PACAP-induced ERK activation in HEK cells expressing PAC1 receptors involves both receptor internalization and PKC signaling

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May V, Buttolph TR, Girard BM, Clason TA, Parsons RL. PACAP-induced ERK activation in HEK cells expressing PAC1 receptors involves both receptor internalization and PKC signaling. Am J Physiol Cell Physiol 306: C1068–C1079, 2014. First published April 2, 2014; doi:10.1152/ajpcell.00001.2014.—The pituitary adenylate cyclase-activating polypeptide (PACAP)-selective PAC1 receptor (Adcyap1r1) is a G protein-coupled receptor (GPCR) that activates adenyl cyclase and PLC. Similar to many other GPCRs, our previous studies showed that the PAC1 receptor is internalized after ligand binding to form signaling endosomes, which recruit additional second messenger pathways. Using a human embryonic kidney (HEK 293) PAC1Hop1-EGFP receptor cell line, we have examined how different PAC1 receptor signaling mechanisms contribute to MEK/ERK activation. Unlike PAC1 receptor-stimulated adenyl cyclase/cAMP production in the plasma membrane, PACAP-mediated ERK phosphorylation was partly dependent on receptor internalization, as determined by treatment with pharmacological inhibitors of endocytosis or temperature reduction, which also suppressed receptor internalization. Stimulation of cAMP generation by forskolin or exposure to the cell-permeable cAMP analogs 8-bromo-cAMP and dibutyryl cAMP had minimal effects on ERK phosphorylation in this system. The ability of reduced temperature (24°C) to consistently suppress PACAP activation to a greater extent than the endocytosis inhibitors Pitstop 2 and dynasore indicated that other mechanisms, in addition to PAC1 internalization/endosome activation, were involved. Inhibition of PAC1 receptor-stimulated PLC/diacylglycerol/PKC signaling by bisindoylmaleimide I also attenuated ERK phosphorylation, and direct PKC activation with phorbol ester increased ERK phosphorylation in a temperature-dependent manner. Inhibition of PAC1 receptor endocytosis and PKC activation completely blocked PACAP-stimulated ERK activation. PACAP augmented phosphorylated ERK staining uniformly over the cytoplasm and nucleus, and PKC signaling facilitated nuclear phosphorylated ERK translocation. In sum, our results show that PACAP/PAC1 receptor endocytosis and PLC/diacylglycerol/PKC activation represent two complementary mechanisms contributing to PACAP-induced ERK activation.

PACAP is a neuropeptide with important roles in developmental and neuronal plasticity. As a neuromodulator, PACAP regulates multiple cellular processes, including survival, proliferation, differentiation, and survival in a variety of tissues and experimental conditions (1). PACAP activates intracellular signaling pathways by interacting with its specific receptor, PACAP receptor (PAC1) (2). The PAC1 receptor is a member of the superfamily of G protein-coupled receptors (GPCRs) (3). GPCRs mediate a variety of biological functions by activating phospholipase C (PLC) and adenyl cyclase to generate second messengers, such as diacylglycerol and cyclic AMP (cAMP), respectively (4). These second messengers activate protein kinase C (PKC) and cAMP-dependent protein kinase A (PKA), respectively, which in turn activate multiple intracellular signaling pathways (5). PACAP activates adenylyl cyclase and PLC. Similar to many other GPCRs, our previous studies have shown that the PAC1 receptor is internalized after ligand binding to form signaling endosomes, which recruit additional second messenger pathways. Using a human embryonic kidney (HEK 293) PAC1Hop1-EGFP receptor cell line, we have examined how different PAC1 receptor signaling mechanisms contribute to MEK/ERK activation. Unlike PAC1 receptor-stimulated adenyl cyclase/cAMP production in the plasma membrane, PACAP-mediated ERK phosphorylation was partly dependent on receptor internalization, as determined by treatment with pharmacological inhibitors of endocytosis or temperature reduction, which also suppressed receptor internalization. Stimulation of cAMP generation by forskolin or exposure to the cell-permeable cAMP analogs 8-bromo-cAMP and dibutyryl cAMP had minimal effects on ERK phosphorylation in this system. The ability of reduced temperature (24°C) to consistently suppress PACAP activation to a greater extent than the endocytosis inhibitors Pitstop 2 and dynasore indicated that other mechanisms, in addition to PAC1 internalization/endosome activation, were involved. Inhibition of PAC1 receptor-stimulated PLC/diacylglycerol/PKC signaling by bisindoylmaleimide I also attenuated ERK phosphorylation, and direct PKC activation with phorbol ester increased ERK phosphorylation in a temperature-dependent manner. Inhibition of PAC1 receptor endocytosis and PKC activation completely blocked PACAP-stimulated ERK activation. PACAP augmented phosphorylated ERK staining uniformly over the cytoplasm and nucleus, and PKC signaling facilitated nuclear phosphorylated ERK translocation. In sum, our results show that PACAP/PAC1 receptor endocytosis and PLC/diacylglycerol/PKC activation represent two complementary mechanisms contributing to PACAP-induced ERK activation.

Materials and Methods

Cell culture. All experiments were done on a stable HEK 293 PAC1Hop1-EGFP receptor-expressing cell line. Preparation of the stable HEK 293 PAC1Hop1-EGFP receptor-expressing cells is described in detail elsewhere (28). Briefly, HEK 293 cells were trans-
fected using TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI) and cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 containing 8% fetal bovine serum and 500 μg/ml G418 (Geneticin) for stable cell selection. Individual cell colonies were selected and expanded; functional expression of the receptor was assessed by green fluorescent protein (GFP) fluorescence and PACAP-stimulated second messenger activation.

Unlike primary neuronal cultures, which express multiple PACAP/VIP receptor subtypes or isoforms in a heterogeneous cell population, the PAC1Hop1-EGFP receptor cell line allowed signaling studies of one prominent central nervous system PACAP receptor without complications from other receptor variants. Furthermore, the enhanced GFP (EGFP) tag permitted ready visualization of PAC1 receptor internalization for direct correlation with second messenger activation.

Chemicals. PACAP27 was used exclusively in this study and is referred to as PACAP. All drugs were obtained from commercial sources: PACAP27 from American Peptide (Sunnyvale, CA); forskolin, BimI (bisindoylmaleimide I), H-89 (N-[2-((p-bromocinnamyl)amino)ethyl]-5-isouquinolinesulfonamide), and PMA from Calbiochem EMD Biosciences (La Jolla CA); Pitstop 2 (N-[5-(4-bromobenzylidene)-4-oxo-4,5-dihydro-1,3-thiazol-2-yl]naphthalene-1-sulfonamide) from Abcam Biochemicals (Cambridge, UK); 8-bromo-cAMP (8-BrcAMP) from Enzo Life Sciences (Farmingdale NY); dibutyryl cAMP from Roche (Indianapolis, IN); KT 5720 from Tocris (Ellisville, MO); dynasore (3-hydroxy-naphthalene-2-carboxylic acid (3,4-

A

Fig. 1. Pituitary adenylate cyclase-activating polypeptide (PACAP)-stimulated cAMP generation is not diminished after blunting of endocytosis. A: human embryonic kidney (HEK) PAC1 receptor cultures were incubated in the absence (Vehicle) or presence of the endocytosis inhibitor Pitstop 2 (15 μM) for 10 min at 34°C before PACAP (P27) treatment (25 nM, 15 min). PACAP increased culture cAMP production ~150-fold compared with control (CTL), regardless of endocytosis inhibition. B: PAC1 receptor cells were maintained at 37°C or ambient room temperature (24°C). Compared with untreated CTL, the increase in PACAP-stimulated cAMP generation was not affected by temperature. Values are means ± SE; n = 4. *P < 0.05.

B

Fig. 2. Forskolin increases cAMP generation without inducing ERK phosphorylation. A: treatment of PAC1 receptor cultures with PACAP (P27, 25 nM) or forskolin (FSK, 10 μM) for 20 min at 37°C increased cAMP levels to comparable levels. Values are means ± SE; n = 4. *P < 0.05. B: representative Western blot showing PACAP (25 nM)-stimulated ERK activation at 15 and 120 min; forskolin (10 μM) had no effects on ERK activation at either time point. pERK, phosphorylated ERK. C: PACAP stimulated ERK phosphorylation ~5-fold at 15 min, and levels were attenuated by 120 min. Forskolin (10 μM) had no significant effects on ERK activation at 15 or 120 min. All data were normalized to total ERK levels on the same blots using a pan-ERK antibody probed with a different fluorochrome-conjugated secondary antiserum. Values (means ± SE) represent fold change from untreated control; n = 3. *P < 0.05 vs. CTL; +P < 0.05 vs. 15-min P27-stimulated response (by 1-way ANOVA with Student-Newman-Keuls post hoc test).

C

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dihydroxy-benzylidene)-hydrazide hydrate) from Sigma-Aldrich (St. Louis, MO); and RO20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] from EMD Millipore (Billerica, MA). All drugs were applied directly to the bath solution from frozen concentrated stocks prepared in DMSO (Biml, forskolin, Pitstop 2, dynasore, H-89, and KT 5720), water (PACAP), medium (dibutyryl cAMP and 8 BrcAMP), or ethanol (RO20-1724). As dynasore is light-sensitive, care was taken to minimize light exposure in all studies.

Fluorescence microscopy. To demonstrate receptor endocytosis, PAC1Hop1 receptor-expressing cell cultures were seeded onto 24-well culture plates and maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 containing 8% fetal bovine serum and 800 μg/ml G418. Assays for PACAP- or forskolin-induced cAMP production were performed as described previously (4, 31). After PACAP treatments in medium containing the phosphodiesterase inhibitor RO20-1724 (50 μM), the cultures were extracted in absolute ethanol containing 100 μM RO20-1724 and processed for cAMP immunoassay using the Biotrak nonacetylation protocol (Amersham GE Healthcare, Piscataway, NJ). All samples and standard curves were performed in duplicates. The cAMP assays were used to assess relative changes in culture cAMP levels following the different experimental manipulations; they were not used to determine absolute cellular quantities or the amount of new cAMP formation. Furthermore, since the assay measured cellular cAMP accumulation over time (15 min), the procedures could not resolve the temporal dynamics of cAMP production or levels within the treatment period.

Western blots for phosphorylated ERK. Control and treated cultures were extracted as described previously (28) with 75 μl of RIPA buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 5 mM EDTA, 1% NP-40, and 0.1% SDS) containing 0.3 mg/ml phenylmethylsulfonfluoride, protease inhibitors (16 μg/ml benzamidine, 2 μg/ml leupeptin, 50 μg/ml lima bean trypsin inhibitor, and 2 μg/ml pepstatin A), and phosphatase inhibitor mix (5 mM EDTA, 5 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and 50 mM sodium fluoride) (15, 27, 28). Total sample proteins were determined using the Coomassie Plus reagent (Pierce Biotechnology, Rockford, IL). For Western blot analysis, protein samples (30 μg) were fractionated on 4–12% lithium dodecyl sulfate polyacrylamide gels, transferred onto Immobilon-FL polyvinylidene difluoride membranes (Millipore), blocked, and incubated with primary antisera for quantitative infrared imaging (LiCor Biosciences, Lincoln, NE). Pan- and phospho-specific antisera to ERK1/2 were obtained from Cell Signaling Technology (Beverly, MA).

Changes in ERK phosphorylation were quantified using a LI-COR Odyssey 3.0 application software package. The immediate area of interest encompassing both p44 ERK1 and p42 ERK2 was circumscribed for fluorescence measurements. All data were normalized to total ERK levels in the same sample.

Intracellular Ca2+ measurements. The Ca2+-sensitive dye fura 2-AM (Life Technologies, Carlsbad, CA) was used to measure PACAP-induced changes in intracellular Ca2+. PAC1Hop1-EGFP cells were maintained in a physiological solution containing (in mM) 121 NaCl, 5.9 KCl, 2.5 CaCl2, 1.3 MgCl2, 25 NaHCO3, 1.2 NaH2PO4, 10 NaHEPES buffer, and 8 glucose (pH 7.4). The cells were incubated in 4 μM fura 2-AM along with 0.02% pluronic acid (Life Technologies) for 30 min at room temperature and then washed for 30 min in dye-free physiological solution at 37°C. Change in the ratio of fura 2-AM fluorescence (F340/F380) was used to indicate changes in intracellular Ca2+ and was monitored using a monochromator system (Photon Technology International) coupled to a Nikon Eclipse E600

Fig. 3. cAMP and PKA are not principal mechanisms of PAC1 receptor-mediated ERK phosphorylation. A and B: PAC1 receptor cultures were treated with PACAP (25 nM), dibutyryl cAMP (dbcAMP or dB, 500 μM), or 8-bromo-cAMP (8-BrcAMP or 8Br, 500 μM) for 15 min at 37°C before quantitative Western blot analysis for ERK phosphorylation. ERK activation by cAMP analogs was modest or not significant compared with PACAP. C and D: PAC1 receptor cells were pretreated with the PKA inhibitors H89 (10 μM) and KT 5720 (KT, 1 μM) for 15 min at 37°C before PACAP addition (25 nM, 15 min) for phosphorylated ERK assays by quantitative Western blot analysis. PKA inhibitors had no effects on PACAP-mediated ERK activation. All data were normalized to total ERK levels on the same blots using a pan-ERK antibody probed with a different fluorochrome-conjugated secondary antiserum. Values (means ± SE) represent fold change from untreated control; n = 3. *P < 0.05 vs. CTL (by 1-way ANOVA with Student-Newman-Keuls post hoc test).
FN microscope equipped with a ×60/1.00 numerical aperture water-dipping physiology lens. PACAP-induced changes in F340/F380 were compared in cells maintained at warm (35–37°C) or room (23–25°C) temperature. Data are expressed as change in F340/F380 as a function of time. F340/F380 is not a direct measure of Ca²⁺ concentration, but it can be used to approximate intracellular Ca²⁺ levels and changes after the various experimental manipulations.

Immunocytochemistry and imaging. PAC1 receptor-expressing cells were cultured on sterile 13-mm glass coverslips. After experimental treatments, the cultures were fixed with 4% paraformaldehyde and washed; for immunocytochemistry, the cells were permeabilized with ice-cold methanol for 10 min before incubation overnight in a 1:300 dilution of rabbit monoclonal anti-phosphorylated ERK1/2 (D13.14.4E, Cell Signaling Technology). Phosphorylated ERK was localized by culture incubation in a 1:300 dilution of Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA), and cell nuclei were stained with Hoechst reagent (1 μg/ml). The coverslips were retrieved and mounted, and cells were imaged on an Olympus fluorescence microscope equipped with GFP, Cy3, or 4′,6-diamidino-2-phenylindole filter sets.

To assess changes in relative intensity across the cells, the micrographs from the experiments were collected at the same time and under the same conditions using ×40 objectives, and a straight line was placed across the diameter of the labeled cells for ImageJ plot profile analyses. For these measurements, the Cy3 micrographs for phosphorylated ERK were not overlaid with Hoechst nuclear staining.

Statistics. Statistics were performed using GraphPad Prism statistical software (version 5.4, GraphPad, La Jolla, CA). Differences among means were compared by a two-tailed Student’s t-test (paired or unpaired) or one-way ANOVA followed by Student-Newman-Keuls post hoc analysis. Values are means ± SE. P < 0.05 was considered significant.
Hence, we first evaluated the PACAP/PAC1 receptor-mediated activation of adenyl cyclase and examined whether the increase in cAMP production augments the levels of phosphorylated ERK. Our previous studies demonstrated that treatment with the inhibitors Pitstop 2 and dynasore or temperature reduction to blunt clathrin- and dynamin-dependent endocytosis did not suppress PACAP-induced cAMP generation in sympathetic neuronal cultures, suggesting that the second messenger was generated primarily at the plasma membrane of primary neurons (31). Thus we evaluated whether the same mechanisms could be observed in the PAC1 receptor-expressing cell lines. When the PAC1 receptor-expressing HEK 293 cells were treated with 25 nM PACAP for 20 min at 37°C, total cAMP production increased robustly (150-fold). Pretreatment of the cultures for 20 min with the small peptide clathrin inhibitor Pitstop 2 (15 μM) (49) to suppress receptor endocytosis had no effect on the PACAP-induced increase in cAMP production (Fig. 1A). Similarly, maintaining cells at 24°C, a condition that significantly blunts vesicular internalization, also failed to suppress PACAP-induced adenyl cyclase/cAMP signaling (Fig. 1B). The efficacy of the PACAP-stimulated cAMP response under the two inhibitory conditions was comparable to that at warm temperatures, supporting our data in primary neurons that PACAP-mediated adenyl cyclase/cAMP signaling was predominantly a plasma membrane-delimited process and was largely independent of receptor endocytic mechanisms.

PACAP can potently stimulate ERK phosphorylation, and characterization studies in the receptor cell lines demonstrated that PACAP/PAC1 receptor signaling could increase phosphorylated ERK levels three- to sevenfold, with an approximate half-maximal response at 5–10 nM peptide; 25–50 nM PACAP generated maximum activation responses within 10–15 min, which gradually declined to a lower, but sustained, level (>1–2 h). These observations recapitulated results obtained in primary neurons (28). To establish the potential roles of adenyl cyclase/cAMP as potential upstream regulators of ERK, the receptor cell line was exposed to forskolin to directly activate adenyl cyclase and elevate cellular cAMP production. Treatment of the PAC1 receptor cells with 10 μM forskolin for 20 min at 37°C robustly increased culture cAMP levels ~80-fold, similar to those observed with 25 nM PACAP (Fig. 2A). However, unlike PACAP, which dramatically and consistently induced ERK phosphorylation, treatment with 10 μM forskolin under the same experimental conditions generated little or, more commonly, no significant effects on ERK, regardless of treatment duration (Fig. 2, B and C). Similar to forskolin, exposure to the cell-permeable cAMP analogs dibutyryl cAMP (500 μM) and 8-Br-cAMP (500 μM) also had a minimal or no effect on ERK activation (Fig. 3A). Treatment with the PKA inhibitors H-89 (10 μM) and KT 5720 (1 μM) also had no effect on the PACAP-induced activation of ERK (Fig. 3B). In aggregate, the results indicated that PACAP/PAC1 receptor activation of MAPK/ERK

Fig. 6. PAC1 receptor endocytosis is decreased at ambient room temperature (RT). A: PAC1 receptors tagged with enhanced green fluorescent protein (GFP) are expressed predominantly on the cell surface of untreated control cells. A few intracellular GFP-PAC1 receptor-containing vesicles were evident at 37°C or room temperature (not shown). B: after 20 min of exposure to 25 nM PACAP at 37°C, internalized GFP-PAC1 receptors are present in numerous endocytic vesicles. C: when cultures were maintained at room temperature (24°C), 20 min of exposure to 25 nM PACAP did not initiate GFP-PAC1 receptor endocytosis. D: when cultures were maintained at room temperature (24°C) and exposed to PACAP for 120 min, GFP-PAC1 receptor endocytosis was evident.
pathways was not primarily due to a direct cAMP-dependent mechanism.

PACAP-induced ERK phosphorylation is mediated by receptor internalization mechanisms. Many GPCR systems have been well described to engage β-arrestin following ligand binding for receptor desensitization, endocytosis, and adaptor protein scaffolding on the resultant signaling endosome to activate other pathways, including ERK (7, 20, 26, 41). To test whether PAC1 receptor internalization and formation of a signaling endosome were critical to the PACAP-induced ERK phosphorylation, the cells were pretreated as described above with the endocytosis suppressor Pitstop 2 (15 μM) to block clathrin terminal domain binding (49) or dynasore (20 μM) to inhibit dynamin I/II GTPase activity necessary for vesicle scission (25) before exposure to 25 nM PACAP for 15 min. As evident from Western blot analysis, pretreatment with the inhibitors Pitstop 2 and dynasore significantly blunted the ERK phosphorylation ~50% and 30%, respectively (Fig. 4). After prolonged (120 min) PACAP exposure during which ERK activation was modestly attenuated, Pitstop 2 remained effective in reducing phosphorylated ERK levels, whereas dynasore lost efficacy under these treatment conditions. Higher concentrations of dynasore could more closely mimic the inhibition by Pitstop 2; pretreatment with 60 μM Pitstop 2 decreased the PACAP-induced ERK activation at 15 min by the same extent as 15 μM Pitstop 2. However, even at the higher concentration, dynasore did not significantly blunt the PACAP-induced ERK activation after 120 min (data not shown).

Fig. 7. PACAP-induced PKC signaling contributes to ERK phosphorylation. A1: representative Western blot from PAC1 receptor cultures pretreated with bisindoylmaleimide I (BimI, 1 μM, 15 min) before exposure to PACAP (P27, 25 nM, 15 min). A2: representative Western blots from PAC1 receptor cultures treated with PACAP (25 nM, 15 min) or PMA (100 nM, 15 min) at 37°C or 24°C. B: BimI pretreatment blunted PACAP-induced ERK activation ~50%. C: direct culture PKC activation with PMA at 37°C stimulated ERK phosphorylation; the PMA response was blunted at 24°C. All data were normalized to total ERK levels in the same samples. Values are means ± SE; n = 4. *P < 0.05 vs. CTL; +P < 0.05 vs. P27.
Our earlier studies demonstrated that reducing treatment temperature from 37°C to ambient conditions (22–24°C) very effectively blunted the PACAP-induced PAC1 receptor internalization (31). Consequently, the temperature dependence of PACAP-induced ERK activation was examined to complement the endocytosis inhibitor studies. Contrary to the robust (>5-fold) ERK activation responses at 37°C, PACAP treatment for 15 min at ambient temperature completely failed to increase phosphorylated ERK, additional results implicating PAC1 receptor internalization for ERK endosomal signaling (Fig. 5). However, after a 2-h PACAP exposure of the cultures at ambient temperature, phosphorylated ERK levels increased 1.5- to 2.5-fold in separate experiments. Although the increase was significant, the fold change was much less that that determined at 37°C (Fig. 5).

Next we determined whether the increase in ERK phosphorylation after prolonged PACAP exposure at ambient temperature was correlated with a gradual increase in vesicle internalization with prolonged 25 nM PACAP exposure at 24°C. As reported earlier (31), PAC1 receptor internalization was minimal in the absence of PACAP but very evident following 20 min of PACAP treatment at 37°C (Fig. 6). Cultures exposed to PACAP at 24°C for 20 min had few internalized vesicles; however, cultures exposed to PACAP at 24°C for 120 min demonstrated significant vesicular receptor internalization (Fig. 6). These imaging data demonstrated that at ambient temperatures the rate of receptor endocytosis was decreased markedly, an effect that could contribute to a delayed increase in PACAP-stimulated ERK activation.

**PAC1 receptor-mediated PKC signaling also contributes to ERK phosphorylation.** The observation that temperature reduction was more effective than treatment with endocytosis inhibitors in suppressing ERK phosphorylation suggested that there may be a parallel PACAP/PAC1 receptor signaling mechanism contributing to ERK activation. Given the potential of PAC1 receptor dual coupling to Goq and Goζ, we examined whether PAC1 receptor-stimulated PLC/diacylglycerol (DAG)/PKC signaling contributed to PACAP-induced ERK phosphorylation, as previously suggested for other cell types (13). In support of this mechanism, pretreatment of the cultures with the PKC inhibitor BimI (1 μM) significantly reduced the PACAP-induced increase in phosphorylated ERK levels (Fig. 7, A1 and B). Furthermore, treatment with the phorbol ester PMA (100 nM) to directly activate PKC stimulated culture ERK phosphorylation (Fig. 7, A2 and C). Chemin et al. (9) reported that PKC translocation is temperature-sensitive and suppressed at room temperature. Accordingly, we examined the temperature dependence of PMA-induced ERK phosphorylation and observed that PMA-stimulated ERK activation was decreased at room temperature (Fig. 7, A2 and C).

In aggregate, our results suggest that two mechanisms participate in PACAP activation of ERK signaling in this model: 1) PAC1 receptor internalization to form signaling endosomes, which in turn recruits the MEK/ERK cascade, and 2) activation of PLC/PKC, leading to ERK activation. To test whether these mechanisms represent parallel and separate, or collinear and coupled, pathways, the cultures were pretreated with Pitstop 2 and/or BimI before PACAP exposure. As described above, Pitstop 2 and BimI each diminished PACAP-induced ERK phosphorylation ~50%. However, treatment with both inhibitors suppressed PACAP-induced ERK phosphorylation to a significantly greater extent than treatment with either drug alone (Fig. 8). These observations suggest that the two mechanisms represent separate, but complementary, pathways contributing to the aggregate cellular levels of PACAP-stimulated ERK phosphorylation.

**Temperature reduction does not blunt PACAP-induced intracellular Ca²⁺ transients.** The ability of the PAC1 receptor to engage Goζ for PLC activation results in the generation of downstream DAG/PKC and IP₃ signaling (4). As the DAG/PKC pathway in ERK signaling is temperature-dependent, we also evaluated whether IP₃-stimulated Ca²⁺ release from the endoplasmic reticulum is similarly temperature-sensitive. With fura 2-loaded cells, PACAP initiated a transient rise in intracellular Ca²⁺ at 35–37°C and at ambient temperature (23–25°C) equally well (Fig. 9). The records shown in Fig. 9 are representative of Ca²⁺ measurements with PAC1 cells from multiple cell passages and indicate that PACAP can initiate a rise in global Ca²⁺ under conditions that effectively suppress receptor internalization.

**Intracellular signaling pathways affect the cellular distribution of PACAP-activated ERK.** The PKC and vesicle endosome/β-arrestin pathways in ERK signaling have been shown to regulate the nuclear translocation or cytoplasmic localization...
of phosphorylated ERK in some cellular systems. In addition to the well-conserved Tyr and Thr phosphorylation sites by MEK at the lip of the ERK molecule for activation, additional phosphorylation sites at the kinase insert domain appear to facilitate nuclear ERK transport (40). Hence, to examine whether the dual PACAP-mediated pathways affected the cellular distribution of phosphorylated ERK, we examined the immunocytochemical localization of activated ERK in the presence of inhibitors. Upon stimulation with PACAP alone, the distribution of phosphorylated ERK in a majority of the cells appeared relatively uniform in the nucleus and cytoplasm; the increase in cellular phosphorylated ERK staining appeared homogeneous, such that the nucleus was indistinct (Figs. 10 and 11). In Pitstop 2-treated cultures, PACAP-stimulated phosphorylated ERK staining was uniformly diminished across compartments. However, with the addition of BimI, the PKC inhibitor inhibited PACAP-activated ERK levels not only in the cytoplasm, but also in the nucleus to a greater extent (Figs. 10 and 11), suggesting that PKC signaling was necessary for phosphorylated ERK nuclear translocation. These results appear consistent with other studies suggesting that activated ERK through β-arrestin-dependent receptor internalization remains cytosolic while PKC-mediated ERK activation facilitates nuclear transport (1, 10, 37, 46). From these results, we suggest that the ability of PAC1 receptors to activate ERK through dual PKC and endosome mechanisms implicates coordinate cytosolic substrate and nuclear transcriptional factor regulation to greatly diversify the cellular responses to PACAP signaling.

**DISCUSSION**

The ability of GPCRs to engage multiple intracellular signaling cascades in a tissue specific manner underlies their extensive regulatory roles in many physiological systems (16, 17, 21, 36, 47). The PACAP-selective PAC1 receptor belongs to a subclass of GPCRs capable of dual coupling to Goα/Goq (4, 11, 38, 44). The dual coupling and resulting downstream events not only allow signal diversification following PACAP binding but, from current work, may also allow signal convergence on ERK pathways for target substrate activation in different cellular compartments.

After ligand binding, the internalization of GPCRs via clathrin-coated vesicles for desensitization/recycling can also result in the formation of signaling endosomes to provide a means for novel signal recruitment distinct from those activated at the plasma membrane (7, 30, 42). As for other GPCRs, our previous work demonstrated that the diverse actions of PACAP are mediated in part through PAC1 receptor internalization (28, 31). Consistent with these observations, our current studies demonstrate that there are multiple modes for PACAP-mediated ERK activation, one of which is principally dependent on receptor internalization and endosome formation. Using a PAC1 receptor/EGFP-expressing cell line, we demonstrate that reduction of incubation temperature or treatment with inhibitors of receptor endocytosis dramatically blunt PAC1 receptor-stimulated ERK phosphorylation. Although GPCR-induced cAMP production has been considered classically to be membrane-delimited, more recent studies have suggested that many GPCRs can activate adenyl cyclase within the plasma membrane and sustain cAMP production following receptor internalization and incorporation into endosomes (6, 7, 8, 14, 19, 33). Interestingly, even with the same receptor, the persistent phase of cAMP production following endocytosis can depend on the cell type and ligand (50). Previous studies have suggested that PAC1 receptor-stimulated ERK phosphorylation is dependent in part on adenyl cyclase activity and cAMP production, a mechanism that may have been associated with endosomal cAMP signaling. As in our previous studies using primary sympathetic neuronal cultures in which suppression of PAC1 receptor endocytosis failed to blunt cAMP production (31), pretreatment of the PAC1 receptor-expressing cells with the clathrin inhibitor Pitstop 2 or incubation of the cultures at room temperature to attenuate PAC1 receptor internalization did not diminish PACAP-induced cAMP production. These observations suggest that the PAC1 receptor-mediated induction of adenyl cyclase activity was localized to the plasma membrane and largely uncoupled to ERK pathways in this cell system. Furthermore, treatment of the cultures with forskolin for 15 or 120 min to directly activate adenyl cyclase had no consistent effect on ERK phosphorylation. Similarly, treatment with cell-permeable analogs of cAMP had a minimal or no effect. Together, these results support a dissociation of
the cAMP/PKA or exchange proteins activated by cAMP (EPAC) pathway on ERK signaling. While some studies have implied that cAMP signaling directly activates ERK (3, 5, 13, 48), the present results illustrate that the PACAP/PAC1 receptor can engage the MEK/ERK pathway using multiple, complementary signaling cascades and conditions in which cAMP production does not directly activate ERK. The present results, however, are not sufficient to preclude the possibility that prolonged cAMP production may support PACAP-induced ERK activation to a small extent and also through presently unidentified mechanisms. We also acknowledge that additional mechanisms, including a cAMP/PKA pathway leading to ERK activation, may be present in other cells types, including neurons (13).

A number of studies have used low experimental temperatures to block GPCR internalization (18, 22). In contrast to prior work in which much reduced temperatures (~4°C) were commonly used, the current studies show that reducing temperature to 24°C is sufficient to suppress PAC1 receptor internalization (31). Similarly, internalization of α-bungarotoxin-labeled ACh receptor α7-subunits was markedly slowed by reducing temperature from 37°C to 20°C (29). These observations suggest that receptor internalization is temperature-sensitive, such that a 10–15°C decrease significantly decelerates the process. Receptor endocytosis was not completely inhibited at these ambient temperatures, as imaging studies revealed that internalization, blocked at 15 min, was evident after 120 min of PACAP treatment, with a concomitant nearly twofold increase in ERK phosphorylation.

We have used the extent of a PACAP-induced elevation of intracellular Ca2+ to demonstrate that activation of PLC/IP3 signaling is independent of temperature. A requisite for this
interpretation is that the PACAP-induced rise in intracellular Ca\textsuperscript{2+} is due primarily to an IP\textsubscript{3}-induced release of Ca\textsuperscript{2+} from internal stores. Support for this view is derived from results generated in ongoing studies in which PACAP induced a rise in intracellular Ca\textsuperscript{2+} in HEK PAC1 cells kept in a Ca\textsuperscript{2+}-containing or a Ca\textsuperscript{2+}-deficient solution, but not in cells depleted of intracellular Ca\textsuperscript{2+} stores by pretreatment with 1 μM thapsigargin (Clason and Parsons, unpublished observations).

While more detailed studies are needed, the current results are consistent with β-arrestin-mediated PAC1 receptor internalization and ERK activation. The roles of β-arrestins in GPCR function have been well studied. First characterized as proteins that bind phosphorylated GPCRs following agonist stimulation for receptor desensitization processes, β-arrestins facilitate receptor endocytosis by binding to clathrin heavy chain, β2-adaptin subunit, and other protein complexes for internalization and endosome formation. The PAC1 receptor is a class B GPCR and, unlike class A GPCRs, contains characteristic Ser residue clusters in the COOH-terminal cytoplasmic tail for high-affinity β-arrestin 1/2 binding (32, 34, 35, 43). In addition to protein recruitment for endocytosis, β-arrestins bind to a variety of kinases, small GTPases, phosphodiesterases, and other regulatory molecules and, hence, behave as molecular scaffolds for signaling components such as Src and Raf/MEK/ERK. The β-arrestin/Raf-MEK-ERK complex in endosomes appears stable, which may be significant for PAC1 receptor-mediated long-term ERK activation and cytosolic substrate phosphorylation.

Inspection of our results suggests that low temperature experiments have consistently more pronounced effects on PACAP-stimulated ERK activation than pretreatment with pharmacological endocytosis inhibitors. The PAC1Hop1 receptor engages dual Go\textsubscript{q} and Go\textsubscript{q}. As Go\textsubscript{q} activation of adenylyl cyclase and cAMP production had no consistent direct effect in ERK signaling, we investigated whether Go\textsubscript{q}-mediated PLC/DAG/PKC signaling participated in the PACAP-induced phosphorylation of ERK. GPCR/PKC stimulation of ERK phosphorylation has been well described (2, 3, 37, 40, 41). In agreement, treatment of the cultures with PMA to directly activate PKC increased ERK activation, and culture pretreatment with the PKC inhibitor BimI diminished PACAP-stimulated ERK signaling. There may be several modes of PKC activation of the ERK pathway, including Pyk2-mediated phosphorylation of ERK, phosphorylation of Raf upstream of MEK, and/or phosphorylation of Raf kinase inhibitor protein leading to Raf kinase inhibitor protein dissociation from Raf-1 for MEK activation (41). The translocation of PKC for target substrate phosphorylation is temperature-dependent (9, 39), and in agreement, PMA-induced ERK activation was reduced at ambient room temperature. These studies in aggregate demonstrate that two temperature-sensitive mechanisms, receptor endosome formation and PKC signaling, contribute importantly to the totality of ERK activation.

The ability of the PAC1Hop1 receptor to coordinate and recruit both mechanisms for ERK activation may be significant for specific substrate phosphorylation in cytosolic and nuclear compartments. As described previously, the β-arrestin/endosome-mediated scaffolding of the Raf/MEK/ERK signaling module appears stable, and several studies have suggested that activated ERK through this mechanism is not translocated into the nucleus and, hence, targets cytosolic substrates. These include phosphatases, phosphodiesterases, phospholipases, the RSK family of protein kinases, cytoskeletal proteins, survival/apoptosis proteins, and related signaling molecules, resulting in the regulation of cell growth, motility, and proliferation for development and cellular plasticity. The PKC-mediated mechanisms for ERK signaling, however, appear different in initiating a series of events that also stimulate nuclear phosphorylated ERK transport. In addition to facilitating the dual Thr/Tyr
phosphorylation at the activation lip of ERK, PKC appears to also promote the phosphorylation of Ser-Pro-Ser nuclear localization signal sequence in the kinase insert domain of ERK via casein kinase 2 for importin-α7 binding and transport through the nuclear pore (40). These nuclear targets of activated ERK have been well studied to include immediate early genes, including c-fos and c-myc, and Elk1/Ets and ternary complex factor families of transcriptional factors to regulate gene expression (40). In concurrence, we have shown that addition of BimL appears to blunt PACAP-stimulated phosphorylated ERK staining in the nucleus.

In summary, PACAP/PAC1 receptor internalization and activation of the PLC/DAG/PKC signaling pathway represent temperature-sensitive, parallel mechanisms contributing to PACAP-mediated ERK phosphorylation. These complementary PAC1 receptor mechanisms appear to differentially route activated ERK signals to cytosolic and nuclear substrates capable of directing diverse regulatory functions over broad temporal windows. Furthermore, along with our previous work (31), these studies reinforce the need for GPCR studies to be performed at physiological temperatures.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

V.M. and R.L.P. are responsible for conception and design of the research; V.M., T.R.B., B.M.G., T.A.C., and R.L.P. performed the experiments; V.M., T.R.B., B.M.G., and T.A.C. edited and revised the manuscript; V.M., T.R.B., B.M.G., T.A.C., and R.L.P. approved the final version of the manuscript.

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