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PACAP-induced ERK phosphorylation in VPAC1 and VPAC2-expressing HEK cells is mediated by receptor endocytosis and PKC signaling

Anne Linden

Research Thesis

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Abstract

Pituitary adenylate-cyclase activating polypeptide (PACAP) is highly conserved signaling molecule in eukaryotes known to regulate a myriad of metabolic processes within the brain as well as the body (Sherwood, Krueckl, & McRory, 2000; Vaudry et al., 2009). The mechanism of action underlying PACAP signaling is known to occur through the PAC1, VPAC1 and VPAC2 G protein-coupled receptors (GPCRs) to initiate signaling cascades that can phosphorylate extracellular signal-related kinase (ERK) proteins to influence many intracellular events including gene expression, long-term potentiation, or induce growth factors, all of which are a specific response to extracellular events (Chang, & Karin, 2001). In the current study, HEK cells expressing VPAC1 or VPAC2 receptors were exposed to PACAP27 (P27) to induce receptor activation and ERK phosphorylation (pERK). The VPAC expressing cells were treated with P27 for varying durations to assess the temporal changes in pERK; further, the cells were pre-treated with signaling pathway inhibitors to determine the predominant mechanisms of P27-mediated ERK phosphorylation. From previous work, it was hypothesized that PACAP-mediated ERK activation of VPAC-type receptors would be elicited via receptor endocytosis and the PKA pathway. Experiments were conducted in triplicate, and the pathway inhibitors included two receptor endocytosis blockers (Pitstop2 and Dynasore), a PKA inhibitor (KT5720), and a PKC inhibitor (Bim1). Relative expression of ERK1/2 and pERK proteins were visualized and compared by quantitative Western analyses. Unlike, PAC1 receptors, PACAP activation of VPAC receptors resulted in transient ERK activation. Contrary to expectations, the experimental results also showed that receptor endocytosis blocker Pitstop2 and PKC inhibitor Bim1 reduced ERK phosphorylation. These inhibitor results are similar to previous experiments conducted on HEK-expressing PAC1 receptors treated with the same pathway inhibitors (May, Buttolph,
Girard, Clason, & Parsons, 2014), providing further sight into how PACAP-mediated mechanisms of ERK phosphorylation may be implicated physiological conditions.
**Introduction**

The endogenous neuropeptide pituitary adenylate-cyclase activating polypeptide (PACAP) is ubiquitous throughout the body and physiologically relevant as a neurotransmitter, neuromodulator, autocrine and paracrine chemical messenger, or neurotrophic factor (Dejda, Sokolowska, & Nowak, 2005). As a highly conserved member of the secretin/glucagon superfamily, PACAP acts as a regulating peptide in a wide variety of functions such as cell division, differentiation, smooth muscle contractility, and metabolic pathways (Sherwood, Krueckl, & McRory, 2000; Vaudry et al., 2009). PACAP is known to be physiologically relevant in diverse array of structures that comprise nervous, immune, gastrointestinal, and endocrine systems. Within the central nervous system, the activity of PACAP in hypothalamic nuclei is capable of evoking many different homeostatic responses. For example, within the ventromedial nucleus of the hypothalamus, PACAP activity has been implicated in the regulation of catabolic processes leading to energy expenditure (Hawke, Ivanov, Bechtold, Dhillon, Lowell, & Luckman, 2009). In addition, increased PACAP expression and utility within the paraventricular nucleus of the hypothalamus has been linked to corticotrophin-releasing hormone (CRH) release to stimulate anterior pituitary gland adrenocorticotropic hormone (ACTH) secretion, evoking the release of cortisol from the adrenal cortex (Stroth, Liu, Aguilera, & Eiden, 2011). Hypothalamic PACAP circuits can also regulate sympathetic nervous system activation. Due to PACAP’s conservation throughout species and cellular processes, it is important to understand the physiological relevance that PACAP-mediated receptor activation has on cellular signaling.

PACAP has the ability to bind to three metabotropic receptors in the body: PAC1 receptor, VPAC1 receptor, and VPAC2 receptor. The PAC1 receptor (PAC1R) possesses selective affinity for PACAP and is expressed in many tissues including nervous system, lung,
liver, and spleen (Sherwood, Krueckl, & McRory, 2000). Contrastingly, the VPAC-type receptors have the ability to bind PACAP and vasoactive intestinal peptide (VIP) with equal affinity. The VPAC1 receptor (VPAC1R) is found within brain tissue such as the cerebral cortex, amygdala and hippocampus as well as in peripheral tissues such as kidney, liver, gastrointestinal tract, and adrenal glands (Dickson & Finlayson, 2009). Similarly, the VPAC2 receptor (VPAC2R) is also expressed in the thalamus, hypothalamus, and brainstem in addition to peripheral tissues such as adrenal glands, blood vessels, smooth muscle, kidney, and lung. The PAC1R and VPAC-type receptors associate with G-protein subunits (Gα and Gβ/Gγ) via transmembrane loops on the intracellular face of the plasma membrane (Harmar et al., 1998). Upon ligand binding, the G-protein complex becomes activated, beginning with the exchange of guanosine diphosphate (GDP), bound to the Gα subunit, for guanosine triphosphate (GTP). The increased energy of the Gα subunit allows for dissociation from both the receptor and the Gβ/Gγ subunits. The G-protein subunits mobilize to activate plasma membrane-delimited intracellular signaling cascades.

Studies that observed second messenger activation downstream of VPAC-type receptors have implicated either adenylyl cyclase (AC) or phospholipase C (PLC) activation depending on if the coupled G-protein α-subunit is Gαs or Gαq, respectively (Langer, 2012). Most commonly, VPAC receptors couple to Gαs subtype to cause adenylyl cyclase activation and catalyze adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) (Vaudry et al., 2009). A significant rise in intracellular cAMP activates protein kinase A (PKA) to exert a broad array of intracellular influences. Contrastingly, Gαq dissociation activates phospholipase C (PLC) to cleave a membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP2) protein into diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 binds to receptors (IP3R) bound to the
endoplasmic reticulum to release intracellular stores of calcium into the cytosol. In the presence of increased intracellular calcium concentrations, DAG can activate protein kinase C (PKC) to cause additional intracellular effects.

Contrastingly, GPCRs including the PAC1R can also initiate G protein-independent- but β-arrestin-mediated intracellular signaling through receptor endocytosis into the postsynaptic cell. This results in the formation of endosomes, vesicles comprised of the receptor complex, where β-arrestin and vesicular proteins can form scaffolds to assemble signaling effectors for the generation of sustained intracellular responses (May, & Parsons, 2017). Ultimately, all three of these signaling mechanisms have the ability to activate mitogen-activated protein kinase (MAPK) proteins, specifically extracellular signal-related kinase (ERK) proteins, by means of phosphorylation. ERK1 and ERK2 proteins, 44 and 42 kDa respectively, each have two phosho-acceptor sites; one on a threonine residue and one on a tyrosine residue (Pearson et al., 2001). Many neurotransmitter, growth factor and cytokine signaling pathways can activate ERK; the ability and mechanisms underlying GPCR activation of ERK is significant in understanding the gene regulatory events, neuroplasticity and neural maladaptations associated with neurocircuit ERK signaling.

With respect to VPAC-type receptor activation in rat brain models, VPAC activation via PACAP in cortical neurons and protoplasmic astrocytes primarily results in PKA pathway signaling (Grimaldi & Cavallaro, 1999). Additionally, studies using somatolactotroph GH4C1 cells expressing VPAC2 receptors observed VIP and PACAP-mediated ERK phosphorylation that was significantly reduced in the presence of PKA pathway inhibitor H89 (Pechon, Magalon, Rasolonjanahary, 2000). Because VPAC receptors are primarily coupled to Gαs and signal
through the cAMP pathway, VPAC-mediated PKA activation is more likely to engage ERK signaling over PKC mechanisms.

Previous work conducted within the May lab observed activity of PACAP upon stimulation of the PAC1 receptor in terms of ERK activation (May, Buttolph, Girard, Clason, & Parsons, 2014). Through use of a stably transfected PAC1R human embryonic kidney (HEK) cell line, ERK activation was determined to be dependent on receptor internalization and PKC signaling. Because PACAP also has affinity for the VPAC receptors, VPAC-mediated ERK responses may be comparable to those for the PAC1 receptor. However, because the VPAC-type receptors preferentially couple to Gαs cascades, it is hypothesized in the current study that PACAP-mediated ERK activation of VPAC-type receptors will be elicited via receptor endocytosis and the PKA pathway.

**Materials and methodology**

**HEK Cell preparation**

Human embryonic kidney (HEK293) cultures were transfected with VPAC1R-EGFP or VPAC2R-EGFP receptor plasmids (gift from Alain Couvineau, INSERM U773 Paris, France) with Lipofectamine 2000 using protocols comparable to those previously described (May et al., 2010; 2014). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 containing 8% fetal bovine serum (FBS) 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. After continued selection for 2 weeks, the VPAC1R-EGFP and VPAC2R-EGFP cell lines were maintained in 8% FBS-supplemented DMEM/Ham’s F-12
containing 50 µg/ml G418; the medium was replaced every 48 h and the cultures were passed weekly upon confluence. For experiments, the trypsinized cell suspension was seeded into 24-well culture plates and cultured until 70% confluence for treatments.

**HEK VPAC Receptor Cell Line Pulse Treatment**

To assess the potential long term activation of ERK via VPAC1R and VPAC2R signaling, the stable cell cultures were pulse-treated with PACAP27 (25 nM final concentration) for 5 min, rinsed (2 x 1 ml medium) and returned to the culture incubation medium for harvest at the indicated times (5, 15, 30, or 60 minutes). All treatment and incubation procedures were performed at 37°C. Upon experimental termination, the control and treated cultures were extracted with 75 µl RIPA buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 5 mM EDTA, 1% NP-40, 0.1% SDS) containing 0.3 mg/ml phenylmethylsulfonylfluoride, protease inhibitors (16 µg/ml benzamidine, 2 µg/ml leupeptin, 50 µg/ml lima bean trypsin inhibitor, 2 µg/ml pepstatin A) and phosphatase inhibitor mix (5 mM EDTA, 5 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50 mM sodium fluoride). All samples were stored at –80°C before assay. Treatments were performed in triplicate.

**Signaling Pathway Inhibitor Treatments**

To examine the signaling pathways mediating PACAP stimulated ERK activation in the VPAC1R and VPAC2R cell lines, the cultures were pretreated with pathway inhibitors before peptide treatments. The experimental cultures were pretreated with a PKA inhibitor (KT5720, 15 µM), PKC pathway inhibitor (Bim1, 1 µM) or receptor endocytosis inhibitor (Pitstop2, 15 µM or Dynasore, 20 µM) for 15 min before 25 nM PACAP addition for 5 min. Pitstop2 inhibits the clathrin protein that is implicated in formation of the endosome, whereas Dynasore inhibits
the dynamin proteins that aid in intracellular vesicle scission to form endosomes. All culture treatments and incubations were performed at 37°C before harvest in RIPA buffer containing protease and phosphatase inhibitors as described above. Each treatment was performed in triplicate.

**Protein Assay Protocol**

The culture extracts were thawed, vortexed, and centrifuged to remove debris, and the concentration of the solubilized proteins were determined using Coomassie Plus protocol. The samples were diluted and aliquoted onto a 96-well plate. Following addition of the Bradford assay reagent (1:1) and incubation for 10 min at room temperature, the samples were analyzed by spectrophotometry at 595nm using a Bio-Tek Synergy microplate reader. Sample protein concentration was determined by interpolation against a BSA standard curve in the same assay. Equal amounts of protein (70 ~µg) were prepared for SDS-PAGE fractionation.

**Western Blot Protocol**

For Western blot analyses, the extracts were prepared in sample buffer containing lithium dodecyl sulfate / sodium dodecyl sulfate (LDS/SDS) and dithiothreitol (DTT). LDS/SDS anionic detergent intercalated within the proteins to provide a greater negative charge; DTT acts as a sample reducing agent to break protein intramolecular disulfide bonds. The samples were boiled for 10 min, cooled, collected by gentle centrifugation and loaded (25 ~µl total) onto NuPAGE 4-12% SDS-PAGE gels for protein fractionation at 150V (constant voltage) in 1X NuPAGE MOPS buffer (50 mM MOPS, 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.7). MOPS was the optimal running buffer to provide a neutral environment that can consistently separate medium to large size proteins throughout the gel to allow for better protein stacking results.
NuPAGE antioxidant was introduced to the buffer to maintain the reduced state of the proteins during migration.

Following fractionation, the cassettes were opened and the gel overlaid onto Immobilon-FL polyvinylidene difluoride membranes, previously rinsed in a methanol bath, for all protein transfers. 1X NuPAGE transfer buffer containing 20% methanol maintained the neutral pH of the experiment as well as prevented amino acid residue modification (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, pH 7.2). The membranes were run at a constant 25V for 1 h 15 minutes. Following transfer protocol, the nonspecific spots on the membrane were blocked with 1:1 1X phosphate buffered saline (PBS) and blocking reagent (Aquablock: EastCoast Bio, product #PP82) to reduce background signal that may interfere the western analyses.

**Antibody application**

The transfer membranes were then incubated overnight at 4°C with 1:500 Antiphospho-ERK (pERK) primary monoclonal antibody (Cell Signaling Technology, product #9106S) containing 10% Tween-20 and 1:1 1X PBS and Aquablock. The blots were washed in 3 x 15 min changes in 1X PBS containing tween 20 (PBST) followed by 1 x 5 min wash in 1X PBS before incubation in 1:20,000 donkey anti-mouse antibody conjugated to IRDye 800nm fluorophore tag (IRDye 800CW, product #ab216774) for 1 h at room temperature. After repeating aforementioned PBST/PBS washes, appropriate bands on the wet blots were analyzed by quantitative infrared imaging at 800 nm (Li-Cor Odyssey) to determine the relative abundance of p44/p42 pERK within each sample.

To normalize the data against total ERK in the same sample, the blots were incubated again overnight at 4°C in 1:1000 primary rabbit anti-ERK1/2 antibody (Cell Signaling Technology, product #9102S) containing 10% Tween-20 and 1:1 PBS and Aquablock. After
repeated PBST/PBS washes, the blot was incubated in 1:20,000 goat anti-rabbit secondary antibody (KPL, product #5230-0342) conjugated to a Dylight 680 nm fluorophore tag for 1 h at room temperature before repeating PBST/PBS washes. ERK bands for each sample were quantitated as above via infrared imaging at 700 nm once the blot was completely dry. The normalized pERK/total ERK ratio was determined for each sample and data are expressed as fold change compared to control values. All treatments were performed in triplicates.

**Statistical analysis**

Statistical analysis was performed using Sigmaplot 12 software for Windows. All experimental data has been reported as mean ± SEM. To determine statistical difference between treatments, one-way analysis of variance (ANOVA) was performed followed by Holm-Sidak post-hoc comparison. p-values, < 0.05 were considered statistically significant.

**Results**

**Time Pulse experiment**

Previous work in the laboratory has shown that brief exposure of HEK PAC1R-EGFP cells to PACAP can result in ERK activation that can be sustained at maximal levels for at least 30 minutes. PACAP can bind to PAC1R, VPAC1 and VPAC2R receptor subtypes; hence the studies examined whether PACAP stimulation of the VPACRs can result in similar long-term activated ERK dynamics. The treatment of PACAP (25 nM), onto the VPAC1R- and VPAC2R-EGFP cultures, was applied for 5 minutes (pulse treatment) before washing and incubation at 37°C for subsequent harvest at various periods of time up to 60 minutes to determine the duration of receptor-mediated ERK phosphorylation. The results of the VPAC1R and VPAC2R receptor pulse experiments have been plotted against previously conducted pulse experiments
that observed HEK-transfected PAC1HOP1 receptor-mediated ERK phosphorylation (Figure 1). To mitigate differences among the culture cell lines, the level maximum of ERK phosphorylation was determined from three samples for each receptor subtype culture and time point. Both VPAC-type receptor cultures demonstrated maximum ERK phosphorylation 5 minutes post-PACAP application. At 15 minutes post application, the VPAC1R receptor pERK levels decayed 40% from maximal levels and 50% by 30 minutes; the activation of pERK at 60 minutes was not significantly different from control baseline levels. Similar temporal changes in stimulated pERK were observed in the VPAC2R-expressing cells. PACAP-mediated ERK activation in the VPAC2R-EGFP cultures was maximal at 5 minutes and declined >50% by 15 – 60 minutes. Due to sample variability, these changes were not significantly different from untreated controls. In contrast, the PAC1HOP1 receptor cells exhibited significant ERK phosphorylation 5 minutes post PACAP-pulse that was sustained for at least thirty minutes, with observed maximal pERK levels occurring 15 minutes post-pulse. Notably, the stimulated ERK activation rapidly returned to baseline levels by after 60 minutes post PACAP application. From these results, the abilities for VPAC receptors to activate ERK appeared transient compared to responses from PAC1 receptors.
Pathway Inhibitor Experiment

Pathway inhibitors were added in the presence of P27 in cultured HEK-cells transfected to express VPAC1 or VPAC2 receptors. Both VPAC-type receptors responded similarly to the presence of P27 and pathway inhibitors (Figure 2). Baseline untreated control samples demonstrated low levels of phosphorylated ERK compared to total ERK proteins available within each sample. In all cells treated with P27, pERK levels were significantly higher (4- to 7-fold) than their respective untreated control cultures. Samples were pretreated with 15 µM Pitstop 2, to inhibit clathrin mechanisms involved in endocytosis, followed by P27 addition, revealed significantly reduced phosphorylated ERK levels by 50% and 40% in VPAC1R and VPAC2R-expressing cells, respectively, when compared to samples treated with P27 alone. Similarly, application of Bim1 to inhibit PKC signaling significantly reduced ERK
phosphorylation by approximately 40% to 60% than when compared to the PACAP-treated samples. Relative pERK levels due to P27 activation were not affected by the presence of the PKA inhibitor KT5720 (15 µM). Although not significant, application of KT5720 + P27 on cells expressing VPAC2R demonstrated slight facilitation in ERK phosphorylation compared to applying P27 on its own. Despite the effects of Pitstop 2, inhibition of the dynamin 1/2 GTPase by Dynasore to also block endocytic mechanisms had no apparent effects on PACAP-stimulated ERK activation which may have reflected the suboptimal dose used in the study (see Discussion). From these results, PACAP-stimulated ERK activation in VPAC1R and VPAC2R cells was dependent on receptor endocytosis and PKC signaling mechanisms, which appeared comparable to mechanisms noted for the PAC1R.

**Figure 2.** VPAC-expressing HEK cell percentage of ERK phosphorylation in the presence of ligand P27 and pathway inhibitors. HEK cells samples treated with 25 nM agonist ligand in addition to Pitstop 2 (15 µM), Dynasore (20 µM), KT5720 (15 µM) or Bim1 (1 µM) to block different potential mechanisms that may lead to ERK phosphorylation downstream of the receptor. Relative ERK phosphorylation was scaled based on peak activation that occurred when 25 nM of P27 was applied in the solution without the presence of pathway blockers. Control samples determined baseline pERK levels were determined by HEK-cultures that lacked agonist P27 stimulation.
Discussion

Ultimately, the results of the current experiment did not support the original hypothesis that PACAP-mediated ERK activation of VPAC-type receptors was predominantly elicited via receptor endocytosis and the PKA signaling pathway. While receptor endocytosis did facilitate ERK phosphorylation as hypothesized, the second mechanism by which VPAC-type receptors phosphorylate ERK was via the PKC pathway, not the anticipated PKA pathway. As previously observed, VPAC1 and VPAC2 receptors could be coupled to Gαq and therefore elicit pERK expression via identical mechanisms. Both duration and relative influence of pathway inhibitors on VPAC1R and VPAC2R-mediated ERK expression were similar. In addition, these results are consistent with previous experiments conducted within the May lab observing PAC1R-expressing HEK cell pERK expression in the presence of P27 and identical pathway inhibitors (May, Buttolph, Girard, Clason, & Parsons, 2014). The results of these experiments, when observed as a whole, indicate that ERK phosphorylation upon PACAP binding occurs via comparable mechanisms in PAC1R and VPACR-type expressing HEK cells.

The time pulse experiments allowed for both comparison between PAC1HOP1 receptor and VPAC-type receptor pERK expression over time. Previous experiments observing PAC1R duration of P27-mediated ERK activation have shown that peak ERK phosphorylation occurs 5 – 15 minutes post application that appeared more robust than the VPAC-type receptors (data not shown). The duration of the PAC1R activity elicited significant sustained ERK phosphorylation between 5 and 30 minutes post-P27 application compared to baseline expression. While VPAC receptor activation elicited similar rapid intracellular response, with maximum ERK phosphorylation occurring 5 minutes post-P27 application, the response diminished significantly within 15 minutes. VPACR-mediated pERK levels declined between 55 – 65% at 30 and 60
minutes post-P27 application and appeared to be maintained. Although long-term pERK activation was significantly reduced from peak levels, the sustained decay response of the receptor still appeared significantly greater than baseline controls without PACAP treatment. The difference in duration and magnitude of intracellular responses between PAC1R and VPAC-type receptors may be physiologically important within endogenous systems. In order for convergent information received from presynaptic cells to be integrated into a single signal within the postsynaptic cell, intracellular responses must be capable of modulating the effect upon ligand binding to the receptors. Having multiple receptors that bind PACAP co-expressed on a specific tissue allows for fine-tuned activation. For example, activation of VPAC-type receptors may moderate the amount of pERK expression in the cell, as the duration of the response has a slow decay but may be compounded on with additional input. However, if the cellular response is dependent on a higher sustained activation of ERK, PAC1R may be more efficient because it provides a robust increase in ERK phosphorylation for at least 30 minutes. Transient cellular ERK activation has been associated with cellular proliferation while sustained ERK activation levels have been suggested to engage transcriptional activation for cellular differentiation (Pellegrino & Stork, 2006). Accordingly, the PAC1R-mediated ERK signaling responses may result in neural remodeling and plasticity underlying many of the homeostatic and even maladaptive physiological responses.

On another important note, the results of VPAC1 and VPA2 receptor pulse experiments gave insight as to the best duration to apply P27 and pathway inhibitors during the second experiment. In order for the pathway inhibitor experiment to observe significant differences in ERK phosphorylation, it was crucial that the expression of pERK be maximized within the samples. Because 5 minutes post-P27 application revealed the greatest percentage of ERK
phosphorylation in VPACR-expressing cells, the pathway inhibitor experiments utilized a 5-minute P27 pulse protocol in all samples. The experimental samples treated with pathway inhibitors could then be compared to both baseline controls lacking agonist stimulation as the minimal pERK expression observed, and PACAP-treated samples as the maximum percentage of ERK activation detected.

The results of the pathway inhibitor experiment revealed statistically significant reductions in ERK phosphorylation when in the presence of Pitstop 2, a clathrin-mediated endocytosis blocker, and Bim1, a PKC pathway inhibitor. Despite VPAC-type receptors coupling more frequently to Gαs, the current results suggest that PKA is not the predominate pathway at which ERK is phosphorylated intracellularly in HEK cells. Rather, the predominant plasma membrane signaling mechanism to activate ERK within VPAC-type receptors is through Gαq coupling to activate phospholipase C and downstream protein kinase C (PKC). Perhaps in the current cell preparation, transfected VPAC receptor cells, due to their transient activation, did not elicit prolonged adenylate cyclase activity, causing intracellular cAMP concentrations to stay below threshold of PKA-mediated ERK phosphorylation. Another possible explanation of why PKC was the predominant G-coupled signaling pathway to cause ERK phosphorylation is because the HEK cells used in the current experiment were derived from the endoderm where the VPAC receptors may have alternative coupling preferences than in ectoderm tissue. In order to determine if the VPAC-mediated ERK phosphorylation is dependent on regionally specific preferences to G protein α-subunit coupling, additional experiments should be conducted in cells derived from nervous or a mesoderm-derived tissue.

An equally likely mechanism within VPAC-type receptors to signal ERK phosphorylation is via endosomal formation as observed by the reduction of pERK expression in
the presence of Pitstop 2, an inhibitor of the clathrin protein that signals for endocytosis. However, not all endocytosis inhibitors blocked the VPACR-mediated activation of pERK; application of 20 µM Dynasore did not appear to produce significant differences in pERK compared to PACAP-treated VPAC1R and VPAC2R-expressing cells. Dynasore blocks the protein Dynamin that is thought to be involved in pinching off endosomes from the plasma membrane (Belcheva, & Coscia, 2002). The described effective concentration of Dynasore in Dynamin inhibition is 80 µM. As the suggested dose may have been potent enough to reduce cell viability, a 20 µM dose of Dynasore was used, which in previous work was shown to be capable of blunting endocytic mechanisms. Further, Dynasore can be labile even in storage at -80°C. Hence, from these considerations, the available dose of Dynasore may have been suboptimal and ineffective in inhibiting the endosomal ERK responses. Because Pitstop 2 was effective in inhibiting VPACR-mediated ERK activation, comparable to the results observed for PAC1R, VPACR endosomal mechanisms were implicated in ERK signaling. Clathrin is likely not the protein that evokes intracellular activity following endosomal formation. Previous speculations of β-arrestin expression on the endosomal membrane may result in pERK expression (May & Parsons, 2017). However, the current study did not investigate the signaling mechanisms that occur following receptor endocytosis. Future studies may benefit from performing experiments that visualize potential signaling proteins present on the endosome such as β-arrestin that may be implicated in the phosphorylation of ERK proteins.

Unexpectedly, although not statistically significant, application of KT5720, a PKA inhibitor, resulted in an apparent enhancement of pERK levels in PACAP treated VPAC2R-expressing cells compared to cultures treated with PACAP alone. While the effect did not appear significant, an increase in pERK expression may have been facilitated by blocking the PKA
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signaling pathway, allowing greater activation of Gαq coupled VPAC2 receptors to activate PKC signaling. While the facilitation may have been fortuitous, additional testing would be required to observe if ERK phosphorylation mediated by the PKC pathway may be further assisted by suppressing PKA signaling mechanisms.

The significance of these results provides more insight regarding VPACR-mediated ERK phosphorylation in response to ligand binding and the possible implications it has on physiological disorders. PACAP and VIP activity have both shown to have neuroprotective functions in a variety of in vivo and in vitro models of brain trauma and Alzheimer’s disease by preventing microglial activation (Delgado, Varela, & Gonzalez-Rey, 2008). Expression of VPAC receptors on microglia has also been shown to prevent morphological changes and mobilization of microglia following brain trauma (Brifault, Gras, Liot, May, Vaudry, & Wurtz, 2015). Both PACAP-mediated events occurred through suppression of microglial activation and maintenance of an M2-type morphology, indicating that PACAP activity in the brain has the capability of reducing M1 microglial phenotype expression, which suppresses release of proinflammatory factors and reduce the overall inflammatory response in brain tissue. Future studies may determine the mechanism of VPAC-receptor activation that reduces microglial proinflammatory activity during neurodegenerative events, and if treatments comprised of agonist molecules may be beneficial in reducing brain inflammation following traumatic brain injuries.

Contrastingly to the neuroprotective effect of VPAC receptor activation on microglia, increase and maintenance of pERK proteins in specific neurons has been shown to mediate the emotional responses to pain. It has been shown previously that sustained ERK signaling via PAC1R-mediated ERK activation in neuronal cells of the central nucleus of the amygdala may be implicated in chronic pain responses (Missig et al., 2017). This phenomenon may be linked to
overexpression or activation of PAC1R in the amygdala, or increased PACAP release over time.

It would benefit future studies to observe the clinical relevance of antagonistic molecules to PAC1R in reducing pain and anxiety in chronic pain or PTSD models, respectively.
References


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*Pharmacological Reviews, 50*(2), 265-270.


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