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Calcium influx, but not intracellular calcium release, supports PACAP-mediated ERK activation in HEK PAC1 receptor cells

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Abstract

In HEK cells expressing GFP-tagged PAC1Hop1 receptors, PACAP augments ERK phosphorylation through two parallel pathways; one through PACAP/PAC1 receptor internalization/endosome MEK/ERK signaling, the other through PLC/DAG/PKC activation. We examined whether elevation of intracellular calcium ($[Ca^{2+}]_i$) was required for either of the PACAP/PAC1 receptor-mediated ERK activation mechanisms. The PACAP (25 nM)-induced elevation of $[Ca^{2+}]_i$ was greater with cells maintained in Ca^{2+} -containing than in Ca^{2+} -deficient solution, suggesting that both calcium release from internal stores and calcium influx contributed to the rise in $[Ca^{2+}]_i$. A thapsigargin-induced increase in $[Ca^{2+}]_i$ also was greater with calcium in the external solution. OAG, the cell permeable analogue of DAG, increased $[Ca^{2+}]_i$, but only in Ca^{2+} -containing solution. Decreasing external calcium or depleting internal calcium stores did not block PACAP-induced PAC1 receptor internalization. Omission of calcium from the external solution, but not thapsigargin pretreatment, significantly blunted PACAP-stimulated ERK phosphorylation. The PKC inhibitor BimI decreased PACAP-mediated ERK activation in both Ca^{2+} -containing or Ca^{2+} -deficient solutions. In contrast, following Pitstop 2 pretreatment to block endocytic mechanisms, PACAP activated ERK only when calcium was present in the external solution. We conclude that the endosome signaling pathway is largely calcium-independent whereas calcium influx appears necessary for the PLC/DAG/PKC component of PACAP-induced ERK activation.

Keywords

Pituitary adenylate cyclase activating polypeptide; PAC1 receptor internalization; ERK phosphorylation; thapsigargin; IP3-mediated calcium release

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AUTHOR CONTRIBUTIONS

RLP and VM designed the experiments and interpreted the data; TRB, TAC, BMG and VM collected and analyzed the data; RLP and VM wrote the paper; RLP, VM, TRB, TAC and BMG reviewed paper. All authors approved the final version.

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INTRODUCTION

Following preferential pituitary adenylyl cyclase activating polypeptide (PACAP; *Adcyap1*) binding, the PAC1 (*Adcyap1r1*) G-protein coupled receptor (GPCR) can transduce multiple intracellular signaling cascades with differential temporal and spatial resolution (Vaudry et al, 2009; Harmar et al, 2012). Among several isoforms, the PAC1Hop1 receptor appears unique in its abilities to activate dual G α s/adenylyl cyclase and G α q/phospholipase C (PLC) pathways through membrane-delimited events, and stimulate other downstream second messengers, including MEK/ERK and PI3K/Akt, through diverse mechanisms (Braas and May, 1999; May et al 2010; Emery and Eiden, 2012).

Using a stably expressing GFP-tagged PAC1 Hop1 receptor HEK cell line, we have shown recently that PACAP/PAC1 receptor signaling activates ERK through two parallel, temperature-dependent pathways (May et al, 2014). One mechanism involves internalization of the PACAP/PAC1 complex and the resulting formation of signaling endosomes for scaffold recruitment of adaptor proteins important for MEK/ERK signal transduction. The second mechanism involves PACAP/PAC1 receptor activation of the PLC/DAG/PKC pathway leading to ERK phosphorylation. Activation of PLC results in phosphatidylinositol hydrolysis resulting in the generation of IP3 for IP3/IP3R-mediated Ca²⁺ release from intracellular stores, and diacylglycerol (DAG) production which has a number of direct actions including PKC regulation and calcium influx through activation of plasma membrane calcium-permeable channels (Clapham, 2007). Both of these ERK activation mechanisms, vesicle endocytosis and PKC activation, could be Ca²⁺-dependent. Accordingly, we hypothesized that either the PAC1 receptor/endosome signaling- and/or the PKC-mediated mechanisms of ERK activation might be dependent on a rise in intracellular calcium ([Ca²⁺]_i) due either to an IP3-induced calcium release from internal stores or calcium influx through plasma membrane channel activation. Consequently, the present experiments tested whether thapsigargin-induced depletion of intracellular calcium stores or omission of calcium from the external medium blunted either PACAP-stimulated PAC1 receptor internalization/endosomal signaling or PKC-mediated ERK phosphorylation. Our results indicate that depletion of internal calcium stores had no effects on PAC1 receptor internalization and did not block PACAP-induced ERK phosphorylation transduced by either endosome or PKC signaling mechanisms. In contrast, the omission of external calcium from the bathing medium significantly diminished PACAP-stimulated ERK phosphorylation levels without suppressing receptor internalization. The results suggest that a PACAP-initiated calcium influx, perhaps through activation of receptor operated channels (ROCs) or store operated calcium entry (SOCE), facilitated PACAP/PAC1 receptor-stimulated PLC/DAG/PKC phosphorylation of ERK.

MATERIALS and METHODS

Cell culture

All experiments were performed using cultured HEK 293 cells stably expressing the GFP-tagged PAC1Hop1 receptor as described previously (May et al, 2010; May et al, 2014). HEK 293 cells were transfected using Mirus TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI) and cultured in Dulbecco's modified Eagle's medium/F-12 containing 8%

fetal bovine serum and 500 µg/ml Geneticin for stable cell selection. Individual cell colonies were selected and expanded and as before, functional expression of the receptor was assessed by GFP fluorescence and PACAP-stimulated second messenger activation. Treatment of the parent HEK293 cells with PACAP (25 nM) had no significant effects on ERK activation suggesting a deficiency in endogenous VIP/PACAP receptor expression.

Chemicals

PACAP27 was used exclusively in this study and is referred to as PACAP throughout the text. All drugs were obtained from commercial sources: PACAP27 (American Peptide Co., Sunnyvale, CA), and BimI (bisindolylmaleimide I), OAG (1-oleoyl-2-acetyl-sn-glycerol) and thapsigargin (Calbiochem EMD Biosciences, Inc. La Jolla CA), Pitstop 2 (N-[5-(4-bromobenzylidene)-4-oxo-4,5-dihydro-1,3-thiazol-2-yl]naphthalene-1-sulfonamide, Abcam Biochemicals, Cambridge, UK). All drugs were applied directly to the bath solution from frozen concentrated stocks prepared in either DMSO (BimI, OAG, Pitstop 2, thapsigargin) or water (PACAP). The concentration of DMSO in the bath solution never exceeded 0.1%.

GFP-PAC1 receptor endocytosis

To evaluate GFP-PAC1 receptor internalization under different treatment paradigms, the cell cultures were maintained in a Ca^{2+} -containing physiological solution (in mM: 121 NaCl, 5.9 KCl, 2.5 CaCl_2 , 1.2 MgCl_2 , 25 NaHCO_3 , 1.2 NaH_2PO_4 , 10 NaHEPES buffer and 8 glucose; pH 7.4) or a Ca^{2+} -deficient solution of similar composition except that MgCl_2 was substituted for CaCl_2 . No calcium chelators were added to the Ca^{2+} -deficient solution. Control experiments indicated that the HEK cells remained completely viable and attached to the culture substrate in these physiological solutions over the entire experimental duration. For drug pretreatments, the cells were exposed to the compounds for 15 – 30 min prior to incubation with 25 nM PACAP27 for 20 min at 37°C. Upon experiment termination, the cultures were fixed with 4% paraformaldehyde, washed and imaged with a DeltaVision restoration microscope equipped with GFP filter sets and a 1.4 NA 60X objective lens.

Western blot analyses for phosphorylated ERK

Control and treated cultures were extracted as previously described (May et al., 2010; May et al, 2014) 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) (May et al, 2002; Girard et al, 2004; May et al, 2010). Total sample proteins were determined using the Coomassie Plus reagent (Pierce Biotechnology, Rockford, IL). For Western analyses, protein samples (30 µg) were fractionated on 4 – 12% LDS-PAGE gels, transferred onto Immobilon-FL PVDF membranes (Millipore, Billerica, MA), blocked and incubated with primary antisera for quantitative infrared imaging (LiCor Biosciences, Lincoln, NE). Pan and phospho-specific antisera to ERK1/2 were from Cell Signaling Technology (Beverly, MA).

Measurement of PACAP-induced elevation of intracellular Ca^{2+}

The calcium-sensitive dye fura-2AM (Life Technologies Corporation, Carlsbad, CA) was used to measure PACAP-induced changes in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) (May et al., 2014). The cells were maintained in either the Ca^{2+} -containing or the Ca^{2+} -deficient physiological solution, incubated in 4 μM fura-2AM with 0.08% pluronic acid (Life Technologies Corporation, Carlsbad, CA) for 30 min at room temperature, and washed in dye-free physiological solution for ~30 min at 37°C. Change in the ratio of fura-2AM fluorescence excited alternatively at 340 and 380 nm was used to indicate changes in cellular calcium and was monitored using a PTI monochromator system coupled to a Nikon Eclipse E600 FN with a 60X 1.00NA water dipping physiology lens. PACAP-induced changes in fura-2AM fluorescence were compared in cells maintained in the Ca^{2+} -containing or Ca^{2+} -deficient solution at 35–37°C. Data are expressed as the change in F340/F380 ratio as a function of time.

Statistics

Statistics were performed using either Excel or GraphPad Prism statistical software (version 5.4; 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 posthoc analysis. Data are presented as mean $\pm$ SEM; $p < 0.05$ was considered significant.

RESULTS

PACAP raises intracellular Ca^{2+} both through release from internal stores and influx

The PAC1HOP1 receptor is commonly coupled to Gas and G α_q /11 for adenylyl cyclase and phospholipase C (PLC) activation, respectively (Braas and May, 1999). The hydrolysis of phosphatidylinositol by PLC leads to the production of diacylglycerol (DAG) and the generation of IP₃; the latter binds to endoplasmic reticulum IP₃Rs initiating release of stored calcium (Clapham 2007). During exposure of the GFP-PAC1HOP1 receptor cells to PACAP (25 nM), the F340/F380 ratio increased, indicative of a rise in $[\text{Ca}^{2+}]_i$. An elevation of $[\text{Ca}^{2+}]_i$ was evident with cells maintained in either the Ca^{2+} -containing or Ca^{2+} -deficient medium. The PACAP-induced rise in $[\text{Ca}^{2+}]_i$ peaked within a minute and then declined towards baseline values upon continued PACAP exposure (Figure 1A, B). The rate of $[\text{Ca}^{2+}]_i$ decline typically occurred more slowly when the cells were maintained in the Ca^{2+} -containing solution and the levels appeared to stabilize at a plateau above baseline (before PACAP treatment) levels. Further, for some cells in experiments completed in the Ca^{2+} -containing solution, the PACAP-induced rise in $[\text{Ca}^{2+}]_i$ exhibited an oscillatory behavior and a more prolonged decay. This oscillatory behavior was not noted in any of the experiments completed in the Ca^{2+} -deficient solution. To estimate the average PACAP-induced change in F340/F380 ratio for multiple cells, data were only quantified from cells in which the increase in $[\text{Ca}^{2+}]_i$ was transient and did not exhibit oscillations. Following PACAP treatment, the averaged peak F340/F380 ratio was significantly greater in cells maintained in the Ca^{2+} -containing solution than that in the Ca^{2+} -deficient medium (Figure 1C). The baseline F340/F380 ratio before PACAP application was also slightly, but significantly, higher in cells kept in the Ca^{2+} -containing than in the Ca^{2+} -deficient solution (Figure 1C). However, this very small difference should not account for the difference in

PACAP-initiated responses. These observations suggested that PACAP-induced an elevation of $[Ca^{2+}]_i$ due to both calcium release from IP₃-sensitive stores and Ca^{2+} influx.

We postulated that the PACAP-induced Ca^{2+} influx may have resulted from activation of store-operated calcium entry (SOCE) (Clapham 2007) channels or calcium permeable channels regulated by PAC1 receptor/Gαq generation of DAG (Vazquez et al, 2004; Pederson et al, 2005; Ramsey et al, 2006). HEK cells express members of the calcium permeable nonselective cationic TRPC channels, a number of which can be activated by DAG (Wu et al, 2000; Wu et al, 2002; Zagranichnaya et al, 2005). To establish whether a DAG-induced calcium influx could potentially contribute to the PACAP-induced rise in global calcium, the response of OAG (100 μM), a membrane permeable analogue of DAG, was determined. OAG could increase $[Ca^{2+}]_i$, with the HEK cells maintained in the Ca^{2+} -containing solution, although the magnitude of the OAG response varied greatly from a robust elevation in $[Ca^{2+}]_i$ as seen in Figure 1E to a much smaller increase in the F340/F380 ratio. In contrast, there was never any OAG-induced rise $[Ca^{2+}]_i$ when cells were maintained in the Ca^{2+} -deficient solution, although a subsequent application of PACAP to these same cells consistently initiated a rise in $[Ca^{2+}]_i$ (Figure 1F).

Prolonged thapsigargin treatment suppresses the PACAP-induced rise in $[Ca^{2+}]_i$

Subsequent experiments tested whether thapsigargin treatment blunted the PACAP-induced rise in $[Ca^{2+}]_i$. Thapsigargin irreversibly inhibits the SERCA pump in the endoplasmic reticulum (ER) resulting in a progressive calcium leakage and transient elevation of $[Ca^{2+}]_i$ (Thomas and Hanley, 1994). Thus, as was expected, thapsigargin application to cells bathed in either a Ca^{2+} -containing or Ca^{2+} -deficient solution initiated a progressive rise in $[Ca^{2+}]_i$ that slowly declined to an intermediate level (Figure 1D). As was found for the PACAP-induced rise in $[Ca^{2+}]_i$, the thapsigargin-initiated increase in F340/F380 ratio was greater in cells maintained in the Ca^{2+} -containing than in the Ca^{2+} -deficient solution (Ca^{2+} -containing, peak F340/F380 = 1.45 ± 0.05 versus Ca^{2+} -deficient, peak F340/F380 = 0.61 ± 0.02 ; $p < 0.05$). The duration of the thapsigargin response was more prolonged and generally did not return to baseline with cells in the Ca^{2+} -containing solution, whereas cells in the Ca^{2+} -deficient solution consistently demonstrated transient thapsigargin-induced calcium responses that returned to baseline (before thapsigargin treatment) values. Also, the baseline calcium levels before thapsigargin application were slightly higher in the Ca^{2+} -containing (peak F340/F380 = 0.413 ± 0.013) than in the Ca^{2+} -deficient (peak F340/F380 = 0.339 ± 0.005) solution. Although significant ($p < 0.05$), this very small difference should not account for the decreased thapsigargin response with cells kept in the Ca^{2+} -deficient solution.

Prolonged thapsigargin application has been used to deplete internal calcium stores (Wu et al 2002) and in other experiments, PACAP was applied to GFP-PAC1Hop1 cells pretreated with 1 μM thapsigargin for 30 minutes with cells maintained either in the Ca^{2+} -containing or the Ca^{2+} -deficient solution. The cells were then washed to remove the inhibitor prior to PACAP exposure. Following thapsigargin pretreatment, PACAP failed to elicit a measurable rise in $[Ca^{2+}]_i$ regardless of calcium absence or presence in the bathing solution; an observation confirming that a significant component of the PACAP-induced rise in $[Ca^{2+}]_i$ was due to IP₃-mediated release of calcium from internal stores.

Unlike previous baseline measurements (Ca^{2+} -containing versus Ca^{2+} -deficient) the baseline F340/F380 ratio values following the 30 minute thapsigargin pretreatment were markedly greater for the cells maintained in the Ca^{2+} -containing solution than in the Ca^{2+} -deficient solution (Ca^{2+} -containing, $\text{F340/F380} = 0.93 \pm 0.33$; Ca^{2+} -deficient, $\text{F340/F380} = 0.32 \pm 0.01$; $p < 0.05$). This 3-fold greater F340/F380 ratio following thapsigargin pretreatment with cells in Ca^{2+} -containing solution is consistent with an ongoing calcium influx through store depletion activated store-operated calcium entry (SOCE) channels.

Depletion of internal calcium stores does not block PACAP-induced PAC1 receptor internalization

Our previous results demonstrated that PACAP signaling resulted in PAC1 receptor internalization for signaling endosome formation (Merriam et al, 2013; May et al, 2014). Taking advantage of similar imaging techniques, we tested whether a PACAP-induced calcium release from internal stores was necessary for PAC1 receptor endocytosis. In affirmation of our earlier work, there were many more internalized GFP-PAC1 receptor-containing vesicles in cultures treated with PACAP (25 nM, 20 min) than control untreated cultures (Figure 2A, B). Similar results were obtained during PACAP challenge with cells maintained in the Ca^{2+} -deficient solution (data not shown). To examine the role of internal calcium release in PAC1 receptor endocytosis, cultures were maintained in the Ca^{2+} -containing or Ca^{2+} -deficient solution, pretreated with thapsigargin for 30 minutes, washed to remove the inhibitor and then exposed to 25 nM PACAP for 20 minutes. In all instances, thapsigargin-mediated depletion of internal calcium stores had no effect on PACAP-induced PAC1 receptor internalization (Figure 2C, D). Since endocytosis occurred in the absence of extracellular calcium with and without thapsigargin pretreatment, these observations suggested that neither calcium influx nor calcium release from internal stores was necessary for the PACAP-induced PAC1 receptor internalization.

Calcium is necessary for the PKC component of PACAP-stimulated ERK activation

PACAP signaling in the GFP-PAC1Hop receptor cell line stimulates ERK phosphorylation through two parallel mechanisms; receptor endocytosis and formation of a signaling endosome to initiate MEK/ERK signaling and activation of the PLC/DAG/PKC signaling cascade (May et al, submitted for publication). Either or both of these mechanisms could potentially be dependent on a PACAP-induced rise in $[\text{Ca}^{2+}]_i$ via release of calcium from internal stores or calcium influx. Consequently, we examined how PACAP-mediated calcium dynamics may be important for ERK activation. For these studies, the cultures were maintained in Ca^{2+} -containing or Ca^{2+} -deficient medium, with a subset of the cultures in each group then depleted of IP3-sensitive internal calcium stores by a 30 minute thapsigargin pretreatment prior to a 15 minute PACAP exposure and phosphorylated ERK measurements. As seen in Figure 3, thapsigargin pretreatment did not block PACAP-induced phosphorylated ERK in cultures maintained in either the Ca^{2+} -containing or Ca^{2+} -deficient solution. Inspection of the data, however, revealed that the magnitude of the PACAP-induced ERK phosphorylation was significantly greater ($p < 0.05$) in the Ca^{2+} -containing (fold increase = 7.78 ± 0.90) than that in the Ca^{2+} -deficient (fold increase = 4.70 ± 0.73) solution. These observations suggested that calcium influx might be a critical cofactor for one of the two parallel pathways. To distinguish which pathway might be

dependent on calcium influx, pharmacological treatments with the clathrin inhibitor Pitstop 2 to block receptor internalization (von Kleist et al, 2011) or BimI to inhibit PKC, were used to evaluate each pathway separately.

To test whether PAC1 receptor internalization and formation of a signaling endosome required calcium influx, the cells were maintained in Ca^{2+} -containing or Ca^{2+} -deficient solution, pretreated with BimI to inhibit PKC, exposed to 25nM PACAP for 15 minutes and assayed for phosphorylated ERK. PACAP alone stimulated ERK activation 6- to 7-fold. Following PKC inhibition, there was a comparable decrease in PACAP-induced phosphorylated ERK level (approximately 50% decrease) regardless of the presence or absence of calcium in the solution (Figure 4). These observations indicated that PAC1 receptor internalization and signaling endosome formation were not significantly affected by diminished extracellular calcium; an observation consistent with the imaging results showing PACAP-induced PAC1 receptor internalization in the Ca^{2+} -deficient solution, with or without thapsigargin pretreatment.

In the converse experiment, the cultures were treated with Pitstop 2 to block endocytosis prior to evaluating PACAP-mediated ERK activation through the PKC pathway. Similarly, the cultures were maintained in Ca^{2+} -containing or Ca^{2+} -deficient solution, pretreated for 20 minutes with 15 μM Pitstop 2 and challenged with 25 nM PACAP for 15 minutes prior to phosphorylated ERK measurements. With cells in Ca^{2+} -containing solution, Pitstop 2 significantly reduced the PACAP-stimulated ERK response approximately 60% (Figure 5); results in agreement with our recent observations (May et al, submitted for publication), which showed that the residual ERK phosphorylation levels reflected PACAP-stimulated PKC signaling mechanisms. However, with cells maintained in the Ca^{2+} -deficient solution and pretreated with Pitstop 2 to block endocytosis, no residual PKC signaling was evident such that PACAP-induced ERK phosphorylation did not exceed control baseline levels. Hence, these results demonstrated that the PKC component of ERK activation by PACAP was dependent on extracellular calcium influx.

DISCUSSION

The interaction of ligands with specific GPCRs not only activate membrane-mediated signaling cascades, but also can recruit additional signaling pathways by internalization of the ligand/receptor complex and formation of a signaling endosome (Calebiro et al., 2010; Scita and Di Fiore, 2010; McMahon and Boucrot 2011; Irannejad et al, 2013). We recently showed that internalization of the PACAP/PAC1 complex and formation of a signaling endosome supports many of the diverse neuronal effects of PACAP, which range from trophic support to regulation of membrane excitability (May et al, 2010; Merriam et al, 2013). Using HEK cells stably expressing the PAC1Hopl receptor, we have demonstrated that one mechanism underlying the activation of ERK by PACAP involves PAC1 receptor internalization and formation of a signaling endosome, which acts as a scaffold recruiting adaptor proteins necessary for MEK/ERK signaling. We also identified PAC1 receptor activation of the PLC/DAG/PKC pathway as a parallel mechanism by which PACAP can increase ERK phosphorylation. These two pathways are temperature-dependent and their combined actions generate total ERK activation by PACAP in this system (May et al, 2014).

PACAP/PAC1 receptor signaling can produce dramatic intracellular calcium transients, and as both vesicular endocytosis and PKC activity can be calcium dependent, the present study examined whether either of the PACAP-mediated ERK phosphorylation pathways required a coincident rise in $[Ca^{2+}]_i$ from either IP3-induced release of calcium from ER stores or calcium influx through plasma membrane channels. Our results demonstrated that neither the endosomal nor PKC pathways activated by PACAP required internal calcium release for ERK phosphorylation. However, the PLC/DAG/PKC component of the PACAP-mediated ERK phosphorylation was significantly diminished when calcium was omitted from the extracellular solution. The latter observation was consistent with the hypothesis that calcium influx is required for PLC/DAG/PKC signaling, which may be important not only to the efficacy of PACAP-mediated ERK phosphorylation but also for the targeting of activated ERK to specific intracellular nuclear or cytosolic compartments.

PACAP-evoked a transient rise in $[Ca^{2+}]_i$ in the HEK PAC1Hop1 expressing cells. One significant component of the PACAP-induced rise in global calcium was due to an IP3-induced release of calcium from internal stores. However, the PACAP-induced calcium transients were smaller and of shorter duration when the HEK PAC1 cells were maintained in the Ca^{2+} -deficient solution than in the Ca^{2+} -containing solution, suggesting that calcium influx contributed to the total global rise in $[Ca^{2+}]_i$. The calcium influx pathway could be through PAC1 receptor-operated calcium channels (ROCs) activated in the plasma membrane by DAG or by activation of SOCE channels following depletion of internal calcium stores. Results of the present study indicated that both pathways could have supported a PACAP-induced calcium influx. Evidence for the presence of ROCs was obtained using the cell permeable DAG analogue OAG, which could initiate a rise in $[Ca^{2+}]_i$ when cells were maintained in Ca^{2+} -containing solution, but not when the cells were kept in the Ca^{2+} -deficient solution. Thus, PACAP/PAC1 receptor signaling via Gq α /11 and the resulting generation of DAG by PLC could have activated ROC channels in some cells, which provided a pathway that allowed Ca^{2+} influx to contribute to the total PACAP-induced rise in $[Ca^{2+}]_i$. However, we noted that the OAG response was variable with a progressive reduction of the OAG response as cell passage number increased. Consequently, we suggest as others have noted that expression of endogenous channels possibly comprising a ROC influx pathway can differ between HEK cell lines and can also change in the same cell line over multiple passages.

Our current results also provide evidence for the potential involvement of SOCE channels proving a calcium influx pathway supporting the PACAP-induced elevation of $[Ca^{2+}]_i$. The presence of SOCE channels in the HEK PAC1Hop1 expressing cells was evident from the response to thapsigargin. For instance, the larger and longer thapsigargin-induced $[Ca^{2+}]_i$ transients evident in Ca^{2+} -containing solution is consistent with calcium influx through SOCE channels as is the markedly greater baseline cellular calcium level following prolonged thapsigargin pretreatment of cells in Ca^{2+} -containing solution. We propose that PACAP likely activated SOCE following ER calcium store depletion as has been described for other GPCRs in HEK cells (Wu et al, 2002) and further that this pathway could also contribute to the total PACAP-evoked rise in $[Ca^{2+}]_i$.

HEK cells express transcripts for multiple TRPC channels (Wu et al, 2000; Wu et al, 2002; Zagranichnaya et al, 2005). The HEK PAC1Hop1 cells used in the present studies express transcripts for TRPC 1, 3, 4, 5 and 6 (Girard and Parsons, unpublished data). While TRPC channels have been reported to contribute to SOCE (Wu et al, 2000; Wu et al, 2002; Zagranichnaya et al, 2005), HEK cells may also express STIM1 and Orai proteins (Liao et al 2007) now thought to be components of the SOCE pathway. Thus, all of the components are present in the HEK cells necessary to support either ROC or SOCE as mechanisms for calcium influx. Furthermore, although our present results do not establish whether only one or both pathways contribute, given the high driving force for calcium, calcium influx through these endogenous channels could add to the PACAP- or thapsigargin-induced elevation in global calcium.

Using deconvolution microscopy, image analyses revealed no apparent differences in PACAP-induced PAC1 receptor internalization with cells maintained in either a Ca^{2+} -containing or Ca^{2+} -deficient solution. In addition, there was no noticeable effect on PAC1 receptor internalization when internal calcium stores were depleted by thapsigargin. In aggregate, these observations indicated that neither PACAP-induced calcium influx nor the PACAP-induced release of calcium from internal stores appeared to be required for PACAP-initiated internalization of the PAC1 receptor.

Delineating the mechanisms underlying PAC1 receptor-stimulated ERK activation is important in understanding the neurotrophic and neuroplasticity functions of PACAP in development, injury and the long term physiological and behavioral consequences of stress. Calcium is a powerful regulator in virtually all physiological processes and accordingly, how changes in cellular calcium levels may regulate PACAP-mediated ERK signaling become important. Of the dual PACAP/PAC1 receptor mechanisms capable of ERK activation, one appeared to require calcium. Depletion of endoplasmic reticulum calcium stores with thapsigargin did not blunt phosphorylated ERK levels in PACAP, indicating that a IP_3 -mediated global rise in $[\text{Ca}^{2+}]_i$ was not required for the PACAP activation of ERK. By contrast, ERK phosphorylation was diminished when extracellular calcium was replaced by magnesium, suggesting that calcium influx supported the ERK activation by PACAP. Between the two independent parallel pathways for PACAP-mediated ERK activation, PAC1 receptor internalization and endosome ERK signaling appeared to be largely calcium independent. Using BimI to inhibit PKC and pharmacologically isolate the endosome signaling pathway, calcium influx did not appear essential for PACAP-stimulated ERK activation. These observations were consistent with imaging studies that indicated neither calcium influx nor calcium release from internal stores was required for PACAP-induced PAC1 receptor internalization and endosome signaling. In the converse experiment with Pitstop 2 to block receptor endocytosis and isolate PKC mechanisms, the omission of calcium in the bathing medium blocked the PKC component of PACAP-mediated ERK phosphorylation.

Prior studies using Chinese hamster ovary cells expressing OX1 orexin receptors also have demonstrated a calcium-dependence of agonist-induced ERK phosphorylation (Ammoun et al, 2006; Ekholm et al, 2007). As found in the present study, receptor activated calcium influx, but not an IP_3 -mediated elevation of $[\text{Ca}^{2+}]_i$, was required for ERK phosphorylation.

Consequently, we postulate along the lines suggested by Ammoun et al (2006) for OX1 receptors that a PACAP-induced calcium influx elevates $[Ca^{2+}]_i$ in cellular domains near the inner surface of the plasma membrane and that the increase in $[Ca^{2+}]_i$ within these domains supports the PACAP-induced activation of the PLC/DAG/PKC pathway leading to ERK activation and signaling.

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Figure 1.

PACAP, thapsigargin and OAG induce a rise in $[Ca^{2+}]_i$. A, B: Traces illustrating the PACAP-induced rise in $[Ca^{2+}]_i$ with cells kept in the Ca^{2+} -containing (A) or Ca^{2+} -deficient (B) solution. The arrow indicates the onset of PACAP application. C: Averaged data from multiple cells showing the peak rise in global calcium was significantly less ($p < 0.05$) when calcium was omitted from the external solution. Note also that the pre-PACAP F340/F380 values were also significantly different ($p < 0.05$), although the difference was very small. D: Traces illustrating the elevation of $[Ca^{2+}]_i$ during a sustained application of 1 μ M thapsigargin. The arrow denotes initiation of thapsigargin application. E, F: Traces illustrating that OAG (100 μ M) initiates an elevation of $[Ca^{2+}]_i$ when cells from the same passage are kept in a Ca^{2+} -containing solution (E), but not when kept in the Ca^{2+} -deficient solution (F). Note in F that even though there was no OAG response, PACAP elicited a rise $[Ca^{2+}]_i$ in these same cells.

Figure 2.

Thapsigargin-pretreatment does not block PAC1 receptor endocytosis. A, GFP-PAC1 receptors were expressed predominantly on the cell surface of control HEK PAC1Hop1 expressing cells; few intracellular GFP-PAC1 receptor containing vesicles were evident at 37°C. B: The internalization of GFP-PAC1 receptors from the cell surface into numerous endocytic vesicles was evident after a 20 minute exposure to 25 nM PACAP. C, D: A 20 minute incubation in 25 nM PACAP initiated internalization of GFP-PAC1 receptors in cells pretreated for 30 minutes with 1 μ M thapsigargin and kept in either the Ca^{2+} -containing (C) or Ca^{2+} -deficient solution (D). Calibration bar in B equals 20 μ m.

Figure 3.

Thapsigargin pretreatment does not block PACAP-induced ERK phosphorylation. A: Representative Western blots showing PACAP-stimulated ERK activation at 15 min. PAC1 receptor cell cultures, kept in either Ca²⁺-containing or Ca²⁺-deficient solution, were pretreated with 1 μ M thapsigargin (TH, 30 min) and exposed to PACAP (P27, 25 nM) for 15 minutes before Western blot analyses for phosphorylated ERK. B: With cells in either solution, thapsigargin pretreatment did not blunt the PACAP- stimulated ERK phosphorylation. All data are normalized to total ERK levels on the same blots using a pan ERK antibody probed with different fluorochrome-conjugated secondary antisera. Data represent mean fold change $\pm$ SEM, n =3. * Statistically different from respective controls (p <0.05).

Figure 4.

PKC inhibition decreases PACAP-induced ERK phosphorylation comparably for cells kept in a Ca²⁺-containing or Ca²⁺-deficient solution. A: Representative Western blots showing PACAP-stimulated ERK activation at 15 min. PAC1 receptor cell cultures, kept in either Ca²⁺-containing or Ca²⁺-deficient solution, were pretreated with 1 μ M Bim1 to inhibit PKC (Bim1, 15 min) and exposed for 15 minutes to PACAP (P27, 25 nM) before Western blot analyses for phosphorylated ERK. B: Following PKC inhibition, the PACAP-induced fold change in ERK phosphorylation was significantly depressed, but remained significantly greater than respective controls. All data are normalized to total ERK levels in the same samples. Data represent mean fold change $\pm$ SEM, n = 4. * Statistically different from respective controls (p < 0.05); + statistically different from P27; all at p < 0.05.

Figure 5.

Inhibition of clathrin-mediated PAC1 internalization decreases PACAP-induced ERK phosphorylation to a greater extent with cells maintained in a Ca^{2+} -deficient than in a Ca^{2+} -containing solution. A: Representative Western blots showing PACAP-stimulated ERK activation at 15 min. PAC1 receptor cell cultures, kept in either Ca^{2+} -containing or Ca^{2+} -deficient solution, were pretreated with Pitstop 2 (15 μM , 15 min) to inhibit clathrin-mediated PAC1 endocytosis and exposed for 15 minutes to PACAP (P27, 25 nM) before Western blot analyses for phosphorylated ERK. B: Pitstop 2 pretreatment decreased the PACAP-induced fold change in ERK phosphorylation. However, following Pitstop 2 pretreatment in Ca^{2+} -containing solution, the PACAP-induced ERK phosphorylation remained greater than the respective control. In contrast, following Pitstop 2 pretreatment in the Ca^{2+} -deficient solution, the PACAP-induced ERK phosphorylation was not greater than the respective control. All data are normalized to total ERK levels in the same samples. Data

represent mean fold change $\pm$ SEM, n = 4. * Statistically different from respective controls (p <0.05); + statistically different from P27; all at p <0.05.