, Falluel-Morel A, Gonzalez B, Vaudry H and Eiden LE (2008) A cAMP-dependent, PKA-independent signaling pathway mediating neuritogenesis through Egr1 in PC12 cells. *Mol Pharmacol* **73**: 1688-1708.

Rybin VO, Xu X, Lisanti MP and Steinberg SF (2000) Differential targeting of beta -adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway. *J Biol Chem* **275**: 41447-41457.

Schneider CA, Rasband WS and Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**: 671-675.

Seifert R, Lushington GH, Mou TC, Gille A and Sprang SR (2012) Inhibitors of membranous adenylyl cyclases. *Trends Pharmacol Sci* **33**: 64-78.

Smith KE, Gu C, Fagan KA, Hu B and Cooper DM (2002) Residence of adenylyl cyclase type 8 in caveolae is necessary but not sufficient for regulation by capacitative Ca(2+) entry. *J Biol Chem* **277**: 6025-6031.

Thangavel M, Liu X, Sun SQ, Kaminsky J and Ostrom RS (2009) The C1 and C2 domains target human type 6 adenylyl cyclase to lipid rafts and caveolae. *Cell Signal* **21**: 301-308.

White JF, Noinaj N, Shibata Y, Love J, Kloss B, Xu F, Gvozdenovic-Jeremic J, Shah P, Shiloach J, Tate CG and Grisshammer R (2012) Structure of the agonist-bound neurotensin receptor. *Nature* **490**: 508-513.

Zhang W, Duan W, Cheung NS, Huang Z, Shao K and Li QT (2007) Pituitary adenylate cyclase-activating polypeptide induces translocation of its G-protein-coupled receptor into caveolin-enriched membrane microdomains, leading to enhanced cyclic AMP generation and neurite outgrowth in PC12 cells. *J Neurochem* **103**: 1157-1167.

Zimmerman B, Beautrait A, Aguila B, Charles R, Escher E, Claing A, Bouvier M and Laporte SA (2012) Differential beta-arrestin-dependent conformational signaling and cellular responses revealed by angiotensin analogs. *Sci Signal* **5**: ra33.

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

This work was funded by the 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 & Behavior Research Foundation [Grant # 21356].

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 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 cells following 40 cycles of RT-PCR amplification using gene-specific primers (sequences and amplification conditions are listed in Table 1).

Figure 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 likely non-specific 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 3-4 independent experiments.

Figure 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 PDE inhibitor IBMX (500 μM). Data points represent means from three determinations and error bars represent the SEM. Curves were fit to data by 4-parameter logistic regressions. This experiment was repeated 2-4 times with similar results. (B) Forskolin (25 μM)-dependent cyclic AMP 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 2-way ANOVA followed by Bonferroni-corrected *t*-tests comparing samples treated with IBMX and vehicle (0.02% DMSO) to those treated with IBMX and forskolin, ***$P<0.001$.

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 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 min with either PACAP-38 (100 nM) or forskolin (25 μM). Following 10 min of treatment, cells were lysed and equal amounts of total protein from each sample were probed with antibodies targeting ERK1/2: dual phosphorylated (Thr^{202}/Tyr^{204}) and total; CREB, phosphorylated (Ser^{133}) and total; and p38 MAPK, dual phosphorylated (Thr^{180}/Tyr^{182}) and total. The images above are representative of results obtained from independent experiments performed 3-5 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 2-way ANOVAs followed by Bonferroni-corrected t-tests comparing within values obtained in untreated control cells to those treated with either PACAP or forskolin, ***P<0.001, NS, not significant.

Figure 5. PACAP requires AC6 to cause PC12 cell differentiation. PC12 cells stably expressing scrambled shRNA, AC6 shRNA, or AC7 shRNA were treated for 48 hours with vehicle (0.02% DMSO), 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 2-way ANOVA, Bonferroni-corrected *t*-tests were used to compare values obtained from to vehicle-treated controls to treated cells within each PC12 cell subline, ****P*<0.001 *NS*, not significant.

Figure 6. PACAP- but not forskolin-induced cAMP accumulation requires cholesterol. PC12 cells, grown in 96-well plates, were pretreated with methyl-β-cyclodextrin (MβCD) in

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

culture conditions. After 1 hour, cells were washed and media were replaced with culture media containing IBMX (500 μM). After 20 min, PACAP-38, forskolin, or vehicle (0.02% DMSO) was added. After stimulation for 20 min, 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% DMSO), forskolin (25 μM), or PACAP-38 (100 nM). Data points represent means from 3 determinations and error bars correspond to the S.E.M. Curves were fit to data by 4-parameter logistic regressions. Each experiment was independently replicated 2-3 times with qualitatively similar results.

Molecular Pharmacology Fast Forward. Published on March 13, 2015 as DOI: 10.1124/mol.115.098087
This article has not been copyedited and formatted. The final version may differ from this version.

Tables

Table 1. Primers sequences and conditions used for RT-PCR

Transcript	Forward (5′-3′)	Reverse (5′-3′)	Annealing Temp (C)
rAC1	CCTTTTGGTCACCTTCGTGT	GGTTTTCCGTGGCTAACTCA	55
rAC2	TTCAACGACTTCAAACTGC	ACCTCTTCATCACTTCTTCC	55
rAC3	CTTCATAACGCTGCCACTCA	TCTGCAAACAGGATGCTGAC	55
rAC4	TGTGGAGAGAGCAGACATGG	GGAGAAGGAAGAGAAGGAAGG	65
rAC5	CGGAAAGAAGAGAAGGCCATG	CCAAATCGGTCATCCACCTGC	65
rAC6	AGTCACACCCCACTGAGGAC	AGGGGTCATGTATTTGAGCG	65
rAC7	AAGGCAGCTGAGGACTGGTA	CCCCAGGTATGCTGTTCACT	58
rAC8	AGCCACACTGGATTGCCTCA	TTATAGTCTTCTGCCGTGGT	55
rAC9	TAATGAAGCAGGGGGATGAG	CGATGGCTTTTATCATGCCT	65
rAC10	ACCACCTACATCACACTC	TCAATCACCTCTCCTTCC	55
rGAPDH	GTTACCAGGGCTGCCTTCTC	GGGTTTCCCGTTGATGACC	55

Fig. 1

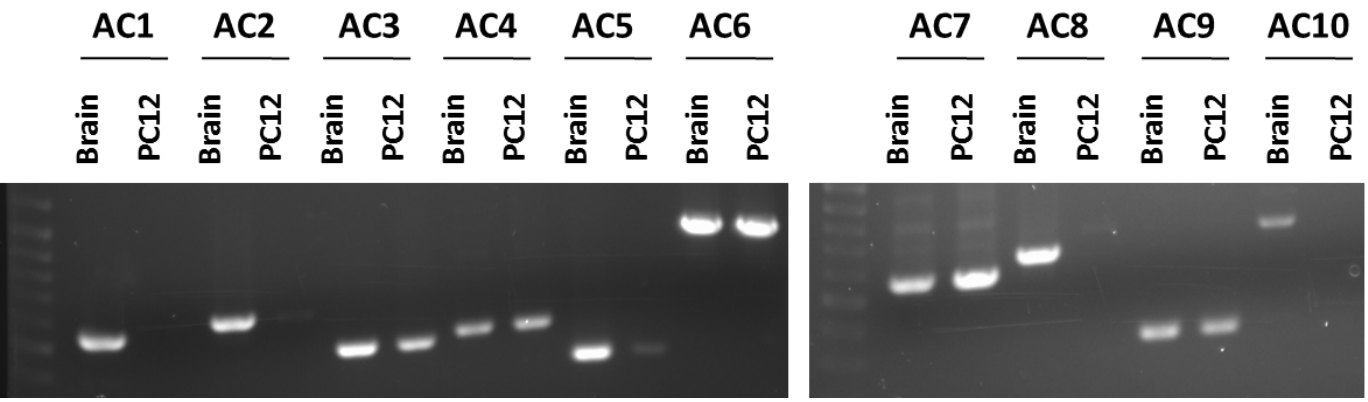


Fig. 2

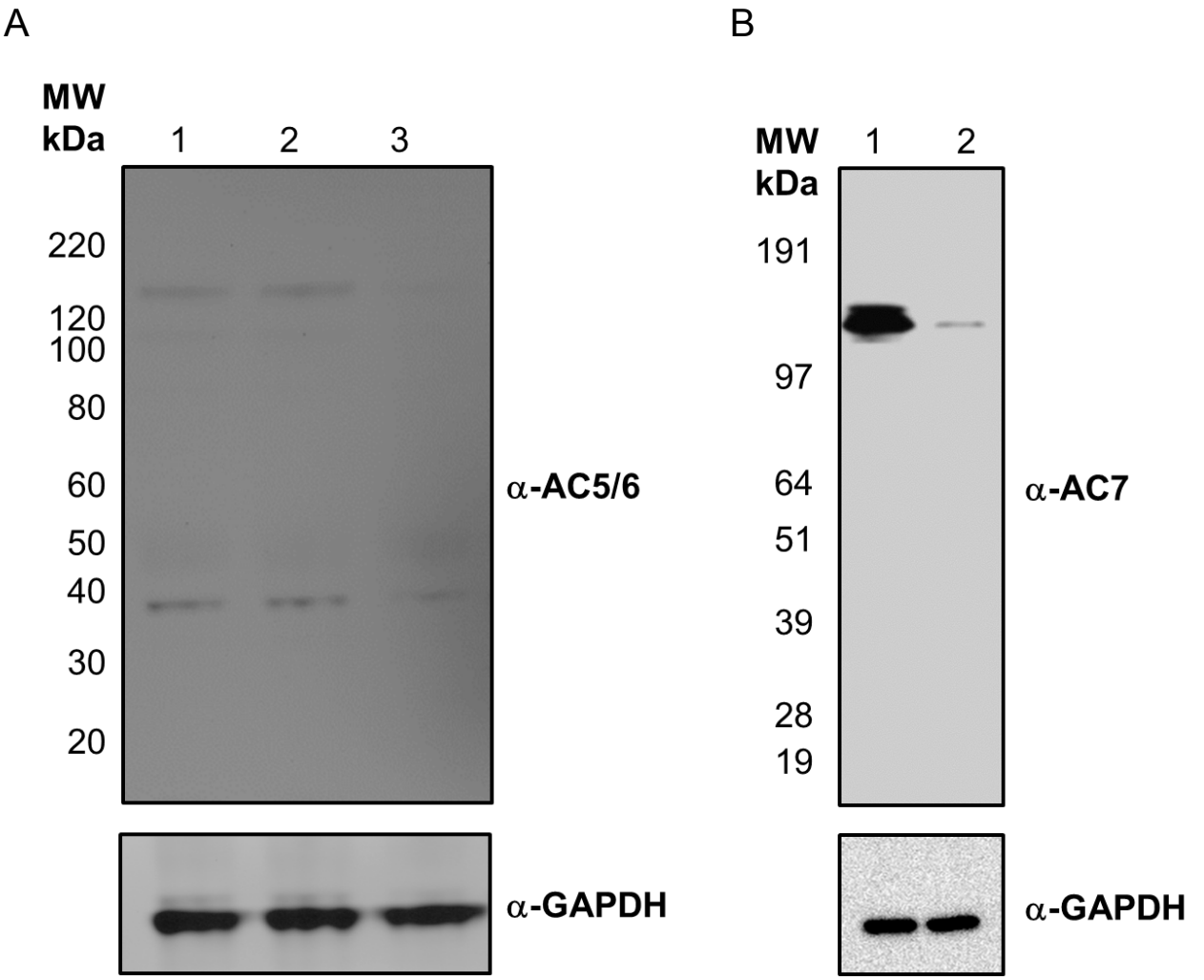
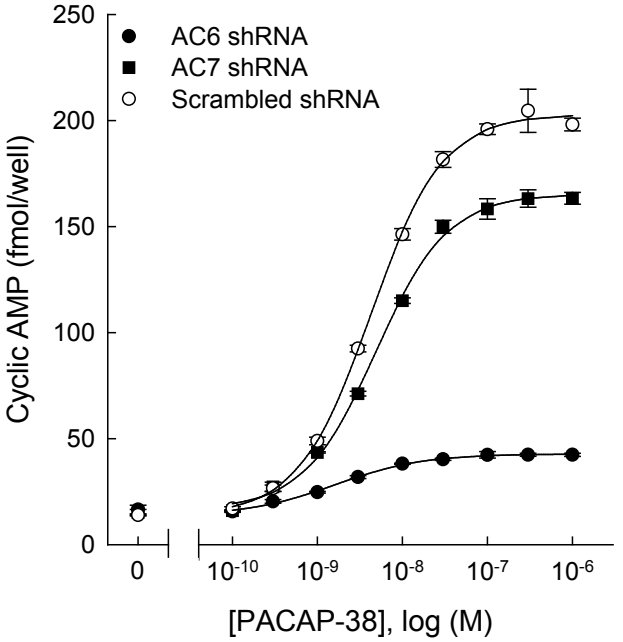

A
MW
kDa
1
2
3
220
120
100
80
60
50
40
30
20
α-AC5/6
α-GAPDH
B
MW
kDa
1
2
191
97
64
51
39
28
19
α-AC7
α-GAPDH

Fig. 3

A


AC6 shRNA
AC7 shRNA
Scrambled shRNA
Cyclic AMP (fmol/well)
250
200
150
100
50
0
0
10-10
10-9
10-8
10-7
10-6
[PACAP-38], log (M)

B

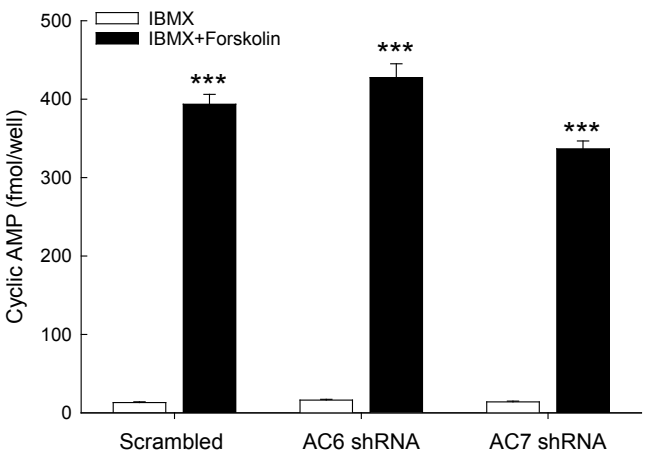

IBMX
IBMX+Forskolin
Cyclic AMP (fmol/well)
500
400
300
200
100
0
***
***
***
Scrambled
AC6 shRNA
AC7 shRNA

Fig. 4

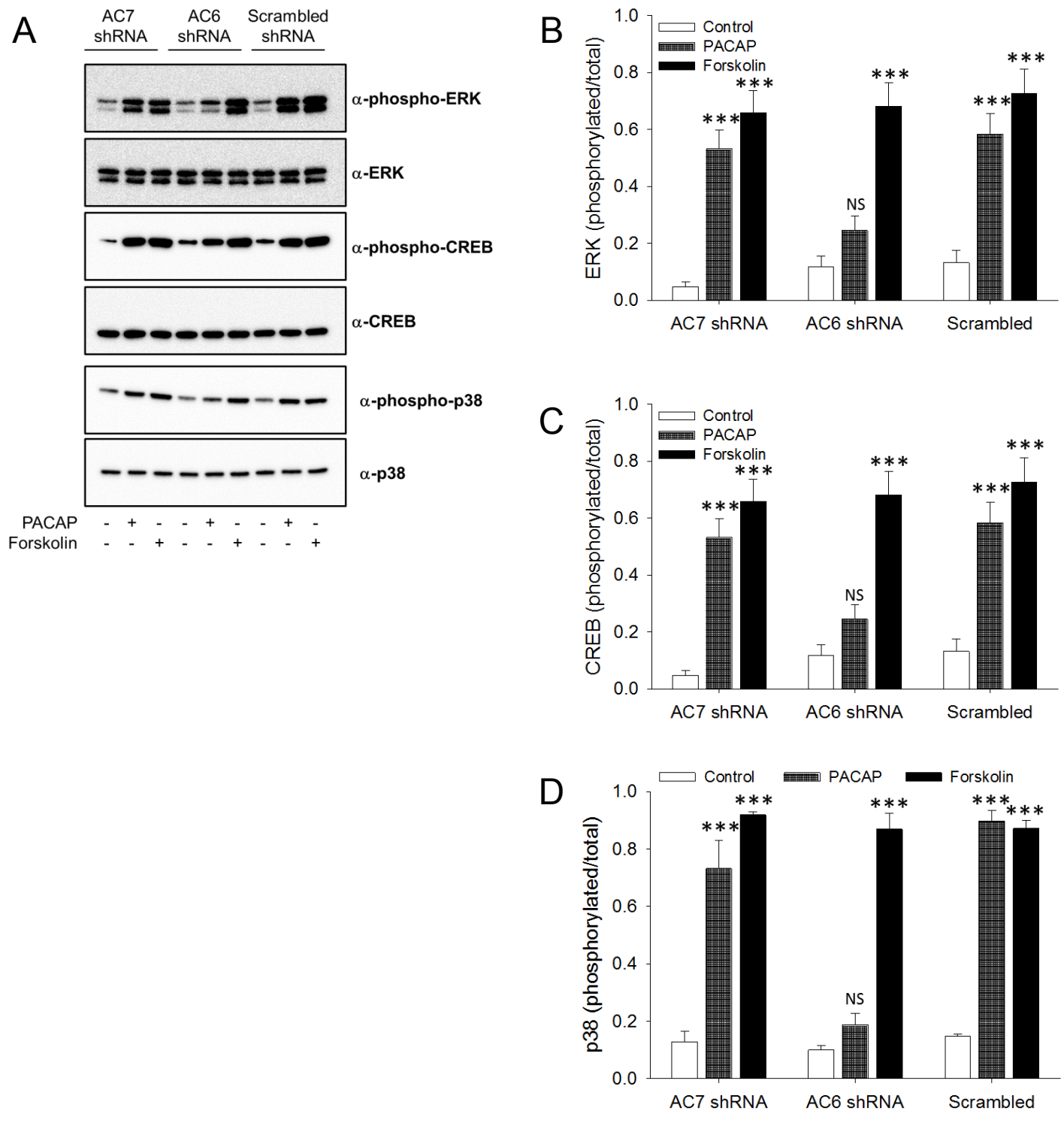
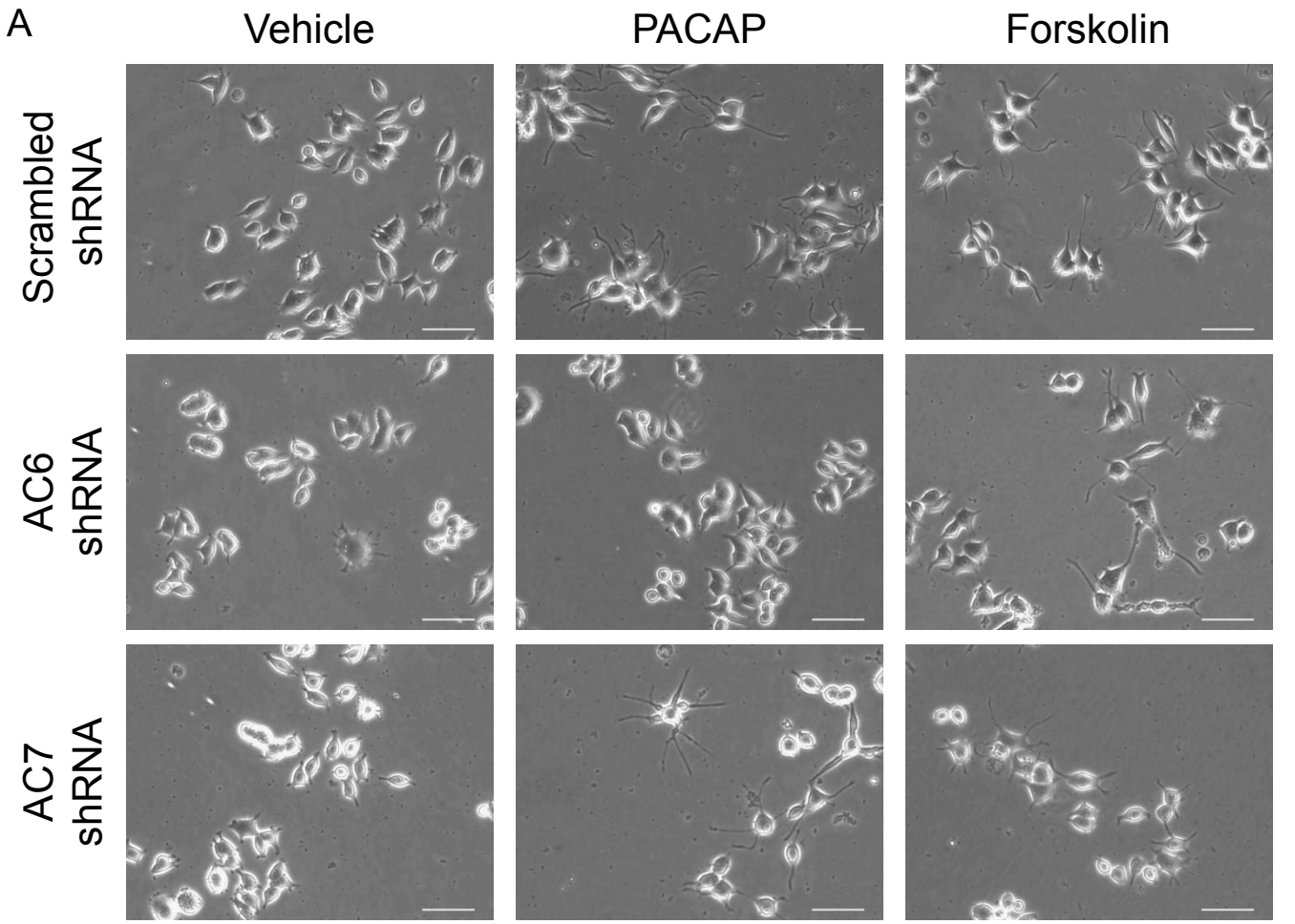

A
AC7 shRNA
AC6 shRNA
Scrambled shRNA
α-phospho-ERK
α-ERK
α-phospho-CREB
α-CREB
α-phospho-p38
α-p38
PACAP
Forskolin
B
ERK (phosphorylated/total)
Control
PACAP
Forskolin
NS
AC7 shRNA
AC6 shRNA
Scrambled
C
CREB (phosphorylated/total)
D
p38 (phosphorylated/total)

Fig. 5


A
Vehicle
PACAP
Forskolin
Scrambled shRNA
AC6 shRNA
AC7 shRNA

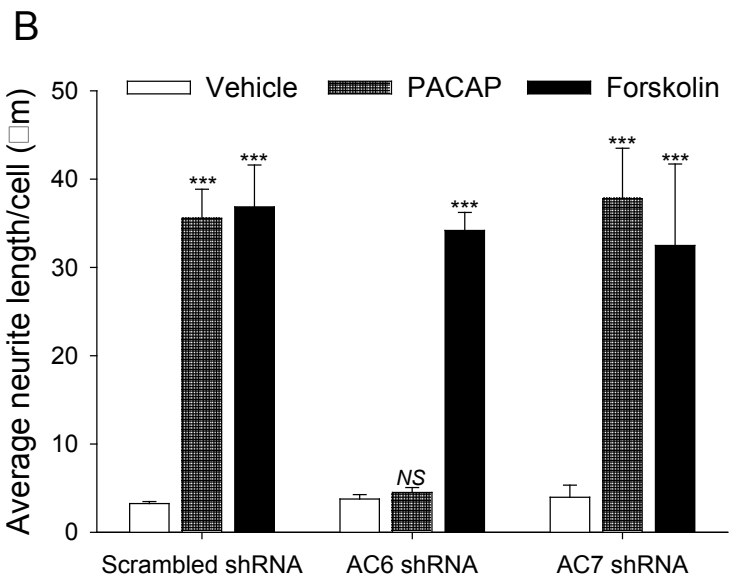
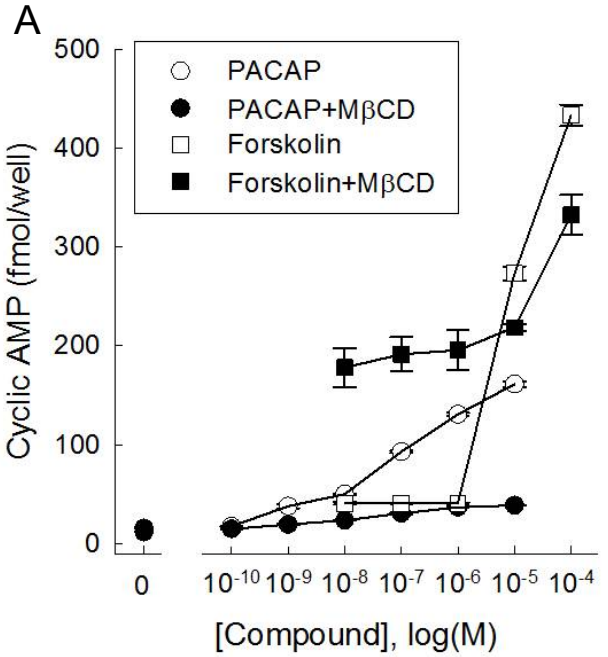
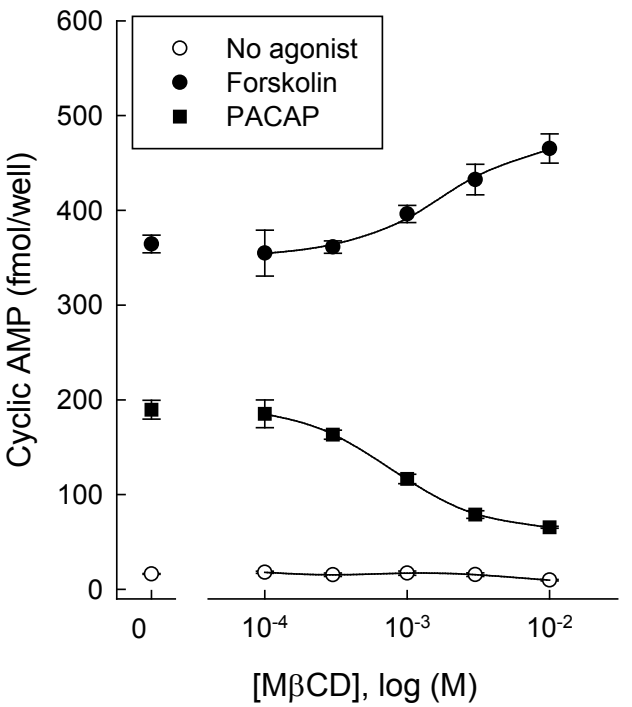

B
Vehicle
PACAP
Forskolin
Average neurite length/cell (□m)
50
40
30
20
10
0
***
***
***
NS
***
***
Scrambled shRNA
AC6 shRNA
AC7 shRNA

Fig. 6

A


PACAP
PACAP+MβCD
Forskolin
Forskolin+MβCD
Cyclic AMP (fmol/well)
500
400
300
200
100
0
0
10-10 10-9 10-8 10-7 10-6 10-5 10-4
[Compound], log(M)

B


No agonist
Forskolin
PACAP
Cyclic AMP (fmol/well)
600
500
400
300
200
100
0
0
10-4
10-3
10-2
[MβCD], log (M)